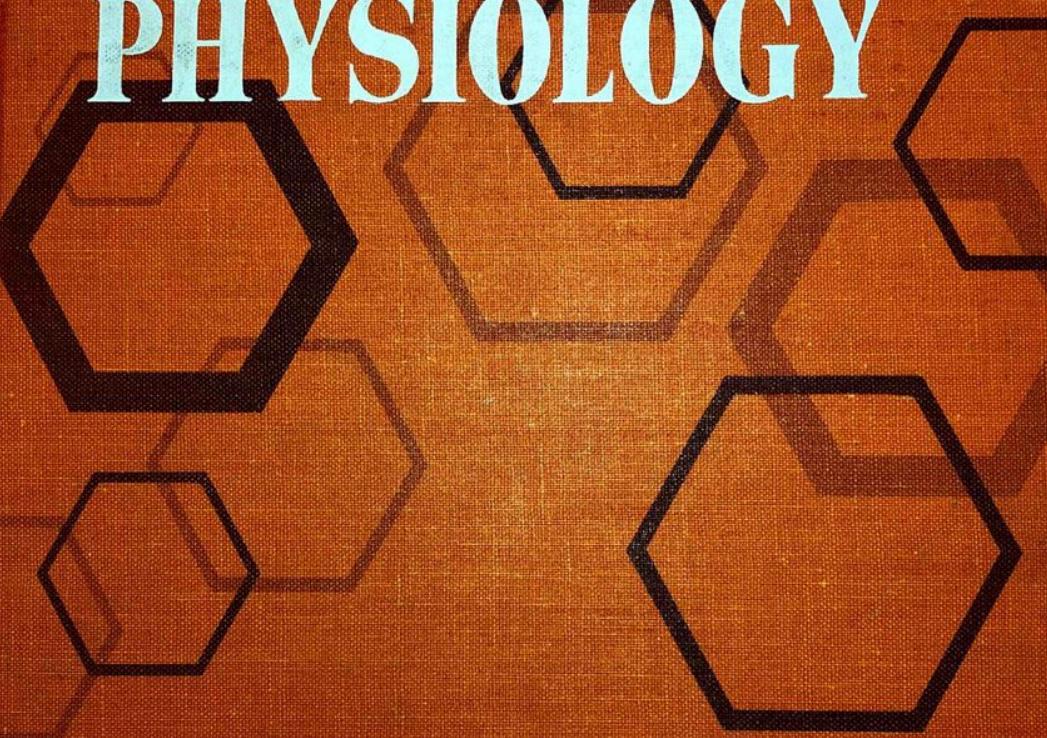


textbook of ENDOCRINE PHYSIOLOGY



TEXTBOOK OF ENDOCRINE PHYSIOLOGY

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PREFACE

This book was initially designed to meet the needs of students enrolled in undergraduate and graduate endocrinology courses at the City University of New York and those preparing for comprehensive examinations in the biological sciences. Suggestions from colleagues and students at other institutions for increasing the versatility of the text have been incorporated.

The courses at the City University emphasize applications of molecular biology to the understanding of whole animal physiology, and are concerned predominantly with the roles of hormones in the regulation of metabolic processes. Most of the discussion relates to mammals, but examples are freely drawn from studies of other vertebrates.

Since the lectures are attended by students majoring in psychology, anthropology, and other disciplines, most of the material has been written for comprehension by those having no preliminary preparation beyond elementary animal physiology and basic biochemistry. Passages concerned with controversial issues, topics of limited interest, and those requiring advanced knowledge of biochemistry have been placed in small type. Beginning students can omit the small-type sections without loss of text continuity. Since the topics are clearly demarcated, the instructor of an elementary course can easily delete portions which do not serve his particular needs; for example, sections on the biosynthesis or metabolism of hormones can be omitted without detracting from the understanding of the functions or mechanisms of action of hormones. The index provides ready access to specific information.

A functional approach has been adopted. Each of the hormones is presented in conjunction with the discussion of a specific physiological process. Thus, the endocrine pancreas is introduced in the section on regulation of plasma glucose concentrations, and thyroid gland physiology appears in the part concerned

with regulation of metabolic rate and body composition. However, a separate section has been devoted to the pituitary gland and hypothalamus because only certain aspects of the discussion of those structures can be conveniently incorporated into such topics as growth and reproduction. The decision to present an entire section on the pineal and thymus glands reflects special interests of the author and the belief that some aspects of this fascinating area have been too long neglected.

A text suitable for student use must be limited in size; this precludes encyclopedic treatment. No attempt has been made to present a comprehensive survey of the literature. The references at the ends of the chapters contain mostly review articles. It is assumed that students will freely utilize the reviews, the papers cited therein, and additional library resources as they prepare term papers and oral reports. They will thereby become familiar with the work of individual investigators whose names do not appear in the bibliography, but without whom the science of endocrinology could not exist.

The presentation has been varied to provide some insight into the kinds of information obtainable elsewhere. The morphology of the pituitary gland is described in detail because of numerous functional implications; but the morphology of most other endocrine structures has been either omitted or presented in abbreviated form. Parathyroid hormone physiology has been singled out for historical review. And comparative aspects receive special treatment in the section on water and electrolyte metabolism. It is certain that any endocrinologist undertaking the task of writing a text with limited scope will make some other selection.

It is, of course, presumptuous for any individual to pretend to be an expert on all aspects of endocrinology. The volume of current literature in each of the areas is overwhelming. It is there-

fore fortunate, indeed, that numerous publications written by specialists in individual fields are available. However, students have a real need for a unified text written by a single author; that is why this book was written. Constructive criticism from those more knowledgeable in specific areas is welcomed.

My husband, Henning Norbom's patience, understanding, sense of humor, and steadfast refusal to entertain complaints of fatigue or frustration contributed substantially to comple-

tion of the project. I am also deeply grateful to Carol C. Halpern for providing both inspiration and help with the literature survey. Many colleagues and students have given encouragement and constructive suggestions, and I especially wish to thank B.R., L.K.M., U.J.B., E.J.D., S.D.J., and M.C.G. It has been a special pleasure working with Trudy Nicholson, who made the original drawings, and with the production staff at the Williams & Wilkins Co.

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I

WHAT ARE HORMONES? WHAT DO THEY DO?

1. General Nature of Vertebrate Hormones

ATTEMPTS TO DEFINE A HORMONE

Until quite recently it was possible to present a simple, straightforward, and totally acceptable definition of the term "hormone." The nine components of the definition are examined in this chapter. The first two still apply to the spectrum of agents with which the vertebrate endocrinologist is concerned; problems which have arisen in conjunction with the seven others reflect changing concepts that have grown out of newer research.

According to the definition, hormones are (1) *physiological regulators* (2) *effective in minute quantities* (3) *synthesized by living cells* (4) *in glands which* (5) *secrete directly into the bloodstream*; the secretions are (6) *transported by the circulating blood* to (7) *specific target organs* located (8) *at a site distant from the site of synthesis* where they (9) *exert specific actions*.

Hormones Are Physiological Regulators

They speed up or slow down biological functions which proceed at a different rate in their absence. (The term "chalone" has been used to designate hormones with inhibitory actions, but it has recently taken on more specialized meaning.)

Heart muscle contracts when no hormones are present, but it contracts with greater force when exposed to adrenalin. Fat is broken down in adipose tissue, but the degradation rate is slowed by insulin.

Some processes appear to be totally dependent upon hormones; for example, mammalian egg cells do not ripen in their follicles, and accessory reproductive struc-

tures remain immature when appropriate hormones are missing. But hormones can only "awaken" existing potential. No quantity or combination of hormones can induce ovulation in gastric mucosa cells or milk synthesis in heart muscle.

While hormones are physiological regulators, many regulators are not hormones. Inorganic ions are ruled out by component 3 of the above definition. Glucose is ruled out by component 2 since large quantities are needed.

But, what about carbon dioxide? Minute quantities produced by living cells travel in the bloodstream to regulate "target organs" such as the respiratory center of the medulla oblongata. Carbon dioxide meets all criteria for a hormone except component 4; it is produced by all kinds of cells. The term "parahormone" is useful for designation of such regulators; a list of parahormones might include histamine, the kinins, and possibly also the prostaglandins (but not glucose).

Distinctions between *vitamins* and hormones are not always obvious. Members of the "B complex" clearly do not qualify as hormones because they are needed in the diet and are distributed to all kinds of cells. But vitamin D can be totally synthesized within specific sites in the body (Chapter 15), minute quantities of its metabolites travel with the blood to "target organs," and the actions exerted are similar to those of "typical" hormones.

Hormones Are Effective in Minute Quantities

Just what is meant by "minute" deserves comment. Daily dietary require-

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ments for the human adult include 40–100 g of protein, $1\text{--}2 \times 10^{-3}$ g of thiamine and perhaps 1×10^{-6} g of vitamin B₁₂. While milligram quantities of certain hormones may be injected (especially when degradation is rapid or when it is necessary to maintain high plasma concentrations), *microgram* (10^{-6} g) quantities of others are quite effective. Pituitary gland thyrotrophs respond vigorously to the presence of a few *picograms* (10^{-12} g) of hypothalamic hormones in the surrounding medium, while *femtograms* (10^{-15} g) of angiotensin II can effectively stimulate the hypothalamic neurons that affect drinking behavior.

Hormones Are Synthesized by Living Cells

This is often true. However, some glands release prohormones which require extracellular metabolic conversion. The kidney secretes renin, a *proteolytic enzyme* which acts on a plasma protein to promote formation of an active hormone.

Hormones Are Secreted by Glands

The term "ductless" is usually inserted to distinguish between *endocrine* glands emptying their products into the bloodstream and *exocrine* glands such as those secreting sweat and saliva. But just what is a gland? Little is accomplished by defining a gland as a structure specialized for secretion.

A search for *morphological* characteristics common to endocrine glands is not rewarding. The thyroid gland has *follicles*; the parathyroid, *cords* of cells; the endocrine pancreas, *clumps* of cells (islets). Three different cell arrangements are characteristically found within the mammalian adrenal cortex, while calcitonin is secreted by cells scattered throughout the thyroid gland.

The problem is further complicated by posing the question, "If a structure is clearly identifiable with other organ systems, can it still be classified as an endocrine gland?" The hypothalamus, in common with other parts of the brain, contains neurons which synapse with other neurons and transmit impulses. Some of these cells synthesize, store, and secrete substances which qualify as hormones.

The kidney synthesizes and secretes

erythropoietin, 1,25-dihydroxycholecalciferol, and prostaglandins, among other humoral regulators. The liver produces its own specialized secretions; it also plays a key role in metabolism of hormones and converts steroid hormones of one type to others having different biological activity. The lungs, heart, salivary glands, stomach, uterus, and intestine have been included in the ever growing list of structures implicated in hormone secretion. Recent research on prostaglandins raises the possibility that all kinds of cells produce hormones. Certainly the evidence grows daily that all cells secrete something.

Hormones Are Secreted into the Bloodstream

This part of the definition rules out *intracellular regulators*, *neurotransmitters*, and *pheromones (ectohormones)* which act on other organisms; but it also raises problems. If cells produce a prostaglandin which acts locally but which also enters the circulation to act elsewhere, is the prostaglandin a hormone in the latter instance but not in the former?

Acetylcholine is clearly a neurotransmitter; it is produced by neurons and it does not pass through the circulation to reach synapses, ganglia, or parasympathetically innervated muscles and glands. But norepinephrine, which is produced by neurons and sent directly to sympathetically innervated muscles and glands, is also secreted into the bloodstream by the adrenal medulla. The heart, gut, blood vessels, and salivary glands give identical responses to norepinephrine from both sources; are they responding to a hormone in one case but not in the other?

Hypothalamic neurons secrete hormones, some of which are directly sent into a restricted part of the circulatory system. The terms "neurohormone" and "neurosecretion" have been used to distinguish such regulators from hormones secreted by "typical" endocrine glands. Recent research findings have blunted the distinction.

Embryonic inducers are usually excluded from the list of hormones, because most act directly on neighboring cells and may complete their function before devel-

opment of the circulatory system. However, some (e.g., medullarin) do enter the circulation.

Pheromones are described in endocrinology texts because their production is usually hormonally controlled, and because their actions are commonly exerted on the endocrine systems of affected individuals.^{1, 4, 7} (The term "primer pheromone" describes such agents, whereas *behavioral or release pheromones* affect neuronal pathways.)

Pheromones were first studied in insects. In addition to practical applications, the knowledge gained has theoretical implications for vertebrate physiology.

Sex attractants released by females of unwanted insect species have been used to gather large numbers of males which can then be sterilized and released to mate with normal females. This permits eradication of a single species without environmental pollution. Vertebrate females are known to release pheromone "trails" which attract males.

Worker bees ingest an ovary-inhibiting pheromone released by the queen. Something released into the urine by female mice inhibits reproductive cycles of other females within the same cage. Similarities between such phenomena and the use of ovarian hormones produced by female vertebrates as oral contraceptives have been noted.¹ Ectohormones have been implicated in reduction of fertility of rodents subjected to crowding (although other mechanisms including alterations of adrenocortical function have been proposed). One may speculate on the possibility that pheromones play a role in recent acceptance of voluntary fertility control among humans concerned with population density, without denying the importance of other factors.

Grouped females of several species (including rats) develop synchronized estrous cycles; could pheromones account for the reported synchrony of menstrual cycles observed in young women residing in the same college dormitory or summer camp cottage? Urine from foreign mice and voles induces abortion in females of the same species inseminated by other males.¹⁰ Can such information be used to develop a human pheromone preparation for voluntary control of human fertility?

Olfactory cues not consciously recognized may affect human behavior. The complexity of neuronal pathways leading from "olfactory" components of the human brain supports the concept. *Copulin* has been identified in genital regions and urine of rodents in estrus and

monkeys in the follicular phase of the menstrual cycle, and it is known to play a role in attraction of the male¹¹. While we like to believe that human sexual behavior is controlled by conscious processes, a pheromone has been identified in vaginal washings of human females during the period immediately preceding ovulation.¹² Some unexplained attractions between individuals and the pleasures of kissing (or nose rubbing) may well have an olfactory component. Infant pheromones stimulate maternal behavior and lactation in rodents; perhaps this will lead to development of a human pheromone preparation which can be taken by women who would like to nurse their infants but are unable to produce sufficient milk.

Pheromones are difficult to study.^{14, 45, 68} Structures implicated in their production include ovaries, uterus, oviducts, testes, pre-putial glands, sebaceous and sweat glands, kidneys, gastrointestinal tract, and lungs. They are produced in minute quantities as components of highly complex mixtures, and available methods for assay are cumbersome, time-consuming, and subjective. Many seem to be released in "inactive forms" (e.g., as conjugates) and require hydrolysis by bacterial enzymes outside the body.

Mammals are known to exhibit changes in sensitivity (e.g., according to the stage of the menstrual cycle) and to respond to both concentration and chemical nature of the pheromone. Stimuli presented with the pheromones also affect responses; e.g., a single agent may arouse one response when present along with barking and another with growling. Even location may be important; "territorial" pheromones released by larger animals high off the ground can be more effective than those released lower by smaller members of the group.

Hormones Are Transported by the Circulatory System

This follows logically from component 5 of the definition. But is it important? Hypothalamic hormones may travel only millimeter distances through a highly restricted portion of the circulatory system, and target sites affected may be no larger than neuromuscular junctions of the toe receiving acetylcholine produced meters away. Functions of "brain hormones" released into the cerebrospinal fluid may not be

qualitatively different from those of hormones entering the blood circulation."¹⁵

Hormone Act on Specific Target Organs

This component of the definition may one day be discarded completely. Thyroid-stimulating hormone has been cited as an example of a specific regulator, since it stimulates epithelial cells of the thyroid gland follicles (with no known influences on neighboring interfollicular cells) and seems to be devoid of action on most other endocrine glands. But it also affects certain hormone-secreting hypothalamic neurons, and large doses promote lipolysis in adipose tissue. Thyrotrophin release factor was once thought to "specifically" promote secretion of thyroid-stimulating hormone, but it also affects prolactin release.

Insulin regulates metabolic processes in liver, heart, skeletal muscle, mammary gland, and probably also neurons affecting glucose metabolism. Its effects on plasma concentrations of glucose, fatty acids, and small ions and its influence on appetite extend to every cell type. Physiological concentrations of testosterone affect testes, prostate glands, seminal vesicles, larynx, skeletal muscle, skin, bone (and bone marrow), liver, kidneys, thymus gland, pituitary gland, and brain; and it would be difficult indeed to find cells escaping the influence of thyroxine. Each day brings new information on the presence of prolactin receptors in tissues not previously regarded as target organs. Moreover, each of the hormones indirectly affects other cells through influences on secretion or metabolism of other hormones.

The Action of Hormones Is Exerted at a Site Distant from the Site of Synthesis

As noted above, distances traveled by some hormones are exceedingly short; except for the possibilities of restricting the quantities of hormone required and the range of action when hormones go directly to their effector sites, little functional significance can be associated with the distance traveled.

Hormones Exert Specific Actions

Acceptance or rejection of this part of the definition depends upon the point of view.

On the positive side, it can be said that thyroid-stimulating hormone exerts obvious influences on the thyroid gland which cannot be mimicked by other known hormones, and that stimulation of protein synthesis by insulin is different from stimulation by growth hormone.¹⁶ However, there has been a growing tendency to seek out common denominators for mechanisms of action of groups of hormones. It will be noted in Chapter 3 that large numbers of peptide hormones enhance the formation of cyclic 3',5'-adenosine monophosphate (cAMP) in responsive cells, and that cAMP functions as a "second messenger." Many of the actions of the hormones can be induced by agents (hormonal or not) which stimulate formation of, or act like cAMP.

The "second messenger hypothesis" suggests that specificity of hormone action resides in the presence within the target cell membrane of a *unique hormone receptor*. Thus, thyroid cells respond to thyroid-stimulating hormone because they have receptors for the latter, but they lack receptors for glucocorticoids, follicle-stimulating hormone, and so forth. Responses to hormones depend upon unique features of cell metabolism. Thus, any agent which stimulates production of cAMP in the thyroid gland can promote synthesis of thyroid hormones, while any agent stimulating production of cAMP in certain cells of the adrenal cortex can promote synthesis of adrenocortical steroids. Each cell type "does its own thing" when appropriately stimulated.

THE CHEMICAL NATURE OF HORMONES

Hormones Synthesized from Amino Acids

Most (but certainly not all) hormones are synthesized from amino acid precursors. The very small molecules (modified amino acids and some low molecular weight peptides) are chemically identical in all vertebrates, although functions subserved may be quite different. Melatonin which affects movement of pigment granules in the skin of cold-blooded vertebrates is the same as melatonin which affects reproductive functions of hamsters.

As peptide molecules increase in size and complexity, species differences emerge; but hormones from one group may be

active when administered to another. Thus, fish insulins are chemically different from mammalian insulins, but fish insulins will affect plasma glucose concentrations of mammals.

Species specificity assumes greatest importance for the large proteins. Growth hormone derived from cattle does not stimulate the growth of humans; this has been attributed to differences in hormone receptors of the two mammalian types.

Modified Amino Acids. These are sometimes called amine hormones. The group includes *epinephrine* and *norepinephrine* derived from the amino acid tyrosine, and *serotonin* and *melatonin* derived from tryptophan. Thyroxine obtained through iodination and condensation of two tyrosine atoms is usually included in this classification.

Small Peptides. *Thyrotrophin release factor* is a tripeptide which may have identical composition in all vertebrates. Several other hypothalamic hormones seem to be small peptides or polyamines. Species variations have not been defined.

The best known *neurohypophysial hormones* (vasopressin, oxytocin, vasotocin, etc.) are octapeptides which are chemically related to one another. Differences in structure and function are described in Section III. *Angiotensin II* is also an octapeptide; at least two forms are known.

Larger Peptides. This group includes active forms of the *gastrins* with 17 amino acid moieties, *glucagons* with 29, and *calcitonins* with 32. *Adrenocorticotrophic hormones* from all species seem to contain 39 amino acid components, but there are well defined species variations. α -*Melanocyte-stimulating hormone* contains 13 amino acids, while the β hormones contain 18 or 22.

Proteins. Authors differ on where they draw the line between large peptides and small proteins. *Insulin* molecules contain 20 or 21 amino acid A chains joined by sulphydryl bridges to 29-31 amino acid B chains. *Parathyroid hormones* have 80-85 amino acids.

Growth hormones exhibit the greatest known species variations. The human form contains 191 amino acids.

Glycoproteins. Some adenohypophysial hormones contain carbohydrate components. These include *thyroid-stimulating*

hormone, *follicle-stimulating hormone*, and *luteinizing hormone*. Chorionic gonadotrophins are also glycoproteins.

Nonprotein Hormones

Steroids. Lipid-soluble hormones secreted by the ovary, testis, adrenal cortex, and placenta are derivates of cholesterol, which contains 27 carbon atoms. *Glucocorticoids* and *mineralocorticoids* from the adrenal contain 21 carbons as does *progesterone*; *androgens* have 19 and *estrogens*, 18 carbon atoms. Vitamin D and its metabolites are chemically related to these.

Other Nonproteins. *Prostaglandins* are cyclic fatty acids. Secretions associated with the hypothalamus of some vertebrates seem to be *mucopolysaccharides*. Some investigators believe that the thymus gland produces heparin or a related mucopolysaccharide.

BIOSYNTHESIS AND STORAGE OF HORMONES

Sites of Synthesis

Most large peptide and protein hormones are synthesized on ribosomes of the rough endoplasmic reticulum, sent through cisternae of the Golgi region (in which carbohydrate moieties may be added sequentially), packaged into membrane-enclosed vesicles which typically contain lipids, enzymes, and ATP, and stored for later release. (A notable exception is described in the section on thyroid hormones.²⁴)

Thyrotrophin release factor (which contains only three amino acid moieties) is synthesized by nonribosomal cytoplasmic enzymes,²⁵ and there is good reason to believe that other very low molecular weight peptides are similarly produced. Slightly larger peptides (e.g., vasopressin) may be split off larger molecules.²⁶

Formation of steroid hormones and of catecholamines requires participation of both cytoplasmic and mitochondrial enzyme systems and the shuttling of intermediates across mitochondrial membranes. Catecholamines are packaged into granules, but steroid hormones are not.

Prohormones

Insulin, glucagon, parathyroid hormone, and other peptides are first synthesized in the form of larger precursors or prohormones which are subsequently split into active principles and residual peptides.

Proteolytic enzymes catalyzing the cleavages have been identified in the Golgi region and in immature secretion granules. Prohormones and residual peptides are released along with the active hormone during normal secretory processes. Additional cleavages of some hormones are known to occur in plasma and target organs. Prohormone formation may not be universal; attempts to find a proprolactin have yielded negative data.⁶⁰ The term "hormonogen" has been applied to certain molecules which participate in hormone formation, for example, a protein produced by the liver (angiotensinogen) later acted upon by the enzyme renin to yield angiotensin.

Prohormones may be better suited than the finished product for the processes of *transport through the cell* or *interaction with lipid molecules*. They may provide a readily recruitable *storage form* for active molecules or may be secreted when requirements for the hormone are minimal (since they usually exert very weak hormone-like activity). Other functions attributed to them include *spatial fixation of proteins* for completion of active components and *provision of a site for regulation* of hormone synthesis.

It has been suggested that prohormone formation reflects a more generalized aspect of ribosomal function which requires that proteins of a minimal size be synthesized; therefore, production of smaller units must depend upon post-translational modification after the protein leaves the ribosome.⁶¹

Secretion Granules

Several important functions have been attributed to the processes of formation of secretion granules. The granules may provide (1) an efficient means for *intracellular transport* of the hormone (one concept is that they travel over microtubules as trolleys move over tracks); (2) favorable conditions for *completion of hormone synthesis*; (3) a mechanism for discharge of discrete packets ("quanta") of predetermined quantity; (4) *protection* of the hormone

against destruction by cytoplasmic enzymes or *against leakage across the plasma membrane*; (5) a site for *rapid uptake of the hormone* when physiological need is reduced, or facilitation of *hormone destruction*, e.g., through interaction with lysosomes (it is known, for example, that actions of catecholamines are rapidly terminated by granule uptake of hormones and that prolactin is destroyed intracellularly when lactation is abruptly terminated); and (6) *protection of the secreting cell* against excessive accumulation of free hormone.

SECRETION OF HORMONES

It is possible that small quantities of *unpackaged hormone* are released directly from granule-forming cells. Recent work on the parathyroid gland supports the concept.

Microtubules and microfilaments have been implicated in movement of secretion granules toward the plasma membrane; one hypothesis states that the filaments bind the granules to the tubules.

Electron microscopy studies have not fully supported the concept; these cytoplasmic structures are not consistently abundant or consistently oriented in the directions that would be needed. A different hypothesis suggests that contraction of the filaments alters the "cytoskeleton" formed by the tubules and thereby influences access of granules to the plasma membrane.

Hormonal discharge is preceded by interactions between secretion granule and plasma membranes. Processes may not be identical in all cell types. In most, there is fusion of the two membranes with loss of some nonhormonal components of the granule along with the hormone, and retention of the remainder within the cell.

Many different kinds of agents (trophic hormones, neurotransmitters, metabolites, high concentrations of potassium ion and certain drugs) and electrical stimuli can promote hormone release. All influence properties of the plasma membrane. In most cases it can be shown that inhibitors of hormone secretion induce changes in the opposite direction; e.g., if an effective stimulus depolarizes, the inhibitor will hyperpolarize the membrane.

Hormone discharge is an active, energy-consuming process. ATP can promote hormone release, while inhibitors of ATP synthesis and of ATPases block the processes.

Most secretion granules contain both ATP and ATPase, but functions have not been elucidated. One hypothesis states that they are involved in promoting contraction of an actomyosin-like protein associated with the microtubules (and a protein of this type has been identified in the adrenal medulla²⁴), and another implicates them in regulation of cytosol calcium ion concentrations. ATP is also a precursor of cAMP, but it is unlikely that intragranular ATP is used in this way.

High concentrations of cAMP (and of its dibutyryl analog, which more readily penetrates the cell) provoke secretion of many hormones; and stimuli for release of those hormones elevate intracellular concentrations of the nucleotide. cAMP can promote phosphorylation of microtubular proteins and of components of the plasma membrane which may be involved in fusion with the secretion granule membrane.

Secretion of many hormones is dependent upon the presence of calcium in the extracellular fluid, and calcium itself has been shown to promote secretion of several hormones in a dose-related manner. Many stimuli for hormone release increase the rate of calcium uptake by the cells, while others promote calcium redistribution (e.g., through release from mitochondria to the cytosol). Some inhibitors of hormone release interfere with calcium uptake.

Calcium is bound by the granules. It has been suggested that such binding alters the surface charge (known to be negative in several kinds of endocrine cells) and thereby facilitates interaction with microtubules or plasma membranes; however, magnesium ions with similar charge often exert opposing influences.²⁵ Calcium is also known to affect polymerization of microtubule subunits and to exert regulatory influences on the enzyme which catalyzes formation of cAMP.

Prolonged application of strong stimuli can lead to preferential release of recently synthesized hormone in some endocrine

cell types. Suggested explanations include more peripheral location of newly formed granules or greater sensitivity of immature granules to stimuli.

TRANSPORT AND METABOLISM OF HORMONES

When hormones enter the circulation, they combine with plasma proteins which facilitate transport, delay degradation, and protect small molecules from loss through the kidney glomeruli. The proteins vary in binding affinities and in number of hormones bound by each type. The binding is reversible and must be broken before the hormone can exert its actions. Usually only a small fraction of the plasma hormone is present in the free (active) form; the protein-bound portion provides a readily recruitable reserve.

Hepatic synthesis of the binding proteins is under hormonal control. Large increases during pregnancy can promote accumulation of high concentrations of hormone for use by the placenta and fetus without disruption of maternal hormone balance, but similar increases during use of oral contraceptives may not be desirable.

Most hormones disappear from the circulation soon after they are secreted. They are taken up by "target organs," liver, lung, and kidney, among others. Half-lives within the circulation vary, but most are measured in minutes. There is no predictable relationship between time of retention in the blood and duration of hormone action.

Liver enzymes inactivate most hormones by oxidation, reduction, deamination, and methylation. Hormones are also conjugated in the liver with glucuronic acid or sulfate; such reactions usually increase water solubility and facilitate renal excretion. Both free and conjugated hormones are passed into the bile, from which they may be lost in the feces or reabsorbed into the blood.

Some hormones are activated by metabolic transformations within target organs; but other reactions within these structures inactivate hormones and limit their duration of effectiveness.

2. Regulation of Hormone Secretion

THE NEED FOR A VARIETY OF CONTROL MECHANISMS

Thyroxine is used in *fairly constant amounts* over *long periods* of time, but un-hurried adjustments must be made to changes in the environment and to changes in living patterns (e.g., periods of sexual activity, lactation, or hibernation). Mechanisms are required to maintain a balance between hormone secretion and degradation for *steady states*, and provisions must be made for *resetting* the system when conditions change. Adjustments can be made slowly since small variations in plasma concentrations of the hormone do not threaten survival.

Norepinephrine is needed in small quantities most of the time, but *large reserves must be readily recruitable* for special purposes, and mechanisms are also required for rapid termination of the actions when appropriate.

Insulin levels must be continuously adjusted to metabolic functions and to quality, quantity, and timing of food intake. Small deficiencies can be tolerated for short periods (with some impairment of body functions); but sudden excesses can be fatal. The quantity of insulin secreted today may be very different from what will be needed tomorrow.

Parathyroid hormone is charged with the responsibility of monitoring a vital function. *Moment-to-moment control* is essential.

Hormones regulating some functions of seasonal breeders are needed in large quantities for certain periods but may not be needed at all at other times. Both timing and quantity of secretion must be controlled, and the mechanisms must be geared to external cues.

It becomes obvious that no one type of regulatory system can solve the many different kinds of problems. Representative mechanisms are described below; additional details are presented in sections dealing with the specific hormones.

REGULATION OF THYROID SECRETION

The unstimulated thyroid gland synthesizes, stores, and releases thyroxine (also

known as T₄ since each molecule contains 4 iodine atoms); but amounts secreted are insufficient for metabolic needs.

Proper function requires tonic stimulation by a hormone of the adenohypophysis (pituitary gland). The thyroid-stimulating hormone (TSH, thyrotrophin) promotes growth of the thyroid gland and T_4 secretion.

High plasma concentrations of T₄ reach the TSH-secreting cells and exert an inhibitory influence. Therefore the system is *self-limiting*. Relationships between the thyroid and pituitary described thus far are summarized in Figure 2-1.

Analogies have been drawn between the regulatory system and thermostatic control of room temperature. A furnace (thyroid gland) supplies heat (T_4); when the temperature of the room rises excessively (accumulation of T_4 in the plasma), the thermostat (pituitary gland) is affected, a circuit is broken, and the furnace is shut off. The room then cools, and the furnace is reactivated. The thermostat is also affected by other sources of heat (e.g., a fireplace); similarly, the pituitary gland is affected by other sources of T_4 , e.g., if the hormone is exogenously administered.

There are obvious differences between

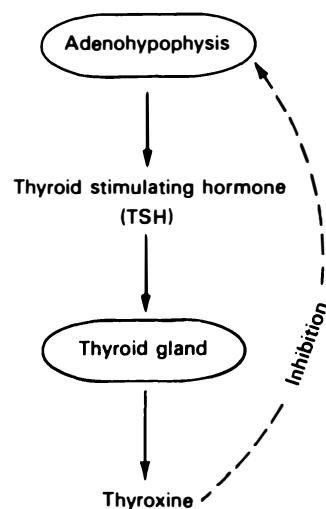


FIG. 2-1. Interaction of the adenohypophysis and thyroid gland in regulation of thyroxine secretion.

REGULATION OF HORMONE SECRETION

the two systems, but the analogy is worth pursuing. It is necessary to have a way to reset the thermostat, for example when the humidity changes or when the room residents go from rest to vigorous activity. Similarly, the thyroid hormone setting must be adjusted to changing needs.

The resetting mechanism for the pituitary gland is located within specialized cells of the hypothalamus. The latter secrete *thyrotrophin release factor* (TRF, thyrotrophin-releasing hormone, TRH, thyroid-stimulating hormone release factor) which exerts a tonic influence on the pituitary cells, promotes TSH secretion, and alters sensitivity of the cells to plasma T_4 . High plasma concentrations of T_4 inhibit the hypothalamic cells. High concentrations of TSH and of TRF can also inhibit the hypothalamic cells. Interrelationships between the hypothalamus, pituitary, and thyroid glands are summarized in Figure 2-2.

Hypothalamic control provides for adjustment of thyroxine levels to changing metabolic needs. But information must be fed into the hypothalamus from a variety of sources including the higher centers of the brain (just as an individual resetting the thermostat may use several kinds of information to determine the suitability of the setting). The higher centers are in turn affected by the levels of thyroxine in the circulating blood. A more complete picture of thyroxine regulation is shown in Figure 2-3.

The described control system provides for effective regulation of a hormone that directly or indirectly influences every organ system of the body. The numerous components with their checks and balances ensure *control within narrow limits*. But the adjustments are *time consuming* since they depend upon changes in secretion of one hormone which must in turn affect secretion of a second and third hormone. They are suitable for control of thyroid function in which fairly large changes from one steady state to another can be made gradually. (A deficiency or excess of as much as 15 or 20 per cent of the optimum persisting for a period of weeks does not threaten survival.) Although the actions of thyroxine are of long duration, there is no critical need for a specific hormone which antagonizes the actions.

The described *negative feedback control*

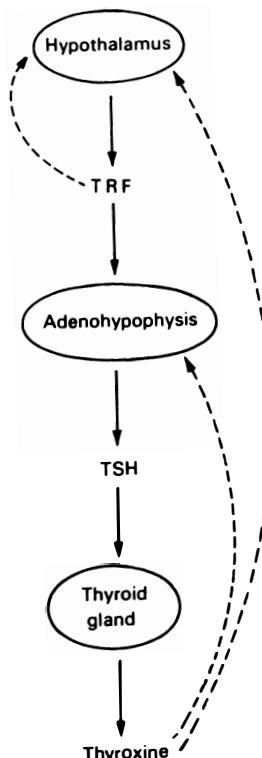


FIG. 2-2. Interrelationships among the hypothalamus, adenohypophysis, and thyroid gland contributing to regulation of thyroxine secretion. Solid arrows, stimulation; broken arrows, inhibition.

system is found in several parts of the endocrine system. Additional examples will be found in sections on the adrenal cortex and testes.

REGULATION OF PARATHYROID HORMONE SECRETION

By sharp contrast, very different mechanisms are required for regulation of parathyroid gland function. The parathyroid hormone acts rapidly on kidney and more slowly on bone to elevate calcium ion concentrations of the blood plasma. A very brief period of calcium ion deficiency leads to heightened neuromuscular excitability which is manifested first in exaggerated reflex responses, but is soon followed in rapid succession by involuntary tremors, muscle spasms, convulsions, and finally

NATURE AND FUNCTION OF HORMONES

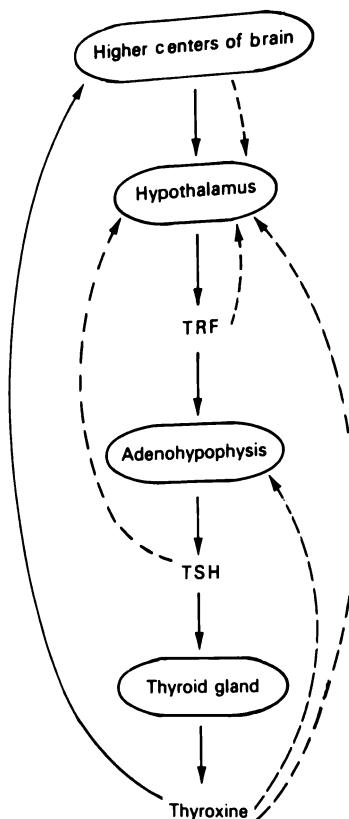


FIG. 2-3. Hormonal control of thyroxine secretion. Solid arrows, stimulation; broken arrows, inhibition.

death because of spasm of the musculature of larynx and diaphragm. Excess calcium in the blood also threatens life. A sudden influx of calcium ions into the circulating blood can quickly arrest the heart in systole, while excessive calcium levels of smaller magnitude maintained over a period of time lead to metastatic calcification especially of the kidney tubules and the smooth muscle of the arterioles. Clearly, a control mechanism is needed which cannot wait for the intervention of several intermediate hormones.

Cells of the parathyroid gland are directly sensitive to changes in the calcium ion concentration of the blood. They respond rapidly to a fall in calcium ion by

release of parathyroid hormone. Within moments, renal excretion of inorganic phosphate is increased, with the result that some of the plasma calcium previously associated with phosphate becomes available as free calcium ion, and renal loss of calcium may be promptly diminished. Parathyroid hormone also acts more slowly to release calcium from bone reserves, and continued calcium deficiency stimulates parathyroid hormone synthesis. High levels of calcium ion in the circulating blood exert an inhibitory influence on cells of the parathyroid gland. The control mechanism is summarized in Figure 2-4.

A second hormone, *calcitonin* (CT, thyrocalcitonin), produced in the thyroid glands of mammals by cells not involved in thyroxine secretion (and in the ultimobranchial bodies of other vertebrates), has been implicated in protection against an excessive rise in the calcium ion concentration of the blood plasma. Its secretion is triggered by the *calcium rise*. CT may be needed under special circumstances (e.g., during absorption of a calcium-rich meal following a period of fasting) because the inhibitory influence of calcium ions on the secretion of parathyroid hormone may not exert sufficiently rapid influences on blood concentrations of the ion. CT favors retention of calcium and phosphate in bone, an influence which is effectively (but not di-

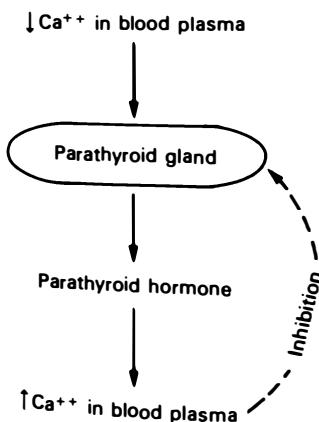


FIG. 2-4. Regulation of parathyroid hormone secretion by calcium ion concentration of the blood plasma.

rectly) antagonistic to the actions of parathyroid hormone.

Additional details on regulation of calcium ion concentrations not directly relevant to this discussion are presented in Section IV.

HORMONES AFFECTING BLOOD GLUCOSE CONCENTRATIONS

Several other hormones are regulated by small, nonhormonal molecules in the blood plasma. When blood glucose levels fall below optimal concentrations, the α -cells of the pancreas secrete glucagon, which rapidly promotes glucose formation from liver glycogen. When the blood sugar rises, the β -cells of the pancreas increase their secretion of insulin. In addition to its many other actions (described in Section II), insulin increases entry of glucose into skeletal muscle cells and thereby lowers glucose concentrations in the blood. The insulin response is especially rapid during intestinal absorption of a carbohydrate-rich meal. But insulin controls a whole spectrum of metabolic activities, and its release can be triggered by other stimuli, including a rise in blood concentrations of certain amino acids.

NEUROENDOCRINE MECHANISMS

Most endocrine glands respond to chemical messages (hormones, inorganic ions, foods, and metabolites) arriving via the circulatory system. Versatility of the sys-

tem is greatly enhanced by *neuroendocrine reflexes* which provide for rapid responses to stimuli arising in the external environment and in structures which do not secrete hormones.

Reduced to its simplest terms, the neuroendocrine reflex requires a *receptor*, a *nerve pathway*, and an *endocrine gland* (Fig. 2-5A). It will be appreciated that the *direct stimulus* to the endocrine gland is still a *chemical messenger* but is now a *neurotransmitter*.

Neurosecretory cells may be directly sensitive to chemical or osmotic stimuli. Hormone synthesized within the cell body is passed down the long axon to be released at the terminals (Fig. 2-5B).

A few examples of neuroendocrine reflexes are cited below, and others will be encountered throughout the text.

Specialized cells within the hypothalamus respond to elevation of the osmotic pressure of the blood plasma or a rise in sodium ion concentration; this leads to release from the neurohypophysis ("posterior pituitary") of *antidiuretic hormone* (ADH). The latter travels through the blood to the kidney where it promotes *water conservation* and thereby corrects disturbances in plasma osmotic pressure and sodium concentration.

But the same hormone is needed when blood *volume* is reduced without changes in ion concentrations, e.g., after hemorrhage. In such cases, volume and pressure receptors located within the vascular sys-

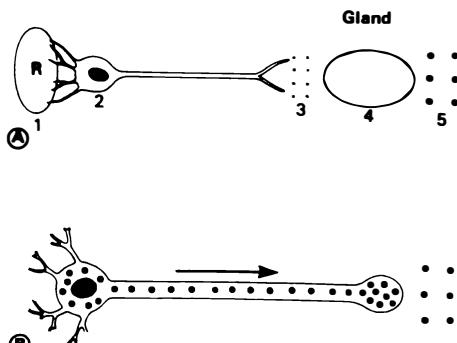


FIG. 2-5. A. Simple neuroendocrine reflex. 1. receptor; 2. neuron; 3. neurotransmitter; 4. endocrine gland; 5. hormone. B. Neurosecretory cell. The cell may be directly stimulated at dendrite; hormone is sent down the axon for release.

tem provide the signal for ADH release. Thus, neural connections provide for appropriate release of one hormone in response to diverse stimuli arising in different locations.

Versatility of the system is still further increased because stimulation of the same vascular receptors activates the *renin-angiotensin system* (Section III). Angiotensin affects hypothalamic receptors which promote drinking; it acts directly on smooth muscle of arterioles and thereby favors redistribution of the limited blood supply so that vital centers continue to function; and it stimulates secretion of *aldosterone*, an adrenocortical steroid that enhances salt and water conservation. Neuroendocrine responses to hemorrhage are summarized in Figure 2-6, A and B.

Hormonal responses to *environmental* stimuli and to sensations arising at the body surface also depend upon neuroendocrine mechanisms. The vasodilation and sweating needed for survival in warm environments require increased circulating blood volume; and temperature sensations relayed to ADH-secreting cells promote water conservation.

CONTROL OF GLUCOCORTICOID SECRETION

Mechanisms for regulation of glucocorticoid secretion analogous to those described for thyroxine are summarized in Figure 2-7.

There are quantitative differences between the two systems. Thus, influences of glucocorticoids on hypothalamic cells are relatively greater than those of thyroxine, while influences of the steroid directly on pituitary gland cells seem to be of lesser importance.

But there are times when glucocorticoid secretion must be rapidly changed. The hormone is required for adjustments to stresses of various kinds (Section II), and noxious stimuli rapidly promote neuroendocrine release of hypothalamic corticotropin-releasing factor (CRF).

Glucocorticoid secretion must also be adjusted to metabolic needs since it plays a key role in regulation of blood glucose concentrations and in adjustments to timing of activity, rest, and food intake. In animals active during daylight hours, plasma concentrations of glucocorticoids

fall during the night and rise rapidly in the early morning, to fall again in the late afternoon. In nocturnal animals such as the rat, concentrations rise in the late afternoon shortly before the usual time of feeding. Major control mechanisms for glucocorticoid rhythms reside in the hypothalamus and are described in Sections II and VII. The rhythms can be slowly adjusted to changes in light-dark schedules; but lags in adjustment can cause difficulties, e.g., in individuals taking long distance east-west airplane flights.

HORMONES REGULATING REPRODUCTION AND LACTATION³¹

Reproduction and lactation are especially dependent upon neuroendocrine reflexes described in Sections VI and VII.

Ovarian cycles require sequential events in which oocytes and ovarian follicles develop before release of ovulation hormones; and these events must be coordinated with preparation of the uterine lining for implantation. In cats, rabbits, and some other mammals, secretion of ovulation-inducing hormones is triggered by stimuli presented during mating; this significantly increases the probability that fertilization will occur, and it extends the period during which the female is sexually receptive.

Survival of some seasonally breeding species depends upon coordination of reproductive functions with changes in the external environment. Mating then occurs at times which lead to birth of the young when conditions such as food and water supply and environmental temperatures are most favorable.

Suckling of the young sets up reflexes which provide for rapid release of pre-formed milk and for synthesis of milk that will be required for the next feeding. Additional mechanisms reduce the chances that fertilization of new ova and embryonic development will take place when energies of the mother are required for care of existing young.

In addition to regulatory mechanisms described earlier in this chapter, the reproductive system utilizes *feedforward* or *positive feedback controls*. For example, pituitary gonadotrophins promote maturation of ovarian follicles and secretion of sex steroids. Low concentrations of the latter promote further secretion of the pituitary

REGULATION OF HORMONE SECRETION

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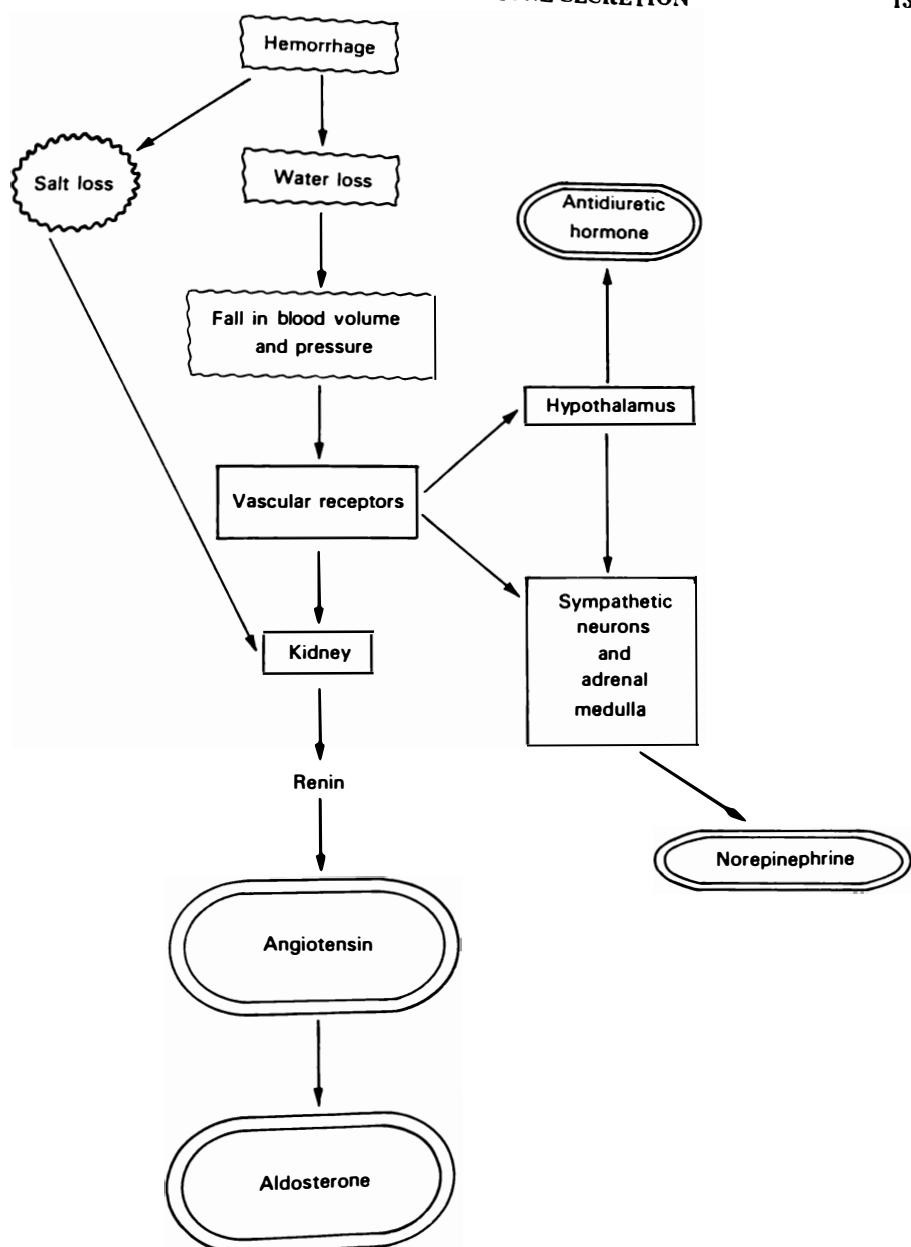


FIG. 2-6. A

FIG. 2-6. A. Release of antidiuretic hormone, norepinephrine, angiotensin, and aldosterone in response to hemorrhage. B. Consequences of changes shown in part A.

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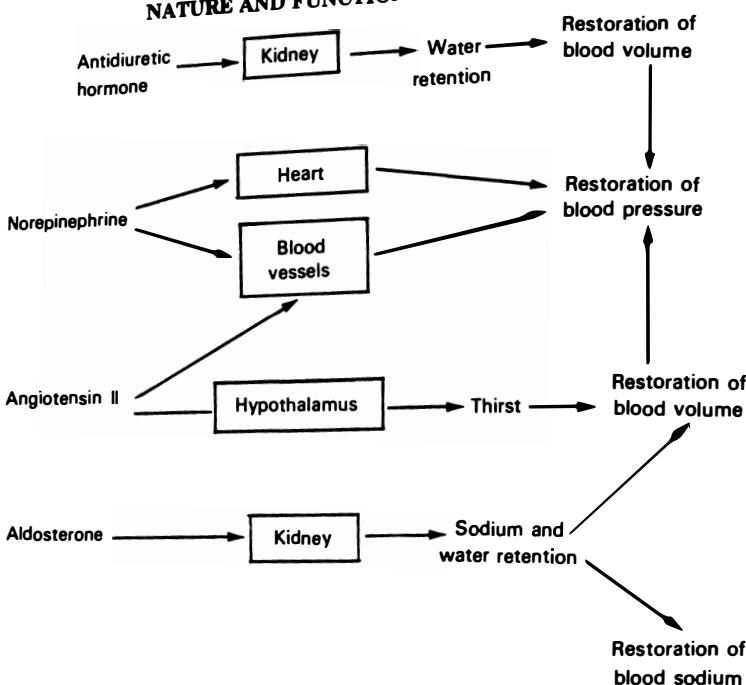


FIG. 2-6. B

hormones, while high concentrations exert inhibitory influences.

DIRECT NERVOUS CONTROL OF THE ENDOCRINE SYSTEM

Certain endocrine glands, e.g., the adrenal medulla and hypothalamus, are directly controlled by the nervous system. Recent research suggests that control of other endocrine structures depends more heavily upon direct innervation than was previously suspected. The fact that an endocrine gland can function when transplanted to another site within the same organism (and, therefore, presumably after complete deprivation of innervation) is no longer accepted as evidence that nervous control is unimportant in the intact organism.

There has been a recent surge of interest in possible roles of the *prostaglandins* in mediation of neuroendocrine reflexes. It is known that prostaglandin release can be triggered by nerve stimulation, and prostaglandins have been directly implicated in mechanisms for secretion of a large number of hormones.

It should be noted that the *quantity* of hormone secreted is only one factor determining the level of hormone *activity*. Other factors include the availability of *specific hormone receptors* (also under hormonal control), *rate of transformation* of the secreted hormone into more active agents, *rate of degradation*, and secretion of *other hormones* whose action is antagonistic or synergistic.

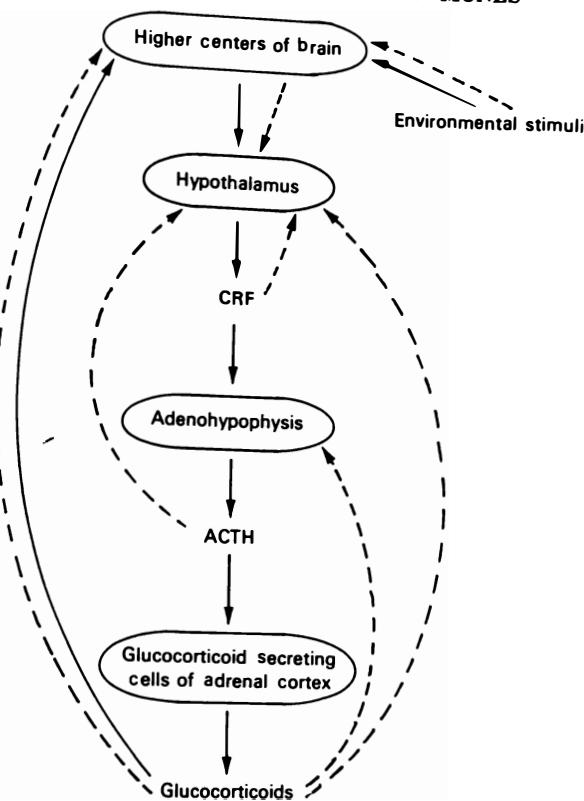


FIG. 2-7. Regulation of glucocorticoid hormone secretion. Solid arrows, stimulation; broken arrows, inhibition; ACTH, adrenocorticotrophic hormone.

3. Mechanism of Action of Hormones

EXAMINATION OF SOME OF THE PROBLEMS INVOLVED IN DETERMINATION OF THE MECHANISM OF ACTION OF HORMONES

In all of endocrinology, the question most frequently posed and least satisfactorily answered may well be, "How do hormones bring about their observed effects?" It is one thing to administer hormones (or to remove an endocrine gland) and describe the obvious results. Countless thousands of carefully controlled experiments have yielded reproducible data. But it is

something else to deduce from such studies, information on the mechanism of action. Decades of work by investigators all over the world (much of it involving ingeniously designed experiments and creative interpretations) have yielded valuable information and fertile concepts; yet, so much remains to be learned.

The "Cascade Phenomenon"

Hormones seem to initiate a *series of interdependent changes* in cellular metabolism, and it is extremely difficult to

sort out *primary* from *secondary*, *tertiary*, and *higher order* events. It may also be difficult to distinguish between effects directly related to and *essential* for hormonal action and those which are merely *coincidental*. Comparison of the small quantities of hormones needed with the magnitude of changes induced is consistent with a "cascade" phenomenon, in which each step of a reaction promotes a succeeding change of greater magnitude. The concept is especially appealing in the case of hormones believed to exert their primary action at some point on the plasma membrane. (The possibility that hormones move rapidly about, thereby acting at multiple sites in rapid succession, has not been completely ruled out but seems less consistent with available data.)

The Latent Period

For most of the hormones there is an obvious "latent period" or *lag* of characteristic duration between the time the hormone is presented to responsive cells and the first appearance of objectively measurable effects. During this time "something" is happening which thus far has eluded understanding. In most cases, thorough washing to remove all traces of the hormone from the surrounding fluids early in the latent period does not interfere with changes which can be readily measured at a later time.

The first event in the cascade may involve changes of such small magnitude they cannot be detected with available techniques; but one is left with the nagging possibility that investigators have failed to ask the appropriate questions. In recent years improved insight and more sensitive methods have made it feasible to uncover earlier and earlier events; more often than not, this has left the investigator with the problem of confronting a merely shortened latent period.

To cite an extreme example, if one measures the influence of thyroxine on elevation of basal metabolic rate, the latent period is measured in days (or even weeks, if maximal effects are studied); but the effects of thyroxine on the *in vitro* incorporation of amino acids into proteins can be demonstrated within hours, while the latent period for thyroxine stimulation of rapidly labeled nuclear ribonucleic acid

(RNA) has a latent period measurable in minutes. The problem of just how thyroxine stimulates synthesis of nuclear RNA remains unsolved, and links between this event and elevation of metabolic rate are just beginning to unfold (Section V).

Indirect methods employing relatively specific *inhibitors* have been useful for making judgment concerning what might be happening during the latent period. If a characteristic hormone effect is consistently blocked by prior administration of *actinomycin D* (which interferes with deoxyribonucleic acid (DNA)-directed synthesis of RNA), there is reason to suspect that the hormone influences synthesis of specific RNAs and that the latter leads to formation of one or more specific proteins which mediate hormone action.

If the effects of the hormone are not influenced by *actinomycin D* but can be impaired by administration of *inhibitors of protein synthesis*, the hormone is suspected of influencing protein synthesis via different mechanisms (e.g., through actions on the ribosomes). The experiment is more convincing if two or more inhibitors known to act at different loci have similar influences. *Puromycin* and *cycloheximide* have been widely used since they inhibit protein synthesis in different ways.

Data from such studies must be interpreted with caution. If the *primary action* of a hormone is exerted, for example, on the plasma membrane or on conformation of preexisting enzymes but *expression* of the primary event requires that the cell continue to produce certain short-lived proteins, the inhibitor may block the end result.

It is not always possible to distinguish between impaired production of a *specific protein* and more generalized disruption of cell metabolism. In whole animal studies it is necessary to be wary of agents which seriously affect general health and food intake. (Large doses of antibiotics can do this.) Moreover, the assumption that a pharmacological agent acts *only* on the pathways that the investigator chooses to inhibit is often not warranted. It is known, e.g., that puromycin can affect cAMP metabolism and that actinomycin D influences iodide uptake by thyroid gland cells.

Many hormones effectively increase the activity of a specific enzyme during the latent

period. Before it can be concluded that action of the hormone depends upon *induction* of new enzyme, it must be shown that (1) the amount of enzyme is actually increased after administration of the hormone; this can sometimes be done by addition of highly specific antibodies to the enzyme; (2) any agent which increases the amount of that enzyme should mimic hormone action (provided of course that the agent does not also block effects of the enzyme); and (3) increased activity of the enzyme in question can be directly related to hormone functions.

Whole Animal vs. *in Vitro* Studies

In the whole animal, hormones interact with numerous regulators present within cells and extracellular fluids; and changes in concentrations of one hormone can lead rapidly to compensatory changes in others. For many purposes it seems simpler to work with isolated organs, tissues, cells, or cell components.

There is uncertainty concerning conditions which must be maintained *in vitro*. Protein buffers present in physiological solutions are often avoided because they are unstable, adsorb to important molecules, or aggregate and gum up narrow tubing. But nonphysiological buffers can produce unpredictable interferences with enzymatic reactions.

It is not always possible (and certainly never easy) to keep *in vitro* preparations in good condition for long term studies and to provide for such things as adequate (but not uneven or excessive) oxygenation or a "physiological" rate of removal of cell products. It may be difficult to determine optimal concentrations of all cofactors required.

The problem of how much hormone to present to excised tissues or cells is multifaceted. The temptation to use high concentrations stems from uncertainties about rates of penetration, hormone degradation, and nonspecific binding, and also from the understandable wish to avoid wasting time gathering negative data. But high concentrations may elicit purely pharmacological actions and may gain access to sites unavailable *in vivo*.

Even when dosages within the "physiological range" are employed, there is the problem of biphasic actions (one at lower physiological dose limits and a very different one at higher concentrations).

Effects observed *in vitro* may be quite different from those seen when the same hormone is given to the intact animal. For example, prostaglandin F_{2α} tends to reduce progesterone secretion *in vivo* because it interferes with blood flow to the corpus luteum; but it also directly promotes steroid synthesis, and only this action will be seen *in vitro*. Some hormone actions can only be elicited from intact animals because the

hormones require metabolic transformations by "nontarget organs" or because observed effects depend upon a complicated sequence of events which cannot be accomplished *in vitro*. In some cases it becomes necessary to administer the hormone to the whole animal and to later excise the tissues for *in vitro* study.

Multiple Actions of Hormones

Insulin promotes glucose uptake by skeletal muscle and adipose tissue cells; but there are reasons to believe that the mechanisms differ. Insulin does not regulate glucose uptake by the liver, yet it stimulates glucose use for glycogen synthesis in both liver and muscle. One effect of insulin on glucose uptake by muscle is rapid; but the same hormone induces long-range adaptive changes in the same process. Glucose uptake is *passive* (along the concentration gradient); but insulin stimulates active transport of amino acids in the same tissue.

The problems of determining just what the hormone is doing are by no means unique for insulin. Glucocorticoids increase the rates of glycine uptake and incorporation into proteins in hepatic cells but decrease both processes in the thymus gland. Thyroxine exerts stimulatory actions on most body tissues but inhibits activity of thyrotrophs. Growth hormone can promote early increases in glucose uptake and later decreases within the same tissue.

It is difficult when interpreting data or selecting working hypotheses to choose between the concepts of *multiple actions* of a single hormone and those invoking some common denominator which finds different expression at different times or in diverse tissues. Perhaps the concept that some hormones act as "messengers" while others function as "maintenance engineers"²⁴ will be useful in sorting out actions; but the same hormone may function in both capacities at different times or in different cells.

A hormone may also exert seemingly opposing actions in closely related species. Prolactin reduces osmotic influx of water across the gills of stickleback fish, apparently by reducing permeability.⁴¹ A similar mechanism has been described for the eel. But an opposite effect of prolactin on water permeability is found in the goldfish. To understand such findings, the endocrinolo-

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gist must know as much about the tissues with which he works as he does about the hormone. A hormone may also exert the same qualitative actions on different species, but the quantitative importance of the action can differ markedly. Pancreatectomy in carnivores leads rapidly to hyperglycemia; in ruminants, the loss of insulin is of much lesser importance.²⁷ Pancreatectomy in birds usually leads to hypoglycemia because the α -cells of birds seem to be more essential to metabolic balance than the beta cells.

Permissive Actions

It has frequently been stated that certain hormones play a "permissive role"; i.e., they do not directly affect reactions but provide conditions which favor the action of other hormones. The smooth muscles of arterioles lose their sensitivity to norepinephrine when there has been a prolonged deficiency of adrenocortical hormones. Administration of glucocorticoids restores the sensitivity. Glucocorticoids have a relatively small direct influence on muscle tone, and no dose-response relationship is seen; but they provide conditions under which a dose-related response to norepinephrine is readily demonstrated. The phenomenon is illustrated in Figure 3-1. Similar influences of glucocorticoids on catecholamine-induced lipolysis in adipose tissue and glucagon-directed hepatic gluconeogenesis have been described.

Thyroxine markedly enhances cardiac responses to epinephrine. Some aspects of the interaction are better classified as synergism or potentiation (see below), although the term permissive has been applied by some authors. The need for pretreatment of tissues with estrogens to elicit some responses to progesterone has been cited as an example of permissive action of estrogens.

It has been stated that the term "permissive action" is nothing more than a catch-all designed to coat a specific body of ignorance with an aura of pseudo-understanding. This seems reasonable, since unfolding of new information often provides insights.

For example, actions of epinephrine have been linked with stimulation of the enzyme

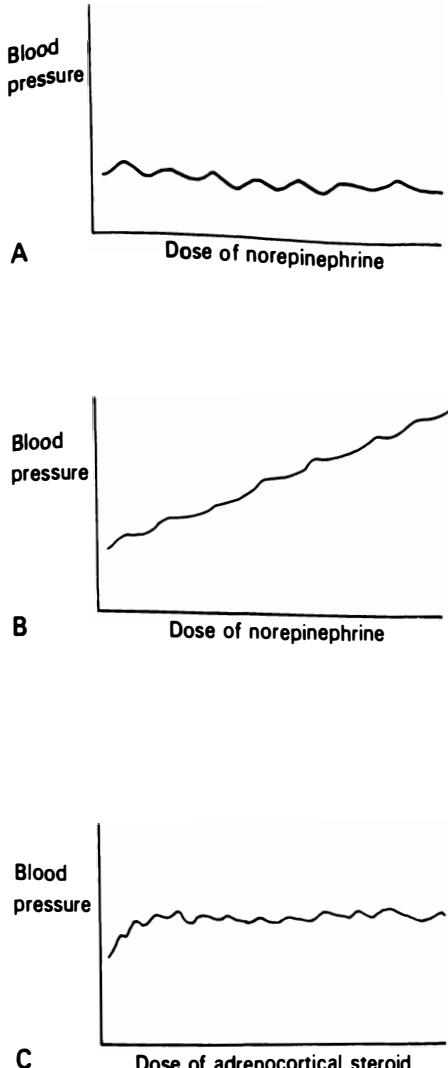


FIG. 3-1. Permissive action of adrenocortical steroids on blood pressure responses to norepinephrine. A. Response to norepinephrine when adrenocortical steroids are deficient. B. Response to norepinephrine in presence of constant level of adrenocortical steroids. C. Response to adrenocortical steroids when norepinephrine is maintained at constant level.

adenylate cyclase and consequent production of cyclic adenosine monophosphate (cAMP). There is evidence that glucocorticoids promote synthesis of proteins acted upon by cAMP, and also that they inhibit enzymatic degradation of the nucleotide.²⁸ Thyroxine can stimulate adenylate cyclase, and it also induces synthesis of

proteins involved in actions of epinephrine. Estrogens promote mitosis of cells which later respond to progesterone, and they also induce formation of progesterone receptors.¹⁰

Synergism and Potentiation¹²

These terms are applied when two (or more) hormones act in the same direction to yield a greater response than can be elicited by one hormone acting alone. Antidiuretic hormone (ADH) increases circulating blood volume by promoting renal conservation of water. Aldosterone promotes water conservation indirectly through effects on sodium transport and on distribution of water and electrolytes between cells and extracellular fluids.

If the response to hormone A is described as equivalent to *a* while the response to hormone B equals *b*, then simple summation of the two actions should yield the response *a+b*. Some authors use the term *additive* to describe effects of combined administration of such hormones; but others describe the actions as *synergistic*.

If the response to administration of both hormones is *greater than a+b*, then the effect of the hormone combination is called *superadditive* by some authors. But some describe the interaction as *synergistic* while others prefer the designation *potentiation*. A few apply the last term to all positive interactions between hormones.

The Importance of Timing

Further complications arise from knowledge that the time of administration of a hormone profoundly influences its actions. Thyroxine is needed early in life for maturation of brain cells; but it cannot exert such influence beyond a *critical period* in development. The finding has been related to differences in enzymes present during *thyroxine-sensitive* as compared with *thyroxine-insensitive* stages.

Prolactin injections administered at midday to white-crowned sparrows promote weight gain and fat storage, whereas injections of the same hormone early in the morning induce fat depletion.¹³ Levels of other hormones present at the time prolactin is given influence the responses.

SITES OF HORMONAL ACTION: EXAMINATION OF THE POSSIBILITIES

Cellular metabolism is controlled by enzymes, and it is reasonable to look for influences of hormones on enzymes controlling rate-limiting reactions. The half-lives of enzymes differ markedly; some are conveniently measured in seconds, others in minutes, and still others in days or even weeks. Hormones have been most often associated with enzymes that have *rapid turnover rates*.

The activity of an enzyme at any given moment depends upon:

1. The *absolute quantity* of enzyme present; this in turn depends upon the balance between synthesis and degradation and is therefore influenced by nucleic acid synthesis, utilization and degradation, ribosomal functions, availability of building blocks (amino acids) and energy sources, and the amount and location of degrading enzymes.

2. The *configuration and chemical state* of the enzyme, including such factors as *aggregation*, association with other cellular components, and in the case of many key enzymes whether it is *phosphorylated* or *dephosphorylated*. The configuration influences not only the activity of the enzyme but also its susceptibility to degradation. Many rate-limiting enzymes can exist in inactive form and are readily activated by hormones or metabolites. The associated processes often involve other enzymes as well as cofactors.

3. The *location* of the enzyme within the cell. This influences access to substrate. Enzymes enclosed within lysosomal membranes are sequestered and therefore temporarily rendered inactive; many different cellular factors affect formation and disruption of lysosomal and other membranes.

4. The *microenvironment* of the enzyme. This includes not only the availability of activators, cofactors, inhibitors, etc., and the presence of substrate (all of which can be affected by permeability of plasma and other membranes), but also factors which may have indirect influences, e.g., hydrogen and other ion contents of the cell.

It is usually extremely difficult to pinpoint a "primary" action of a hormone. Often the first effect induces a change of a magnitude too small to be detected by available methods. The smallest perturbation of cell metabolism leads rapidly to secondary, tertiary, and higher order changes. The product of one reaction can serve as the substrate, inhibitor, activator, cofactor,

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or even enzyme for the next reaction, or it can influence the uptake or binding of essential cell components.

Any compilation of mechanisms for influencing enzyme activity necessarily includes duplication and overlap (as well as omission of unknown factors). Ribosomes are essential for synthesis of proteins, but protein synthesis is needed for formation and function of ribosomes. Proteins synthesized on the ribosomes direct the formation of membranes, but membrane transport provides materials used by ribosomes. The mitochondria supply ATP for all kinds of cellular processes, but ATP supports some mitochondrial functions. Nuclear functions provide information for synthesis of cell membrane components, but the membranes affect availability of molecules needed for nuclear function.

Some of the proposed sites of action of hormones are listed below.

1. Availability of substrate for enzymatic reactions via effects on
 - a. Membrane "permeability," structure, electrical properties
 - b. Quantity and distribution of carriers for specific ions or molecules
 - c. Structure, affinity of the carrier, presence of competing molecules
 - d. Intracellular redistribution
 - e. Rate of blood flow and therefore delivery of substrate
 - f. Concentration of substrate in the plasma (through influences on other cells)
 - g. Degradation of substrate
 - h. Diversion of substrate to other reactions
 - i. Direction of energy sources for active transport
2. Removal of reaction products
 - a. Cellular redistribution and sequestration
 - b. Mechanisms similar to those listed under 1
3. Alterations in activity of existing enzymes
 - a. Conformational changes, aggregation of subunits, binding to other molecules and to organelles
 - b. Availability of cofactors, inhibitors
 - c. Direct participation of the hormone in the reaction
4. Changes in the quantity of enzyme within the cell
 - a. Availability of template for formation of specific RNA molecules
 - b. Ability to bind and utilize the template, effects on RNA polymerase
 - c. Movement of RNAs, proteins from nucleus to cytoplasm

- d. Changes in number, composition, distribution, aggregation, binding of ribosomes
- e. Synthesis of membrane lipids
- f. Availability of cofactors for ribosomal functions
- g. Effects on rates of degradation of specific RNA sequences
- h. Effects on degradation of the enzyme
- i. Availability of "building blocks" for RNA, protein synthesis
- j. Effects on initiation, elongation, termination, release of enzyme proteins from the ribosomes, effects on folding of the protein, and on post-translational modification.
5. Changes in availability of energy sources
 - a. Number, size, distribution, structure of mitochondria
 - b. Availability of oxidizable substrate, coenzymes, enzymes
 - c. Use of energy by other reactions
 - d. Availability of oxygen
6. Long range influences on cell number
 - a. Influences on DNA synthesis, microtubular functions
 - b. Changes in cell differentiation linked to cell division

ACTIONS OF HORMONES ON MEMBRANES

Entry of Hormones into the Cell

There are good reasons to believe that *peptide* and *protein* hormones do not penetrate into the cell interior. Mechanisms for entry of large protein molecules are difficult to conceptualize. Micropinocytosis is possible, but there is little support for the idea that hormones are characteristically taken up in this way.

The belief that protein hormones exert their influences at *cell surfaces* is strengthened by several kinds of observations. While the conclusiveness of evidence obtained from each kind of experiment has been questioned, the collective data present a strong case, and few investigators doubt that protein hormones act primarily on membranes.

Radioactively labeled hormones have been observed to bind to specific components of *cell membranes* under conditions when radioactivity is not detectable inside the cell. (But the possibility that some hormone enters the cell after separation from its radioactive marker is difficult to rule out.)

Intact cell membranes are required for expression of many hormone actions. When the

membranes are disrupted but all cell components are retained, many characteristic hormonal actions cannot be elicited. (But the problems of artificial redistribution of enzymes, cofactors, inhibitors, binders, etc. are difficult to evaluate.)

Influences of the hormone on cell metabolism have often been shown to be preceded or accompanied by objectively measurable changes in the cell membrane (hyperpolarization, depolarization, increased rate of ion uptake, etc.). Pharmacological agents known to exert similar actions on the membrane may imitate hormone effects, while those causing changes opposite in direction may inhibit.

Protein hormones have been covalently linked to very high molecular weight polymers which cannot enter the cell, and they still exert hormonal actions. In some cases it has been demonstrated that the hormone effects can be blocked by addition of specific antibodies to the hormone, and it is reasonable to believe that the antibody combines with the hormone extracellularly.

By contrast, there is very strong evidence that most actions of steroid hormones require penetration into the cell interior prior to induction of hormonal influences.

Effects of Hormones on Plasma Membrane Configuration

Direct Effects on Permeability. Hormones could theoretically act directly on the plasma membrane to induce conformational changes and thereby affect "pore" size. Admittedly, no one has ever seen the pores, much less the change in pore size in response to a hormone. But indirect methods are available for measurement of diffusion rates of small molecules in the absence and presence of hormones, and the information can be compared with calculated changes that would be consistent with a "pore" theory.

Detailed suggestions have been presented of mechanisms whereby interaction of the hormone with some membrane component could directly affect cell permeability. While a few hormones could conceivably act in such ways (e.g., parathyroid hormone which very rapidly promotes calcium uptake), most regulators affecting membrane transport must do so indirectly.

Insulin increases the rate of uptake of glucose, galactose, and some other sugars

by skeletal muscle cells. Explanations are needed for failure to affect uptake of closely related molecules, including stereoisomers of glucose and some smaller pentose sugars. ADH increases passive uptake of water in the kidney; the uptake is at the mucosal surface, whereas the hormone is effective only when presented to the opposite (serosal) side of the cells.

Hormonal Alteration of Cell Membrane Charges.⁷⁰ Insulin influences on glucose uptake are associated with membrane hyperpolarization and movement of inorganic ions. Several other hormones depolarize membranes. In some cases hormone effects can be mimicked by chemically unrelated agents which exert similar changes in membrane polarity.

Secondary Influences. Passive movement of small molecules or ions through the membrane can be accelerated by a variety of mechanisms initiated within the cell interior. Actions of aldosterone on sodium transport^{21, 57} (Section III) clearly depend upon synthesis of new protein, while ADH stimulation of water transport has been linked with production of cAMP.

Insulin-sensitive cells possess an ATP-dependent "barrier" which resists inward diffusion of glucose. It has been suggested that insulin effects on glucose uptake depend upon diversion of ATP away from membrane sites.

Hormones can accelerate efflux as well as influx of small molecules. In some cases this seems to be directly dependent upon elevation of cytosol concentrations of the extruded substances. Hormones can also inhibit uptake; this is usually associated with synthesis of specific new molecules that affect the plasma membrane.

The same hormone may promote influx of some molecules and efflux of others. Thyroid-stimulating hormone promotes sodium, glucose, and iodide uptake by thyroid cells, although it promotes thyroxine release. Parathyroid hormone enhances renal phosphate excretion, but it promotes calcium retention. In many cases, different sites of action can be demonstrated.

Interactions between hormones can be complex. Although epinephrine, somatotrophin, and glucocorticoids elevate blood glucose concentrations (thereby increasing the gradient for passive entry), and epinephrine also enhances blood flow through skeletal muscle, all three may (under certain physiological conditions) actually decrease net glucose uptake.

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Effects of Hormones on Transport Carriers

Specific carriers for small molecules have thus far eluded attempts at isolation and purification; but strong evidence for their existence derives from kinetic studies, and from studies of mutant forms of microorganisms exhibiting defects in transport mechanisms.

Although glucose enters most cells down a steep concentration gradient, its transport is carrier-mediated. Insulin has been implicated in both activation of an existing carrier and in induction of its synthesis.

A provocative suggestion for which evidence has been presented is that a hexokinase isozyme functions as the glucose carrier (Section II).

Influences of Hormones on Active Transport Mechanisms

Hormones may influence synthesis, location, or activation of carriers needed for active transport. They have also been implicated in direct stimulation of existing cellular "pumps," in synthesis of new pump proteins and lipids, and in mechanisms leading to increased passive entry at one cell surface of molecules that are actively extruded at the opposing surface. Some affect ATP availability.

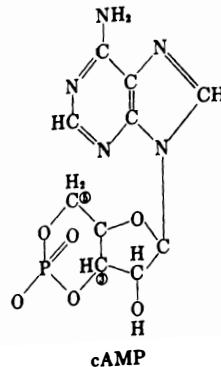
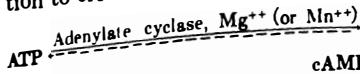
THE SECOND MESSENGER HYPOTHESIS^{4, 26, 28, 51, 56}

Numerous references are made throughout the text to influences of hormones on the membrane-bound enzyme which catalyzes formation of cyclic 3',5'-adenosine monophosphate (cAMP). The enzyme was originally named adenyl cyclase, but the term "adenylate cyclase" is now more commonly used.

Statement of the Hypothesis

It is believed that actions of a very large number of hormones are initiated by combination of the hormone with a specific receptor on the plasma membrane, and that formation of the hormone-receptor complex leads to activation of adenylate cyclase. The enzyme catalyzes synthesis of cAMP, and the latter mediates subsequent actions of the hormone. The hormone is said to be the "first messenger" and cAMP the "second messenger" for cell activation.

The reaction utilizes ATP, requires magnesium (or more rarely manganese) as a cofactor, and yields pyrophosphate in addition to cAMP:



The hypothesis does not require that all actions of a hormone that elevates cAMP concentrations be mediated by the nucleotide (and there are good reasons to believe that some are not); nor does it require that adenylate cyclase be sensitive only to hormones.

But it does require that the hormone elevate cAMP concentrations before actions attributed to the nucleotide are manifested, and that increased cAMP concentrations form an *essential link* in the train of events. Moreover, any agent which activates adenylate cyclase should mimic hormone actions (provided it does not also interfere with later events).

Only those hormones capable of *binding with the specific receptor* can be expected to elevate cAMP concentrations in a particular type of cell. (Thus, glucagon will elevate cAMP concentrations in the liver because it combines with hepatic cell receptors; but thyroid-stimulating hormone does not affect liver adenyl cyclase, although it stimulates the related enzyme in cells of the thyroid gland.) The *effects* of elevation of cAMP depend on the kind of cell stimulated. (cAMP increases glycogenolysis in the liver but promotes release of thyroid hormones from the follicular cells of the thyroid gland.)

Some cells may have more than one type of receptor. Adipose tissue cells can be activated by norepinephrine, certain "lipolytic hor-

mones," and very high concentrations of thyroid-stimulating hormone and of adrenocorticotrophic hormone. All of the preceding increase cAMP concentrations and promote lipolysis.

There are unresolved questions concerning whether cells contain adenylate cyclase in more than one location and whether activation at one site induces a different response than activation at another. Some tissue preparations which seemed to function in this way were later shown to contain two or more different cell types.

Activation of Adenylate Cyclase

It is likely that cells differ in mechanisms for activation of the enzyme. It is known, for example, that calcium ions are required for activation of adrenocortical adenylate cyclase but not for stimulation in several other cell types. Very high calcium concentrations inhibit the enzyme; but sensitivity to the ion varies greatly in one cell type as compared with another.

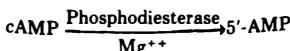
Some hormone actions may depend upon *reduction* of adenylate cyclase activity. Insulin and prostaglandins have been suspected of functioning (partly) in this way; they may also promote rapid destruction of cAMP. The second messenger hypothesis has been extended to actions of this type.

Maintenance of Intracellular Concentrations of cAMP

The cytosol concentration of cAMP at any given moment depends primarily upon the balance between synthesis and degradation. Some cell types release small amounts into the surrounding medium, and others may sequester minute quantities in a metabolically unavailable form.

Synthesis of the nucleotide is inhibited by high concentrations of pyrophosphate and calcium. Under some conditions it may be limited by ATP availability.

The major pathway for degradation is rupture of the bond between carbon 3 of the ribose moiety and the phosphate, yielding adenosine 5-monophosphate (AMP). The reaction is catalyzed by phosphodiesterase enzymes present in the cytoplasm:



Insulin and other hormones may enhance phosphodiesterase activity.

Third and Higher Order Messengers

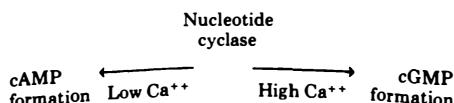
cAMP influences calcium metabolism in several ways, and many actions of cAMP depend upon such influences. It has been proposed that calcium functions as a "third messenger;" but this can lead to confusion. It has been pointed out²⁶ that the concept of multiple messengers leads to problems of semantics. Thyrotrophin release factor (a first messenger) can increase cAMP (second messenger) concentrations and thereby affect movements of calcium (the third messenger). One consequence is secretion of thyroid-stimulating hormone (the fourth messenger) which travels to the thyroid gland and stimulates its adenylate cyclase. cAMP then functions as a fifth messenger to participate in release of the sixth messenger, thyroid hormone.

Cyclic Guanosine 3',5'-Monophosphate (cyclic GMP, cGMP)^{8, 22, 50}

cGMP is formed from GTP, and the enzyme catalyzing its formation has been found in every animal tissue in which it has been sought. The distribution differs from that of adenylate cyclase; it has been found within the nuclei and also associated with microsomal elements. cGMP is degraded by cytosol phosphodiesterases and may compete with cAMP for such enzymes.

Guanylate cyclase is activated by concentrations of calcium ions high enough to inhibit activity of adenylate cyclase, and there are numerous examples of situations in which cGMP concentrations increase as those of cAMP fall.

It has been suggested that a single enzyme, *nucleotide cyclase*, catalyzes formation of cAMP after cell stimulation when the calcium concentrations are below a certain level and then "switches" substrate to promote formation of GMP when the calcium concentration rises excessively:



An arrangement of this kind could protect cells against excessive stimulation by cAMP, and it is known that some actions of cGMP are opposite in effect to those exerted by cAMP. However, the concept is not readily reconciled with differences in distribution of the enzyme in

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most cells, the fact that cGMP can prolong the life of cAMP by competing with it for the phosphodiesterases, the rise in cytosol calcium after stimulation in cells which contain high "resting" levels of cGMP, and observations that cGMP mimics some of the actions of cAMP. The possibility that cAMP itself regulates formation of cGMP must be considered.

Some kinds of observations suggest that the two nucleotides subserve very different functions, while others are consistent with interactions in regulation of the same functions. cGMP concentrations rise in response to administration of acetylcholine and related agonists and to stimulation of nerves releasing the neurotransmitter. It has been proposed that cGMP mediates cholinergic responses in heart, brain, and possibly in other tissues. Many actions of epinephrine and of sympathetic nerve stimulation are antagonistic to those elicited by cholinergic nerves. cAMP concentrations rise after stimulation of β -adrenergic receptors; but they may fall after stimulation of α -adrenergic receptors. (The receptors are described in Chapter 9.) cGMP and cAMP seem to exert antagonistic actions on inflammatory responses.

Certain observations on the endocrine system have not been adequately explained. cGMP concentrations are fairly high in unstimulated cells of the mammalian cortex, and they tend to increase after administration of very low doses of adrenocorticotrophic hormone. Very high doses of adrenocorticotrophic hormone (ACTH) provoke elevation of cAMP concentrations and depression of cGMP levels, while hypophsectomy (which removes the source of ACTH) induces prompt changes in the opposite direction. It has been proposed that cGMP mediates responses to low levels of stimulation whereas cAMP mediates responses to strong (pharmacological) stimulation. The observations have not been reconciled with the finding that the adrenal cortex of the crocodile responds to ACTH stimulation with a rise in cGMP and fall in cAMP.

Concentrations of both nucleotides rise in the urine after administration of ADH (Section III); different functions have been associated with each. Mechanisms for interaction of cAMP and cGMP may not be identical in all cell types.

Hormones Known to Influence Formation of cAMP²³

The list of hormones known to increase activity of adenylate cyclase and believed

to have actions mediated via cAMP is exceedingly long. It includes ADH, parathyroid hormone, catecholamines, thyroid-stimulating hormone, follicle-stimulating hormone, some hypothalamic release factors, gastrin, secretin, angiotensin, and histamine.

It was once believed that hormones could be conveniently divided into two groups: peptides, proteins, and certain amines which act via adenylate cyclase, and steroids which do not. Recent research suggests that some actions of steroids may also require cAMP. Steroid hormones have also been implicated in inhibition of phosphodiesterase activity, in induction of proteins which mediate responses to cAMP, and in alterations of cytosol electrolyte concentrations that affect sensitivity to cAMP.

Target tissues exhibiting responses to hormone-mediated changes in cytosol cAMP include liver, adipose tissue, kidney, bone, testis, ovary, heart, and most hormone-secreting cells.

Experimental Support for the Second Messenger Hypothesis^{24, 55}

Intracellular concentrations of cAMP are in fact elevated following administration of many hormones.

It is necessary to demonstrate that elevations of concentrations of the nucleotide are not merely coincidental to effects of hormonal stimulation but that they form an essential link in the chain of events leading to expression of hormone influences. The rise in cAMP concentrations should precede other effects and should be of sufficient magnitude to bring about the hormonal actions.

It has been possible to mimic effects of hormonal stimulation by administration of cAMP alone.

In some studies, the low rate of penetration of the nucleotide into the cells has presented problems, and better results have been obtained with N⁶ O^{2'}-dibutryl cyclic AMP (D-cAMP), and N⁶-butyryl-cAMP. The analogs penetrate more rapidly and are resistant to degradation by the phosphodiesterases. (They may even reduce the rate of degradation of endogenous cAMP.) But it is also known that hydrolases can remove the butyryl groups, and that the latter are not without influence on cell metabolism. There are also situations in which effects of cAMP and its dibutyryl analog appear to be qualitatively different.²⁵

Some hormone agonists bind specifically with hormone receptors and this leads to activation of adenylate cyclase, while specific inhibitors interfere with the activation by competing with hormones for their receptors or in other ways prevent hormone binding.

Agents which inhibit phosphodiesterases (and therefore prolong the life of cAMP) can potentiate effects of submaximal hormone dosages or increase the duration of action.

The methyl xanthines, caffeine and theophylline, have been extensively used for this purpose. But such agents also exert different actions; they can directly affect membrane permeability.

Agents chemically unrelated to hormones which stimulate adenylate cyclase may elicit hormone-like actions.

Cholera toxin is a powerful stimulant for intact cells. Fluorides have been used in studies on broken cell preparations, but they are unsuitable for use with intact cells since they penetrate poorly and are also well recognized poisons for key enzymes.

Agents which activate phosphodiesterase (e.g., imidazole and nicotine) can reduce effectiveness of some hormones and shorten duration of action.

Actions of cAMP^{14, 26, 27}

Effects on Cytosol Calcium Ion Concentrations. In some cell types cAMP seems to directly alter properties of the plasma membrane in a manner leading to increased rate of uptake of calcium from the extracellular fluids; hormone effects usually cannot be elicited if such cells are bathed in a calcium-free medium (or if chelating agents such as EDTA are added to the fluids). Artifacts resulting from nonspecific membrane damage must be considered under these conditions.

cAMP promotes extrusion of calcium from mitochondria, possibly through direct interaction with mitochondrial membranes. Effects on cytosol calcium ion concentrations can be substantial, since mitochondria store large amounts and the extrusion rate is rapid. Mitochondrial release of calcium is accompanied by release of inorganic phosphate and uptake of hydro-

gen ions. Therefore cytosol content of both phosphate and hydrogen are simultaneously altered. Cell ATP content may be secondarily affected, since ATP is utilized for reuptake of the calcium.

cAMP may also promote release of calcium from the plasma membrane. It is likely that effects of cAMP which can be elicited in cells bathed in calcium-free media depend upon actions of the nucleotide on intracellular redistribution of calcium.

Cytosol calcium ion concentrations affect activity of many key enzymes. Examples will be presented throughout the text and especially in the section on parathyroid hormone action. Cytosol calcium also affects the rate of uptake of sodium ions from the extracellular fluids.

cAMP and Phosphorylation Reactions. Many regulatory enzymes exist in two forms, one phosphorylated and the other dephosphorylated. For some, addition of phosphate activates the enzyme, while for others, it deactivates.

cAMP-dependent kinases which catalyze transfer of a phosphate moiety from ATP to a regulatory protein have been identified in large numbers of cell types. Important consequences of such activation of a kinase system are considered in detail in Section II. A "cascade" leading to increased activity of phosphorylase enzymes provides cells with a supply of glucose-phosphate for fuel and secondarily with nicotinamide adenine dinucleotide phosphate (NADP) for synthetic processes.

Other cAMP-dependent kinases have been implicated in regulation of triglyceride metabolism, in phosphorylation of ribosomal, nuclear and microtubular proteins (and therefore in protein synthesis and mitosis), and also in phosphorylation of plasma membrane and other lipids. cAMP inhibits release of lysosomal enzymes, exerts several important actions on immune processes and inflammatory reactions, and seems to play a role in aggregation of platelets and other surface phenomena.

There is good evidence that the cAMP-dependent kinases are present in cells in combination with regulatory subunits which inhibit their activity, and that attachment of cAMP to the regulatory subunit releases the kinase in active form (Fig. 3-2A). Inhibitors which combine with the

activated kinase have also been found (Fig. 3-2B).

Functions of cAMP are described in every part of this text, and information on the subject increases each day. However, a definitive summary of cAMP importance awaits new insights. The ubiquitous distribution of the nucleotide (in all animal cells thus far studied, vertebrate, invertebrate and unicellular, including forms not known to be responsive to hormonal stimulation) suggests that many of its functions remain to be discovered; but the same kinds of information raise the possibility that changes in intracellular cAMP may represent a *nonspecific* response to cell stimulation (rather than an essential component in the train of events initiated by hormones).

HORMONAL ACTIONS WHICH MAY NOT DEPEND UPON ADENYLYL CYCLASE OR ON PREVIOUSLY DISCUSSED INFLUENCES ON THE PLASMA MEMBRANE

Effects on Nucleic Acid Metabolism

It is certain that cAMP influences on nuclear proteins affect gene transcription. But there is strong evidence that gene transcription is affected in other ways by *steroid hormones* which are known to gain access to the nucleus and to bind (in combination with specific peptides) to the chromatin.

Steroid hormones have been implicated in exposure of specific template sites (by direct actions and through influences on preexisting repressor molecules), in stimulation of RNA polymerase activity, in regulation of RNA degradation, and in control of transport across nuclear membranes. Specific details are presented in appropriate sections of the text.

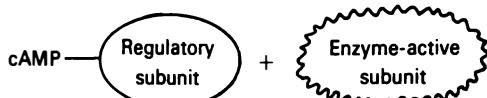
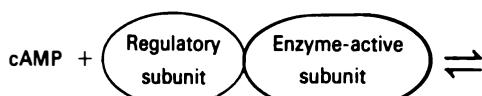
Information on exactly what the steroids and their associated peptides are doing is now being assembled, some of it from studies of chick oviduct, mammalian uterus, and amphibian urinary bladder. Application of new research methods (molecular probes, specific recovery of messenger RNAs by adsorption of polyadenylate sequences) should provide new insights.

Much of the earlier work on nucleic acid functions was carried out on prokaryotic systems. The information gained has been useful, but there is still only partial comprehension of just what can (and what cannot) be carried over to explain events in eucaryotic cells.

Protein molecules which bind to specific DNA sites and thereby repress transcription have been identified in bacterial cells. Under certain conditions smaller molecules interact with the proteins and effect derepression by interfering with the binding to the DNA sites.

Application of this information led to formulation of a hypothesis which states that hormones interact with repressors to

A



B

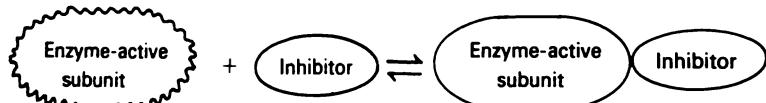


FIG 3-2. Diagrammatic representation of mechanism for activation and inhibition of cAMP-dependent kinases. Jagged outline indicates active forms. A. Activation of cAMP-dependent kinase. B. Inactivation of cAMP-dependent kinase.

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unmask specific DNA sites, thereby permitting transcription of specific messenger RNAs which code for the proteins that mediate hormone action. The concept is esthetically appealing and is consistent with observations that inhibitors of DNA or protein synthesis can block actions of steroid hormones. It is possible that some mechanisms, but others affecting RNA and protein synthesis do not.

Histones are small basic proteins or peptides rich in arginine, lysine, or histidine which are synthesized in the cytoplasm of eucaryotic cells and transported to the nucleus. They are also synthesized by bacterial cells and associate with DNA in both cell types. In certain systems they have been shown to inhibit ability of DNA to serve as a template and have been implicated as the repressors. The histones undergo metabolic transformations including phosphorylation, methylation, and acetylation, and such transformations affect their ability to bind to DNA. There have been numerous suggestions that hormones can affect transcription either through direct binding to histones in the nucleus or through activation of enzymes catalyzing histone modification.

Serious doubt has been cast on the concept that histones directly regulate gene transcription in animal cells. The histones exhibit limited variation in chemical makeup, exhibit no striking cell or species specificity, and no obvious changes (qualitative or quantitative) which can be related to changes in cell function. (A histone present in pea seedlings differs from one in calf thymus glands by only two amino acid residues.)

It seems more likely that "acidic" nuclear proteins rich in aspartic and glutamic acids function in regulation of transcription. Unlike the histones, acidic proteins exhibit cell and species specificity, and variations in quantity and composition have been related to changes in cell activities. The acidic proteins may combine with histones and alter binding of the latter to DNA. It has been proposed that steroid hormones interact with the acidic nuclear proteins.^{47, 50}

The acidic proteins are known to be susceptible to modification by cAMP-activated kinases and have therefore also been implicated in influences of the nucleotide on gene transcription.

The exact sequence of events leading from hormone stimulation to production of specific proteins has not been worked out yet. Elucidation will require full appreciation of the special properties of animal cells.

Differences of major importance between prokaryotic and eucaryotic cells include the presence of a nuclear membrane in the latter, differences in structures of chromatin (and of ribosomes), and in life spans of messenger RNA and protein molecules. Mechanisms for regulation of synthesis and function of ribonucleic acids are probably quite different; but very recent findings suggest that bacterial mechanisms are far more complex than was previously believed.

Eucaryotic cells produce "giant" nucleic acid molecules which become rapidly associated with nuclear proteins. The terms "heterogeneous nuclear RNA (hnRNA)" and "premessenger RNA" have been used to designate such molecules which contain not only messenger components but also nucleotide sequences that participate in regulation of RNA utilization.¹³ They also seem to contain components which function as receptors for information imparted by hormones and other molecules. In some systems the earliest recognizable influence of a hormone is that of stimulation of precursor incorporation into the premessenger RNA. Information is needed on functions of the large RNA molecules and mechanisms whereby hormones can alter such functions. It is also necessary to know more about the relative importance of RNA molecules as compared with post-transcriptional and post-translational events in formation of proteins mediating hormonal responses.

Known influences of hormones on DNA synthesis take a long time to develop and seem to be secondary to earlier influences on cell growth and other cytoplasmic events. A role of hormones in "gene amplification" (leading to increased DNA content per cell) has been proposed, but there is no convincing evidence to support the concept that hormones function in this way. Enhancement of DNA synthesis seems to be consistently associated with cell proliferation. In addition to obvious changes in tissue growth and in the numbers of cells capable of synthesizing key proteins, factors promoting cell proliferation can affect certain aspects of cell differentiation which are intimately linked with mitotic processes. It is known, for example, that long-range influences of estrogens on the endometrium result in production of new populations of cells which differ in many ways from cells receiving the initial hormonal stimulus. There are indications that some hormonal influences on synthesis of key proteins can be accomplished only during specific phases of the mitotic cycle.

Direct Influences on Ribosomes

Cell fractionation studies utilizing cytosol and ribosomal components derived from normal, hormone-deficient, and hormone-injected animals have revealed impaired *in vitro* synthesis of proteins associated in the case of insulin with the larger (60 S) subunit of the ribosome. One hypothesis states that a conformational change impairs binding of aminoacyltransferase I in ribosomes of insulin-deficient animals.⁶³ Growth hormone-deficient animals seem to have defective smaller (40 S) ribosomal subunits.¹⁶

Several examples are known of hormone deficiencies which lead to increases in the numbers of *free* ribosomes within the cytoplasm accompanied by decreases in numbers and sometimes sizes of *polysomes*. Administration of the missing hormone leads to reestablishment of normal ribosomal "profiles." According to some investigators, the hormone deficiency can be directly linked with defective peptide chain initiation if polysome formation can be restored by administration of cycloheximide.

Several hormones are known to influence incorporation of phosphorus into membrane phospholipids. The chemical make-up of the lipids and the attachment of ribosomes to membranes of the endoplasmic reticulum are probably of crucial importance. Many thyroxine effects have been related to stimulation of production of *new populations of ribosomes* which exhibit firm attachment to membranes.^{60, 61} Geographical sequestration of ribosomes within specific cell loci may also influence ribosomal functions. Hormones may also affect availability of small molecules utilized by ribosomes.

INFLUENCES OF HORMONES ON MITOCHONDRIA

Mitochondria directly regulate synthesis of a few proteins. Chloramphenicol is a useful tool for studying such processes, since it inhibits mitochondrial protein synthesis without exerting similar influences on functions of 80 S ribosomal particles. Thyroid hormones may exert rapid and direct influences on mitochondrial protein synthesis or on mitochondrial release of regulatory molecules (Section V). The

same hormones have been implicated in regulation of oxidative phosphorylation. Thyroxine, aldosterone, and other hormones indirectly promote synthesis of mitochondrial components. Parathyroid hormone, vitamin D metabolites, and cAMP promote mitochondrial extrusion of calcium and phosphate and uptake of hydrogen ions.

INFLUENCES OF HORMONES ON PREEEXISTING ENZYMES

When changes in activity of a given enzyme can be demonstrated soon after exposure of tissues to hormones *in vitro*, it is usually assumed that the hormone has affected either properties of the enzyme or substrate availability. Similar conclusions may be drawn if a hormonal influence on enzymatically controlled reactions is not blocked by administration of inhibitors of protein synthesis.

The most obvious effects of hormones on preexisting enzymes are those mediated via cAMP, but numerous attempts have been made to demonstrate other kinds of influences.⁴⁸

The older thyroid literature is especially rich in descriptions of such studies. Much of the data are of a negative nature. In many cases, no reproducible influences could be found; in others, positive findings have been traced to artifacts of the *in vitro* system utilized. An influence of thyroid hormones on diphosphoglycerate mutase activity of erythrocytes can be shown *in vitro*. But most actions of thyroid hormones take a long time to develop and depend upon new enzyme synthesis. Usually it is necessary to administer the thyroxine to whole animals and to study excised tissues at a much later time.

Data suggesting that steroid hormones can directly influence aggregation and activity of glutamic dehydrogenase⁶⁵ have not been related in any way to physiological functions of the hormones. Similarly, studies which demonstrate that steroid hormones with an interconvertible alcohol-ketone group can participate in transhydrogenase reactions⁶⁷ probably provide no useful information on physiological functions of the hormones.

Glucocorticoids have been reported to affect rates of degradation of existing enzymes. Such actions may result from influences on lysosomal membranes leading to release of catalytic enzymes.

There is a considerable body of literature suggesting direct influences of hormones on

enzymes directing carbohydrate and lipid metabolism which can be separated from demonstrated influences of the hormones on enzyme induction. Some are clearly related to intracellular

changes in concentrations of cAMP or of small ions. Some of the more rapid influences of insulin may be linked with reduction of cytosol cAMP.

4. What is an Endocrine Gland?

Elementary biology texts present a simple three-step procedure for determining if a structure is an endocrine gland: (1) remove the structure, (2) observe the effects, (3) reverse the effects by administration of a preparation from the excised tissue. If step 3 can be accomplished, it is concluded that the structure in question is an endocrine gland. For those believing that matters can be readily settled by following this protocol, a few comments of a practical nature are appropriate.

Extirpation of a little-known organ can present problems. Some endocrine glands exist singly and others in pairs. The number, size, shape, and location of still others is highly variable, and it is not unusual to find small groups of hormone-secreting cells embedded within other kinds of tissue. It is important to remove all cells of the kind to be studied, because any that are left behind unnoticed will probably increase their secretory functions within hours (or minutes) and rapidly undergo compensatory hypertrophy.

The surgical procedure and the painful healing process which follows can be traumatic. Sham operations (in which control animals are subjected to the same handling, anesthesia, cutting, etc. without actual removal of the structure) are useful for distinguishing between nonspecific effects of trauma and "stress" and the specific consequences of removal of the tissue. However, sham operations do not solve all of the problems. Sometimes nerves or blood vessels supplying unrelated organs are inadvertently damaged only in the experimental group.

If experimental animals change their voluntary food consumption, it is important to distinguish between effects of altered food intake and effects of possible hormone deprivation. The sham-operated animals can be "pair-fed," but this means

that experimental animals eat as much as they choose while "controls" go hungry or endure forced feeding.

Endocrinologists have a variety of agents which specifically inhibit production of certain hormones or interfere with their access to receptor sites. However, such agents are developed *after* information has accumulated on the chemical nature and function of the hormone.

The deficiency syndrome can be difficult to define. Effects of removal of all cells secreting insulin, parathyroid hormone, or antidiuretic hormone will be obvious soon enough. However, removal of thyroid glands from adult animals may not induce recognizable changes for many days, and effects of ovariectomy might escape detection completely if no information were available on where to look.

While some deficiency syndromes develop slowly, problems arise if effects are sought too late. The endocrine system utilizes a variety of compensatory mechanisms whenever it is perturbed.

Assuming that deficiency effects can be demonstrated, the reasons for attempting to reverse the effects by administration of a tissue preparation are obvious. (Characteristic systems will reproducibly emerge following removal of a large number of "non-endocrine" organs, e.g., the heart.)

What should be administered, and how? The least traumatic procedure could be the addition of minced, homogenized, or powdered tissue to the usual diet. Thyroid glands can be effectively presented in this way because they contain large quantities of thyroglobulin which is digested to yield thyroxine. But most endocrine glands store so little hormone that amounts conveniently added to the diet are insufficient to alleviate deficiency syndromes. Moreover, peptide and protein hormones are destroyed by gastrointestinal secretions,

while steroids are inactivated by the hepatic enzymes they encounter soon after leaving the intestine.

Oral administration also presents problems of uncertain absorption, and it is difficult to maintain adequate blood levels even when large amounts are fed. Moreover, some of the commonly used laboratory beasts (e.g., rats) are quite capable of sorting out the smallest particles of foreign matter from the most finely powdered diets; they may choose to leave all of the test tissue behind while consuming almost 100% of the usual food.

Sometimes test substances can be dissolved or finely suspended in drinking fluids presented in calibrated bottles with nonspill devices. The animals are then faced with the choice of taking in the test substance or going thirsty. Usually they prefer the first alternative—but not always. The decision about hormone dosage resides with the animal.

Excised structures can be put back into animals with the hope that the transplant will "take" and develop a blood supply before necrosis sets in. Care must be taken regarding the site of implantation. It is known for example that secretory processes of the ovary are markedly altered if the structure is implanted at a superficial site in which the temperature is lower than that which obtains internally. If the blood supply of the transplanted organ drains directly into the hepatic portal system, secretions may be metabolically degraded before they reach the general circulation.

Most endocrine organs will function when deprived of their nerve supply. However, it is becoming increasingly apparent that nerves can have important influences on the blood supply to the gland and therefore on delivery of hormone precursors, oxygen and metabolic fuels, and on removal of waste products and secretions. Nerves to some glands have even more direct influences on secretory processes. The pineal has gained respectability as an endocrine gland in recent years; it is highly dependent on innervation and will not perform functions approaching those of the intact gland if it is transplanted.

The tissue can be homogenized and suspended for parenteral injection. Usually, this leads to no end of difficulties. Proteins that are sequestered within an intact organ can act as potent antigens when injected.

Homogenates may contain enzymes which destroy hormones, proteins which bind active sites, or mixtures of components with antagonistic effects. (The pancreas contains trypsin which digests protein hormones, insulin which lowers blood glucose concentrations, and glucagon which elevates blood glucose.)

Extracts are usually better tolerated. But should the tissue be extracted with water? salt solutions? acids? alkalies? alcohol? acetone? ether? And how much should be given and where?

The long process of obtaining a suitable extract usually starts with crude trial-and-error procedures. Early phases are especially frustrating because so many variables must be considered. The writer is firmly convinced that expected results of pilot studies should never be discussed within earshot of test animals. Repeated (but unconfirmed) observations suggest that each carefully selected group of animals appoints one or more individuals to assume the function of reacting differently from the others.

If crude extracts can be prepared which reverse effects of tissue extirpation, then belief that the structure in question is an endocrine gland is strengthened. The possibility that the extract contains *pharmacological* agents which reverse in unnatural ways the effects of removing the structure is not easily ruled out. It is important to demonstrate that the purported hormone is in fact *secreted* by the structure and that the quantities which must be administered bear some reasonable relationship to amounts which can be secreted by the animal.

Information on the chemical nature of the active principle can be gathered by subjecting large quantities of the tissue to fractionation and purification procedures. Usually materials from cattle, sheep, and hogs are studied first (since they can be obtained at low cost from the slaughterhouse), and extracts are tested on small laboratory animals which presumably require very small amounts. One soon discovers that the content of active principle varies with age of the donors (which is difficult to determine), the sex, previous dietary history, and season of the year, and that the hormone from one species may differ from that of another.

A bioassay must be developed to provide

a measure of the suitability of purification procedures. This almost always involves solving unanticipated problems in addition to expected ones relating to choice of a suitable vehicle, the route and timing of administration, and appropriate pretreatment of test animals. It is not always possible to obtain dose-response curves with crude extracts, and regulators with biphasic actions can be especially troublesome.

Some investigators will feel that the whole project has been unworthy of the time consumed if purification and subsequent chemical analysis reveal that the active substance is identical with agents produced elsewhere in the organism, or if no advantage to the animal can be attributed to secretion of the substance in question.

If the investigation is continued, attempts will be made to elucidate the mechanism of action of the hormone. This

should provide more problems than all of the preceding combined.

The search for specific hormone receptors is usually first made on structures implicated in the deficiency syndrome. High affinity receptors will probably be found elsewhere by different investigators, and this can lead to a quest for previously undisclosed functions. If they are found, reexamination of crude extracts may reveal that the purification procedures remove most of the principle effective for the secondarily disclosed activities. A "new hormone" may then be discovered which has functions unrelated to the "deficiency syndrome" first observed. At this stage, the investigator may resume perusal of the literature (neglected during the period in which so much time was spent in the laboratory); he may discover that this new endocrine gland provides but a minor source of the new hormone.

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II

HORMONES AND BLOOD SUGAR

5. Insulin

This section describes endocrine control of blood glucose concentrations and the associated metabolism of carbohydrates, proteins, and lipids. Hormonal regulation of food intake, body weight, and body composition is presented in Section V.

BLOOD GLUCOSE REGULATION AND SURVIVAL

Need for Protection against Hypoglycemia

Glucose is an essential fuel for neurons, erythrocytes, and vigorously contracting skeletal muscle cells. The higher centers of the brain are especially sensitive to even short term deficiency.

Since glucose enters most cells via passive processes (down a steep concentration gradient), it is necessary to maintain adequate concentrations in the blood plasma. It is therefore not surprising that several hormones (including epinephrine, glucagon, glucocorticoids, and growth hormone) are charged with the responsibility of elevating blood sugar concentrations when the need arises.

Regulatory hormones assume greatest importance in intermittent (meal) eaters, in which a discontinuous food supply must be apportioned for ever present but rapidly changing fuel needs. Frequent nibbling reduces requirements for some of the hormones; but evolutionary emergence of a powerful musculature which demands more fuel than can be immediately delivered by the intestine, and development of mechanisms for efficiently coping with recurrent periods of fasting, have led to formation of hormone-dependent systems. The dependence is most obvious in carnivores and of lesser importance in ruminants.⁴

Hypoglycemia and Mental Functions

Although neurons require a continuous supply of glucose, there is no experimental support for the widespread notion that mild glucose deficiency leads to drowsiness, or that the latter can be alleviated by ingestion of sugar. Infants awaken when hungry and sleep after a feeding; adults may find it difficult to fall asleep when hungry and equally difficult to remain alert after a sizeable meal. Food-deprived laboratory animals tend to become restless (or even vicious) rather than drowsy.

Eating does more than affect blood glucose concentrations; but effects on blood glucose contribute heavily to the physiological state which follows. It would be unfortunate indeed, if drowsiness were an inevitable consequence of food deprivation. Consider the plight of an animal which hunts for its breakfast; it would fall asleep and soon starve to death. Clearly, the time of food deprivation is the time when it is necessary to be maximally alert to the potential presence of the next meal.

The concept that "sugar provides quick energy" probably stems from (1) psychological factors, including the power of suggestion so expertly promulgated by the manufacturers of carbohydrate-rich foods, and the pleasure and diversion from boring tasks provided by snacking; and (2) influences of carbohydrate ingestion on insulin functions (described later in the chapter) which can lead to increased efficiency of muscular contraction.

Patients with untreated diabetes mellitus (and high blood glucose concentrations) often have difficulty concentrating on mental tasks, and improvement follows administration of sufficient insulin to re-

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duce glucose concentrations to normal levels. When too much insulin is taken, the patient experiences restlessness and sensations of anxiety which can be relieved by ingestion of glucose-containing foods. Larger doses of insulin (that severely lower blood sugar levels) provoke involuntary tremors; still larger doses have been given to psychiatric patients for the purpose of inducing "shock," which is invariably accompanied by convulsions. One method for bioassay of insulin depends upon determination of the dosage required to precipitate convulsions in the rabbit; the convulsions can be prevented by administration of glucose along with the insulin.

Severe sustained hypoglycemia invokes symptoms different from those seen in mild deficiencies. Functions of forebrain neurons are impaired, and this leads first to mental confusion and in time to loss of consciousness. Tremors, convulsions, and activation of the sympathetic nervous system have been attributed to loss of inhibitory control normally exerted by higher brain centers over lower ones. More drastic glucose deprivation affects the vital lower brain centers and can lead ultimately to death.*

The Need for Protection against Hyperglycemia

The importance of supplying sufficient glucose is obvious. The reasons for avoiding excessive elevation of blood glucose concentrations are more subtle. Glucose as such is not toxic; large quantities can be injected intravenously with few if any ill effects.

Theoretically, it is possible to administer glucose in quantities which dangerously elevate the osmotic pressure of the blood and thereby draw fluid from the body tissues. But the glucose concentrations in severe cases of untreated diabetes mellitus seldom cause serious difficulties in this way.

The osmotic pressure exerted by mammalian blood plasma is approximately equivalent to 310 milliosmoles per liter; this is largely attributable to the content of inorganic salts. The normal contribution of glucose is approximately 6 milliosmoles; it is obvious that doubling or even tripling of glucose concentration exerts only minor influences, even if it is assumed that

compensatory changes in water content are not made.

During periods of hyperglycemia, glucose metabolism in nervous tissue is controlled by the enzymatic machinery of the neurons, not by the surrounding concentration of glucose.¹⁷

The devastating effects of chronic hyperglycemia are related to the fact that glucose exists in the form of small, water-soluble molecules which are readily filtered by the glomeruli of the kidney and lost in the urine.

Under normal conditions all of the filtered glucose is reclaimed by the proximal tubules. But the reabsorption process requires energy (since work is done against a concentration gradient), and the rate of reabsorption is limited.

Glycosuria (sugar in the urine) need not be associated with renal damage. The situation may be compared with one in which a bricklayer performs a finite amount of work each day. If he is supplied with exactly the number of bricks he can use, none are left over. But if he is given too many, some will remain unused.

Hyperglycemia then, leads to glycosuria. Therefore there is a *wastage of potential fuel*; if there is no compensatory increase in glucose intake, *body tissues will be broken down* to supply carbohydrate requirements of neurons and erythrocytes. (An early symptom of diabetes mellitus is increased appetite accompanied by loss of body weight.) But an unlimited food supply does not solve all of the problems.

Urinary excretion of glucose *draws large volumes of water* from the circulation. (Two additional symptoms of developing diabetes mellitus are severe thirst and decreased urine volume.) The term *diabetes* is derived from a Greek word meaning syphon, and therefore refers to the large urine volume. Mellitus (honey) denotes the high sugar content; diabetes mellitus is therefore "sugar diabetes."

Diabetes insipidus is a hormone-related condition (described in Section III) in which a large urine volume results from inability to conserve water. *Renal diabetes* involves excessive urine volume resulting from a kidney defect.

Circulating blood volume can be partially sustained through recruitment of

nonvascular extracellular fluids; however, this leads to *cellular dehydration*. In time, *blood volume is substantially reduced with consequent lowering of systemic blood pressure*, impaired delivery of food and oxygen to the cells, and delayed removal of metabolic wastes.

Since glomerular filtration depends upon maintenance of appropriate hemodynamic relationships, *renal function is compromised*, and metabolic wastes accumulate. Some of the dire consequences of glycosuria can be alleviated by ingestion of large quantities of water and of the minerals which tend to be washed out in the urine.

Hormonal Protection against Hyperglycemia and Glycosuria

Insulin is the only hormone which lowers blood glucose and thereby protects against hyperglycemia, glycosuria, and associated tissue destruction. It promotes rapid transfer of glucose from the blood to insulin-sensitive tissues, stimulates glucose-utilizing mechanisms, and inhibits actions of hormones which promote glucose synthesis from protein. It provides for conservation of food materials when they are available (so that reserves are built up to supply needs during fasting), and it promotes growth and repair.

Insulin not only instigates rapid shifts in metabolism in accordance with immediate needs, but it also promotes long range *adaptive changes* to timing, quality, and quantity of the diet. In addition, it stimulates the appetite. In one way or another, this vital hormone affects the function of virtually every cell of the body.

THE METABOLIC FUNCTIONS OF INSULIN

Tissues differ widely in their *requirements for glucose*, in major *pathways of glucose metabolism*, and in *responses* to the various *regulatory hormones*. It is convenient to consider some of the important structures individually against a background of information on the manner in which they interact with other structures.

Role of Insulin in Maintaining Carbohydrate Metabolism in Nervous Tissue

As noted above, neurons require a continuous supply of glucose, and the need is

inflexible. Hormonal (or other external) limitation of glucose uptake or utilization could therefore not be advantageous, since such limitation would lead to impaired function. During periods of food deprivation, other tissues must do without while glucose availability to the nervous system is maintained.

Some recent studies indicate that neurons can "learn" to utilize fatty acids for fuel during periods of starvation, provided that minimal quantities of glucose are made available via tissue catabolism. But this is an extreme situation in which some localized functional loss may be traded off in the interests of survival of the whole individual. Obese adults subjected to weeks of food deprivation characteristically display psychological disturbances and disruption of functions of the autonomic nervous system even when measures are taken to avoid acidosis, vitamin deficiency, and dehydration. And glucose-deprived immature animals develop defective brains.¹⁷

While a few specialized groups of neurons (e.g., those involved in regulation of food intake and of growth hormone secretion) may be directly sensitive to insulin, and there are indications that the hormone can affect the rate of disappearance of glucose from the cerebrospinal fluid, there is little evidence for a quantitatively important direct regulatory role for insulin in glucose uptake or utilization by nervous tissue. (Insulin does, however, affect neurons indirectly through influences on concentrations of several constituents of the blood plasma; and it has been implicated in regulation of lipid synthesis in nerve tissue during developmental stages.) On the other hand, the central nervous system directly affects insulin secretion.

Actions of Insulin on Resting Skeletal Muscle

Effects on Glucose Uptake. By sharp contrast with the situation in nervous tissue, there is considerable *flexibility* in the glucose requirements of resting skeletal muscle. Resting muscle *can* use glucose directly as a metabolic fuel; it *can* also store appreciable quantities for later use in the form of muscle glycogen. In addition, ATP energy derived from glucose is utilized in several ways to support protein and lipid synthesis.

But resting skeletal muscle *does not require* a continuous supply of glucose for

fuel. It can effectively use short-chain fatty acids as a source of maintenance energy, and can minimize storage and anabolic processes when necessary.

Since the metabolic mass of skeletal muscle is very large, small per gram changes in the relative quantities of glucose and fatty acids taken up at any given time can have profound effects on the over-all body use of glucose.

Skeletal muscle is, therefore, an excellent candidate for target organ regulation by hormones. When the glucose supply is limited (as it would be several hours after absorption of the last meal), curtailment of nonessential glucose utilization by muscle tissue can spare the sugar for use by the brain. On the other hand, when very large quantities of glucose enter the circulating blood (during and just after ingestion of a carbohydrate-rich meal), increased muscle uptake and utilization reduce the dangers of hyperglycemia, glycosuria, and the attendant consequences, while metabolic adjustments within the muscle provide for future needs.

Since the blood supply to skeletal muscle derives from the same system which supplies the brain, limitation of muscle use of the sugar cannot be accomplished by reduction of plasma concentrations. But rates of glucose uptake can be controlled.

When plasma insulin levels are low (during fasting), muscle cell membranes have a "barrier" which resists inward diffusion of glucose. (Concentrations of free sugar within the cells are below levels detectable with sensitive methods when the plasma contains 80–100 mg of glucose/100 ml; plasma concentrations three or four times as great are needed for entry of appreciable quantities of glucose.)

Ingestion of a carbohydrate meal or injection of glucose leads to prompt elevation of plasma insulin concentrations. By acting on muscle cell membranes, insulin "lifts the barrier" (Table 5-1).

Insulin increases the rate of uptake of several other small molecules (including galactose) which cannot be metabolized by skeletal muscle. It clearly alters properties of the membrane; but it does not simply increase "pore" size; uptake of stereoisomers of glucose and of some smaller pentose sugars is unaffected.

Insulin induces hyperpolarization of the membrane and affects transmembrane move-

ment of small ions.¹¹ (Potassium uptake can be demonstrated even when glucose is removed from the medium). Phospholipases A and C and low concentrations of some proteolytic enzymes which are known to act directly on membranes^{12, 13} can mimic insulin actions when no hormone is present, while prolonged exposure to membrane-disrupting influences of trypsin blocks subsequent insulin influences.

Hypotheses suggesting that sulfhydryl groups on the insulin molecule combine with components of the plasma membrane and thereby induce configurational changes have been presented along with evidence that thiol blocking agents impair insulin action.⁸ But reexamination of actions of the blocking agents and newer evidence for participation of tyrosyl and histidyl (rather than cysteinyl) moieties in insulin binding have led to rejection of such concepts.^{14, 4, 5}

Insulin action is initiated by binding of the hormone to specific high affinity receptors on the target cell membrane. Indirect evidence that the receptor is a short-lived peptide or protein derives from observations that pretreatment of cells with trypsin abolishes insulin responses, and that insulin sensitivity can be restored within an hour after removal of the proteolytic enzyme in the absence but not in the presence of inhibitors of protein synthesis.⁹ A

TABLE 5-1
Importance of Insulin Action on Skeletal Muscle Cells in Regulation of Blood Glucose Concentrations

A. During fasting	No glucose entry from gastrointestinal tract Relatively low blood glucose concentrations Low blood insulin levels Barrier interferes with glucose uptake by skeletal muscle cells; skeletal muscle utilizes short chain fatty acids and previously stored reserve materials Glucose spared for use by nervous tissue cells
B. After feeding	Glucose enters blood stream in increased quantities Blood glucose concentrations slightly elevated Islet cells of pancreas stimulated Blood insulin levels rise Insulin lifts barrier, permitting increased glucose uptake by skeletal muscle cells and preventing excessive elevation of blood glucose concentrations Skeletal muscle uses glucose for fuel, and for formation of muscle glycogen and creatine phosphate

protein with high affinity for insulin has recently been identified.⁹

Insulin-sensitive cells seem to contain more receptor molecules than are needed to promote maximal hormonal effects. The presence of large numbers of receptors may enhance insulin sensitivity by increasing the probability of hormone binding when low concentrations of insulin are present. Binding of some insulin molecules apparently reduces the affinity of unoccupied receptors; specific amino acid sequences of the hormone have been implicated in such phenomena. There is evidence that formation of insulin receptors is stimulated by glucose and inhibited by prolonged presence of high concentrations of insulin, but conflicting interpretations have been attached to some of the experimental data.²²

The finding that agents (streptozotocin, neuraminidase) which permit insulin binding can interfere with subsequent hormone actions, suggests that hormone-receptor interaction triggers a secondary response leading to (but separable from) effects on glucose transport.

All aspects of glucose transport (stereospecificity, competition of one sugar with another, counter-transport phenomena and saturation kinetics) indicate that glucose transport is carrier mediated.¹⁴ Insulin could be affecting availability or structure of carriers with specific affinities. An intriguing concept is that a hexokinase isozyme (which catalyzes glucose phosphorylation) also functions as the glucose carrier.⁴

Glucose transport does not directly utilize ATP; in fact, ATP depletion signals the need for uptake of glucose fuel. The "barrier" requires ATP for its maintenance; conditions which interfere with ATP availability (anoxia, presence of inhibitors of oxidative phosphorylation) tend to increase the rate of glucose entry.

It has been proposed that insulin diverts ATP from the membrane to internal cell sites, e.g., those concerned with glucose phosphorylation or protein synthesis. Insulin does indeed change the use of ATP, but the concept is difficult to test experimentally.

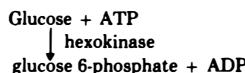
The carrier appears to exist in two forms;⁴ it has been suggested that ATP is required for its phosphorylation, whereas the hormone promotes dephosphorylation. The dephosphorylated form seems to have a lower affinity for glucose; therefore the sugar can be more readily dissociated at the inner membrane surface, leaving the carrier free to return to the outer surface to pick up more glucose. Since glucose concentrations outside the cell are high enough to permit binding to a carrier with lower affinity (while glucose within the cell is immediately metabolized), this could explain more rapid uptake.

Several other actions of insulin have been linked with dephosphorylation of regulatory

molecules (e.g., see pp. 41, 55) while they may simultaneously affect addition of phosphate groups to substrate molecules.

Influences of Insulin on Glucose Phosphorylation in Skeletal Muscle. The preceding discussion indicates that processes of glucose uptake and glucose phosphorylation are intimately related. When plasma glucose concentrations are within the low-normal range and little insulin is available (e.g., just before a well-nourished individual eats a meal), ability to take up the sugar is the limiting factor for glucose utilization.

Glucose uptake is rapidly accelerated soon after the presence of carbohydrate in the small intestine signals release of insulin. Under these circumstances, the rate of glucose utilization is controlled by the hexokinase reaction, in which a high energy bond from ATP is transferred to the sugar molecule:



The reaction is strongly exergonic and is, for practical purposes, irreversible. It is an obligatory step for all subsequent glucose utilization. It also represents an investment, since ATP energy is utilized but no useful cell energy is obtained until later.

While an effect of insulin on the activity of existing muscle hexokinase has not been unequivocally demonstrated (apart from influences on glucose availability and glucose-phosphate removal), an effect on induction of enzyme synthesis is well established.^{11, 16, 15} Habitual ingestion of a high carbohydrate diet leads to more rapid glucose utilization, while starvation and insulin deficiency impair entry of glucose into metabolic processes. This is one of many examples of adaptive interrelationships between dietary intake and insulin functions.

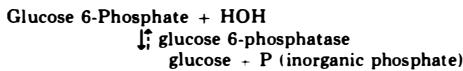
Hexokinases exist in multiple forms (isozymes) which have been studied under a variety of experimental conditions.⁷ Normal skeletal muscle contains relatively large quantities of hexokinase II (the isozyme implicated in glucose transport) and smaller amounts of hexokinase I.

Correlations have been found between insulin sensitivity and the ratio of hexokinase II:hexokinase I. The ratios are low in tissues not dependent upon insulin for glucose uptake

(liver, kidney, brain, erythrocytes) and high in insulin-dependent fat pads of young animals. Animals subjected to prolonged fasting or to damage or removal of pancreatic β -cells, exhibit progressive loss of hexokinase II which can be restored upon refeeding of intact animals or administration of insulin. Emergence of insulin sensitivity during embryonic development parallels appearance of hexokinase II.

As soon as it is formed, most of the glucose 6-phosphate arising from the hexokinase reaction enters one of two pathways: *glycolysis* or *glycogenesis*. Cellular needs determine which pathway predominates. Unlike free glucose, the phosphorylated form cannot readily diffuse out of the cell.

Muscle cells cannot convert glucose phosphate back to free glucose because the hexokinase reaction is irreversible, and because muscle cells lack the glucose 6-phosphatase enzyme which catalyzes the following reaction:



Although hexokinase promotes formation of glucose 6-phosphate from glucose while the phosphatase promotes formation of glucose from glucose 6-phosphate, it should be pointed out that the reactions involved are quite different.

The absence of the phosphatase enzyme is crucial to muscle economy. It will be recalled that muscle receives its glucose only during times when the sugar is abundant, and that it utilizes ATP energy for the hexokinase reaction. If the muscle were to lose glucose phosphate to the blood and other tissues during periods of fasting, it would suffer depletion at the time of greatest need, i.e., when muscular activity is required for acquisition of the next meal.

Influences of Insulin on Formation of Muscle Glycogen. Muscle cells store up their glycogen at times when glucose and insulin are abundant. Insulin enhances activities of enzymes used to synthesize glycogen, and it also inhibits activities of enzymes which catalyze glycogen breakdown. The topic is discussed in a later section of the chapter.

Influences of Insulin on Glycolysis in Skeletal Muscle. Glycolysis provides ATP energy used directly for muscle contrac-

tion, for active transport of amino acids, and for a variety of synthetic reactions. During periods of rest, some of the ATP energy is stored in the form of creatine-phosphate:



The glycolytic sequence leads to production of two molecules of pyruvate from each glucose (Fig. 5-1). During periods of vigorous activity pyruvate is converted to lactate, much of which diffuses out of the muscle cell and travels to the liver. At other times (when oxygen supply keeps pace with pyruvate formation), the pyruvate is decarboxylated and sent through the tricarboxylic acid cycle (and electron transport chain) to yield CO_2 , H_2O , and additional ATP energy.

Insulin stimulates glycolysis in several ways. In addition to providing glucose phosphate, insulin promotes synthesis of a key enzyme of the glycolytic sequence, *pyruvate kinase*^{3, 9, 15} (Fig. 5-1). It also enhances conversion of pyruvate to acetyl coenzyme A. (In addition it promotes utilization of glucose and the ATP derived from glycolysis for protein and lipid synthesis.)

All metabolic pathways for utilization of glucose are enhanced in the presence of insulin. Paradoxically, chronic insulin deficiency leads to accumulation of high concentrations of glucose in the plasma, but very little can enter the cells and that which does enter is poorly utilized.

Actions of Insulin on the Liver

Parenchymal cells of the liver provide a second control site for regulation of glucose metabolism because they, too, have flexible needs.

Glucose Uptake. The liver receives much of its blood supply directly from the gastrointestinal tract via hepatic portal vessels which drain the small intestine and related viscera. Therefore during absorption of a carbohydrate-rich meal, glucose-laden blood reaches the liver before it enters the general circulation.

The rate of glucose uptake increases with the concentration of the sugar in the blood. The liver performs a kind of buffer function, modulating the rise in systemic blood glucose which would otherwise be excessive after feeding. Hormonal control is unnecessary; and insulin has no direct influence on

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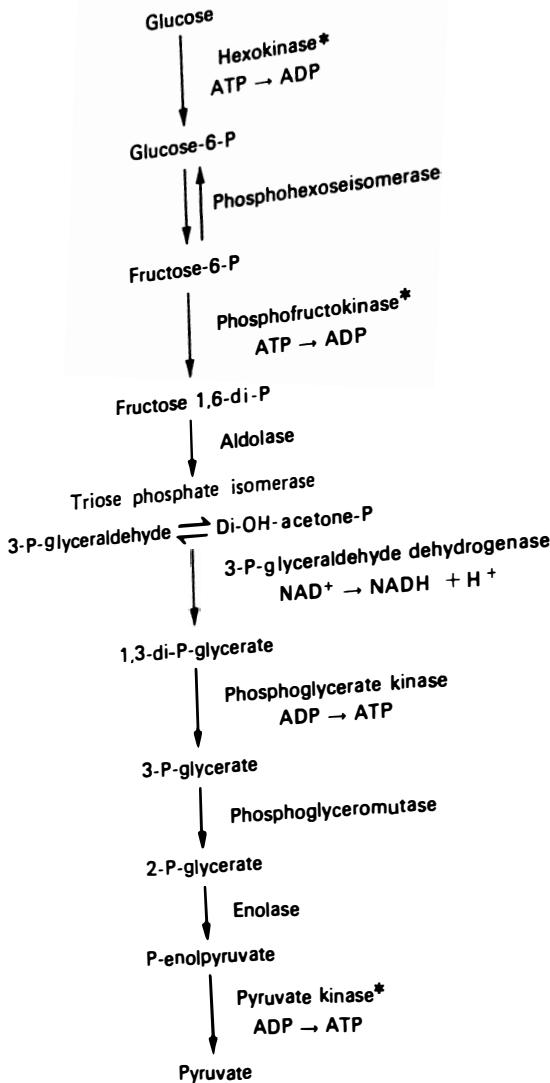


FIG. 5.1. Glycolytic pathway. *Influenced by insulin.

hepatic uptake of glucose. Most of the glucose is rapidly converted to liver glycogen, and reserves continue to build up as long as glucose concentrations remain elevated.

When systemic blood glucose concentrations begin to fall (during the early stages of fasting), liver glycogen is rapidly converted first to glucose-phosphate and then to free glucose which is released into the circulating blood. Thus, the liver provides protection against hypoglycemia during

fasting and against hyperglycemia after feeding.

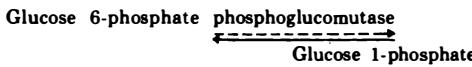
Effects on Hepatic Hexokinases. The rate-limiting step for glucose utilization in liver is the hexokinase reaction. Unlike the situation for skeletal muscle, there is some evidence that insulin can activate preexisting enzyme. But influences of the hormone on enzyme synthesis are more important. Animals chronically maintained on high carbohydrate diets "learn" to rapidly utilize glucose entering the liver.

Most hexokinases are inhibited by the high concentrations of glucose 6-phosphate which build up in the liver during absorption of a carbohydrate meal. Insulin induces synthesis of a special isozyme, hexokinase IV, which functions best at very high glucose concentrations and is minimally inhibited by the sugar phosphate. After prolonged fasting or chronic insulin deficiency, activity of the enzyme falls to undetectable levels. It can be restored in hours by administration of glucose and insulin. Inhibitors of protein synthesis block the restoration. Liver also contains the insulin-sensitive hexokinase II and some insulin-insensitive hexokinase I. In addition it contains small quantities of a special isozyme (hexokinase III) which permits liver to utilize glucose for its local needs when glucose concentrations are very low.

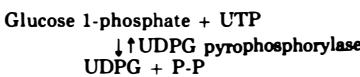
Insulin deficiency creates a condition in which hepatic cells can utilize small amounts of glucose for their own survival but one in which capacity to store large amounts (as glycogen) for future export is impaired.

Effects on Glycogen Synthesizing Enzymes. Liver hexokinases regulate the formation of glucose 6-phosphate but have little influence on the direction of glucose-phosphate utilization. Additional controls are required to channel the sugar phosphate into glycogen formation when conditions are appropriate.

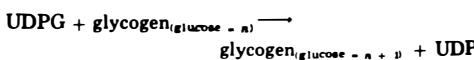
Glycogen is synthesized from glucose 6-phosphate in three steps. Glucose 6-phosphate is first converted to glucose 1-phosphate by a readily reversible reaction which is controlled by substrate concentrations:



Glucose 1-phosphate then reacts with uridine triphosphate (UTP) to form uridine diphosphate glucose (UDPG) and pyrophosphate (P-P):



In the final step the hexose component of UDPG is transferred to the end of an existing glycogen chain:



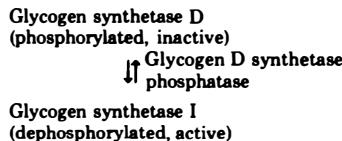
The enzyme catalyzing the final step is the one influenced by insulin. The full

name of the enzyme is UDPG: α -1,4-glucan α -4-glucosyltransferase. It is most commonly known as glycogen synthetase, but some authors prefer to call it glycogen synthase and others, glucosyl transferase.

In addition to activating glycogen synthetase, insulin promotes inactivation of enzymes which catalyze glycogen breakdown.¹¹

When insulin levels are low, glycogen synthetase exists predominantly in the inactive or D form (so named because of its strong dependence for function on high concentrations of glucose 6-phosphate). It can be converted to the more active glycogen synthetase I. (The I form was so named because maximal function is obtained independent of high glucose 6-phosphate concentrations; activation seems to increase sensitivity to low concentrations of the sugar phosphate and also to increase affinity for UDPG).

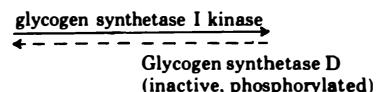
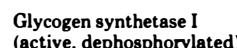
Conversion of inactive to active forms involves removal of phosphate:



Insulin activates the phosphatase and therefore favors glycogen synthesis. The action may depend in part upon reduction of cyclic adenosine monophosphate (cAMP) concentrations.

During insulin deficiency, both activity of glycogen synthetase and the needed substrate for glycogen formation are inadequate. Therefore only minimal glycogen synthesis can occur even when blood glucose concentrations are high.

A phosphorylating enzyme catalyzes conversion of glycogen synthetase to the D form:



Insulin reduces the activity of the glycogen synthetase I kinase.

Effects on Hepatic Glucose-6-Phosphatase. Insulin not only promotes formation of glucose 6-phosphate and channeling of the sugar phosphate into glycogen synthesis; it also opposes actions of hormones

which promote conversion of glucose 6-phosphate back to glucose for escape into the circulating blood. The glucose 6-phosphatase reaction was described above.

Insulin also antagonizes actions of other hormones which promote glucose synthesis from glycolytic pathway intermediates and from amino acids.

Other Actions of Insulin on the Liver. Insulin promotes glycolysis in liver, just as it does in skeletal muscle. It also channels carbohydrates and the energy derived from their metabolism into a variety of synthetic pathways. Under the influence of the hormone greater quantities of lipids and proteins are formed.

All of the actions of insulin work in the direction of reducing blood glucose concentrations and enhancing synthesis of macromolecules. Some of the influences are summarized in Table 5-2.

It is useful to once again consider the problems arising from insulin deficiency. After feeding, when glucose levels are high, glucose can enter the liver parenchymal cells. But glucose utilization is poor: glycogen synthesis is inadequate, less glucose is channeled into protein and lipid synthesis,

TABLE 5-2

Role of the Liver in Regulation of Blood Glucose Concentrations and Sites of Insulin Control

A. During fasting

Limited glucose uptake by liver cells because of low glucose concentrations in blood entering liver

Preformed glycogen, glucose-phosphate, amino acids and other precursors converted to glucose

Secretion of glucose into circulating blood protects against hypoglycemia

B. After feeding

Glucose enters liver in high concentration from hepatic portal veins; glucose concentration somewhat elevated in general circulation

Liver cells increase glucose uptake and decrease glucose export; glucose converted to glycogen; some glucose used to provide ATP for other processes

Insulin promotes glucose phosphorylation, glycogen synthesis, glycolysis; inhibits glucose formation

and reduced glycolysis diminishes the ability to acquire ATP energy. At a time when reserves should be building up, excess glucose is wasted by the kidney. The problem is compounded by tissue destruction because of incomplete suppression of glucose synthesis.

Actions of Insulin on Adipose Tissue^{1M, 1P}

Glycogen storage in the liver is very important for insuring that glucose will be available to nervous tissue (and other cells) during the intervals between feedings. But the quantities that can be stored are limited; the supply cannot satisfy needs for more than a few hours, and even this assumes minimal glucose uptake by tissues with flexible needs. Other kinds of fuel must be stored for use by skeletal muscle, heart, etc., and for some synthetic processes in the liver.

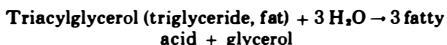
Adipose tissue presents a third site for adjustment of metabolic needs to intermittent availability of food. As anyone who enjoys eating sweet foods can testify, sugar is readily converted to fat and retained as such. Fat is a highly efficient storage form for potential energy. When metabolized it yields 9 calories per g (compared with 4 calories per g for carbohydrate), and its deposition requires less water retention than does glycogen storage. Moreover, facilities are available for long-range storage of large quantities of fat which can be slowly withdrawn in accordance with metabolic needs. During periods of prolonged food deprivation, adipocytes can give up most of their reserve contents and severely curtail glucose uptake without sustaining injury.

Influence of Insulin on Glucose Uptake in Adipose Tissue. When glucose concentrations are low to moderate, relatively little sugar is taken up by adipose tissue. A barrier to glucose diffusion serves a purpose comparable to that described for skeletal muscle. Uptake of glucose may be somewhat increased when blood sugar levels rise, and this is an appropriate response to postfeeding situations. But insulin is needed to promote still greater glucose uptake during and soon after absorption of a meal.

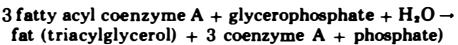
The action of insulin on glucose uptake by adipose tissue is similar in many ways to the action on muscle cells. In both cases, there is membrane hyperpolarization, and both systems exhibit stereospecificity for hexose sugars, competitive inhibition, saturation kinetics, and counter-transport phenomena. But differences have also been noted, for example in some of the kinetic data, and in the fact that insulin has been reported to increase formation of microvilli (and possibly promote pinocytosis) in fat cells.¹⁹

Increased glucose uptake by adipose tissue contributes to the protection against postfeeding hyperglycemia. But it has other important consequences.

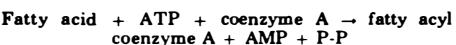
Relationships between Glucose Uptake and Triglyceride Synthesis. Fat molecules are continuously synthesized and degraded. Adipose tissue lipases catalyze the following reaction:



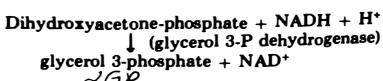
The liberated fatty acids can leave the cell, or they can be reutilized for fat synthesis. But the lipase reaction cannot be directly reversed. Synthesis of new fat molecules requires activation of the fatty acids and formation of glycerophosphate and is accomplished as follows:



Fatty acids are readily activated within adipose tissue if sufficient ATP (derived from glucose) is available:



The limiting factor for fat synthesis is the availability of glycerophosphate. In liver this intermediate can be directly synthesized from glycerol; but adipose tissue does not contain sufficient glycerol kinase to utilize glycerol. It is dependent upon glycolysis which yields the necessary intermediate (Fig. 5-1) and the following reaction:



When glucose readily enters adipose tissue, conditions are favorable for fat synthesis. This occurs during and soon after

absorption of a meal, and the process can be accelerated by administration of insulin and glucose.

During insulin deficiency states, glucose cannot readily enter the fat cell. The glucose that does enter is not efficiently phosphorylated (since insulin affects adipose tissue hexokinases), and the glucose which is phosphorylated is not readily utilized for glycolysis. Therefore, fat degradation predominates over fat synthesis; and fatty acids and glycerol enter the circulating blood. A buildup of fatty acids in the blood aggravates other problems of insulin deficiency since it affects plasma membranes and further reduces the low rate of glucose uptake by skeletal muscle cells.

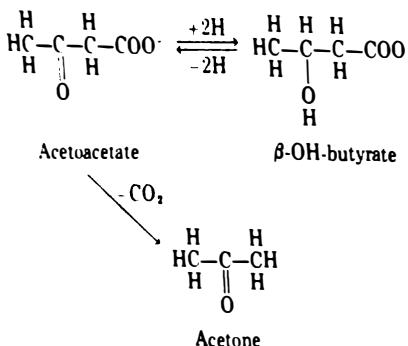
The conditions imposed by insulin deficiency are appropriate in normal individuals during periods of food deprivation because they help to conserve the limited glucose supply for use by neurons and erythrocytes; but they are highly undesirable in the presence of abundant glucose.

Insulin and Lipolysis. In addition to promoting fat synthesis through mechanisms just described (and others considered in Section V), insulin *opposes fat degradation* through inhibition of lipase activity. Therefore, during starvation or insulin deficiency, fatty acids are released into the circulation at an even more rapid rate than would result from just inability to synthesize glycerophosphate.

The long chain fatty acids leaving adipose tissue travel to the liver where they are enzymatically degraded to short chain fatty acids and released for use as fuel by peripheral tissues (especially muscle cells). Under normal metabolic conditions, oxidation of the short chain fatty acids to CO_2 and H_2O keeps pace with the rate of formation in the liver. Small quantities are also used to synthesize new fat molecules.

Ketosis. Any condition (starvation, insulin deficiency, high fat low carbohydrate diet, excessive secretion of lipolytic hormones) which substantially elevates plasma fatty acid concentrations leads to overproduction of short chain fatty acids in the liver. The capacity of peripheral tissues to oxidize the fatty acids is limited, and the excess accumulates in the blood plasma in the form of acetoacetate, β -hydroxybutyrate and acetone:

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The substances are known collectively as *ketone bodies* or *ketones*, and the terms, "ketosis" and "ketonemia" denote excessive accumulation of such substances in the blood plasma. In common with most small molecules, they are excreted by the kidney and their appearance in the urine is referred to as "ketonuria." Acetone is also removed by the lungs and is responsible for the "fruity" odor recognized in the breath and urine of patients suffering from uncontrolled diabetes mellitus.

Acetoacetate and β -OH-butrate combine with plasma cations and are excreted as sodium salts. This osmotically draws large quantities of water from the blood and reduces the plasma $\text{NaHCO}_3:\text{HHCO}_3$ ratio; the results are metabolic acidosis and dehydration which aggravate previously described conditions arising from insulin-deficiency impairment of glucose metabolism.

As with most metabolic disorders, a "spiral" evolves. Ketosis develops because disturbances in lipid metabolism lead to accumulation of fatty acids in the plasma and liver. High fatty acid concentrations in the liver interfere with use of ketones for fat synthesis,³ and this adds to the ketosis. High fatty acid concentrations in the blood plasma interfere with glucose transport across membranes and reduce use of glucose for synthesis of the tricarboxylic acid intermediates which are needed for ketone oxidation.

Consequences of severe insulin deficiency on fat metabolism are summarized in Table 5.3.

Body fat stores are depleted even when food intake is high. Fat destruction contributes minimally to glucose supplies for the brain, since only the glycerol component can be used for net synthesis of carbohydrate.

Insulin Deficiency and Obesity. Several kinds of explanations have been offered for the occurrence of obesity in patients suffering from maturity-onset diabetes mellitus. The first relates to "pancreatic reserve." Excessive food consumption increases the need for insulin and provides the stimulus for its secretion. (Most forms of obesity in experimental animals are known to be associated with hypersecretion of insulin, and the hormone concentrations in the blood usually return to normal limits when food intake is restricted.)

Up to a certain point the β -cells of the pancreas are capable of meeting the increased demand; the islets enlarge and secrete larger quantities of the hormone. In some individuals this can be sustained indefinitely, but in others (presumably suffering from genetic defects) the islets become exhausted. (Forced feeding of high glucose diets can damage islet cells in experimental animals.)

It is possible therefore that obesity develops during the period in which high insulin

TABLE 5-3
Consequences of Insulin Deficiency Effects on Adipose Tissue

Decreased triacylglycerol synthesis because of unavailability of glycerophosphate, combined with unopposed action of hormones which increase adipose tissue lipase activity

↓

Release of large quantities of long chain fatty acids into bloodstream

↓

Uptake of long chain fatty acids by liver cells; conversion of long chain fatty acids to ketones; release of ketones into blood stream in greater quantities than utilized by peripheral tissues; inhibition of ketone use for fatty acid synthesis

↓

Ketonemia and acidosis; neutralization of fatty acids with sodium bicarbonate of blood plasma

↓

Ketonuria, sodium depletion, diuresis

↓

Death from acidosis and dehydration

secretion is maintained. If the patient seeks out treatment soon after insulin secretion lags behind metabolic requirements, then he may retain the preestablished obese condition and avoid lipid depletion because of exogenous insulin. In some patients simple dietary restriction is all that is needed to restore metabolic balance, presumably because this lowers insulin needs to levels that can be supplied by the pancreas.

The situation is probably not so simple in most cases. Development of obesity has often been attributed to "gluttony" and "laziness." The possibility that mild *insulin deficiency contributes to the development of the obese state* is worth considering. Fat cells are especially sensitive to actions of insulin. It is conceivable that amounts of insulin secreted are sufficient for maintaining lipid stores, but that insulin-stimulated glucose uptake by other tissues is inadequate. This could lead to inappropriate hunger and muscle weakness, and also drowsiness because of elevation of blood glucose concentrations. A related concept suggests that *adipose tissue cells of some individuals possess greater than normal sensitivity to insulin* (while liver and muscle cells do not), or that adipose tissue is excessively efficient at liberation of insulin from binding to plasma proteins. The problem could be further aggravated by the buildup of adipose tissue, since the large surface of the latter can bind large quantities of the hormone.

Other kinds of explanations are required to reconcile the finding that many patients with maturity-onset diabetes have high circulating concentrations of insulin and reduced insulin sensitivity. (Insulin sensitivity can often be increased in obese diabetics when food intake is restricted.) Current research on physiology and pathology of insulin receptors will probably supply answers to many questions.

It has become increasingly apparent that diabetes mellitus is a disease which involves more than simple insulin deficiency. Genetic defects which lead to secretion of less insulin than is required for metabolic needs could simultaneously create disorders unrelated to insulin deficiency. Pancreatectomy of experimental animals induces metabolic defects mentioned above and others to be described; however, the

animals do not develop a number of pathological conditions commonly found in diabetic patients. Many patients suffer from defects in capillary and muscle membranes which could contribute to insulin resistance. (Abnormalities have been confirmed with electron microscopy).

Epidemiological data accumulated over several decades is consistent with an infectious etiology of at least some forms of diabetes mellitus.^{18, 21} Associations have been made with the mumps virus, and there are indications that the infection may exert long-range influences with delayed onset of clinical symptoms of relative insulin deficiency. It is possible therefore, that some individuals who incidentally happen to be obese have acquired the disease for reasons unrelated to previous metabolic history. It is also possible that individuals with high caloric intake or with other reasons for obesity have a predisposition which is realized in the presence of the virus.

Other ideas have been presented, but have received little experimental confirmation. These include the possibility that *abnormal insulins*^{1d} (with exaggerated affinity for adipocytes and reduced binding to other cells) are elaborated in some individuals, and that genetic differences in target organs affect the ability to utilize insulin.

A recent suggestion is that diabetics suffer from defects in synthesis or activation of adenylate cyclase in adipose tissue, and therefore have difficulty mobilizing lipids for metabolic use. (However, *accelerated lipolysis has been found in cells from some obese subjects*).

It is likely that the clinical combination of obesity and diabetes mellitus has multiple origins. In some individuals, reduced secretion of lipolytic hormones or defective sympathetic innervation to adipose tissue may exaggerate the lipogenic and antilipolytic actions of insulin. In others, excessive secretion of growth hormone or of glucocorticoids or some growth hormone abnormality⁹ may contribute substantially to hyperglycemia and insulin resistance under conditions in which fat reserves are permitted to accumulate.

Long range Adaptive Functions of Insulin. In normal individuals, a sudden increase in the carbohydrate or caloric content of the diet can precipitate temporary hyperglycemia. But insulin secretion

soon increases and is maintained at a higher rate if the diet is continued. In a relatively short period of time insulin-induced enzyme synthesis in liver, skeletal muscle, and adipose tissue provide, for more efficient food utilization and storage. It then becomes more difficult to induce hyperglycemia; and intense hunger may be experienced for a time if food intake is precipitously decreased.

Conversely, prolonged periods of fasting or ingestion of low calorie, low carbohydrate diets reduce the need for insulin, and insulin levels in the blood decline. Soon the ability to rapidly utilize food decreases, and the body becomes more dependent upon endogenous lipids for fuel. For a time only minimal quantities of carbohydrates are used by muscle and adipose tissue, while glucose supply to neurons is maintained. During prolonged starvation insulin secretion falls to undetectable levels, and even neurons begin to burn fatty acids (but their function may be compromised). Some individuals experience loss of appetite and distortion of the sense of taste, irritability, and difficulty concentrating on mental work. Under these conditions ingestion of even small quantities of carbohydrate may evoke hyperglycemia and glycosuria. The term "hunger diabetes" has been applied to this reversible condition.

Insulin and Protein Metabolism^{1k, 1q}

Insulin directly enhances the uptake of certain rate-limiting amino acids (and also of non-metabolizable AIB), even in the presence of inhibitors of protein synthesis. Influences on appetite, ATP availability, ribosomal functions (p. 28) and on synthesis and activities of key enzymes are described in several places. Synergisms with anabolic functions of androgens and somatotrophin, and antagonism of gluconeogenic actions of glucagon and glucocorticoids are associated with decreased non-protein nitrogen content of the blood and urine.

BIOSYNTHESIS, CHEMISTRY, AND METABOLISM OF INSULIN^{1c, 1d, 1o, 2}

All of the known vertebrate insulins consist of a 20 or 21 amino acid *A chain* joined by two disulfide bridges to a 29-31

amino acid *B chain*; a third disulfide bridge is formed by cysteine moieties of the *A chain*. Approximately 25 of the amino acid components appear to be identical in all species (from hagfishes to man).

Species differences in amino acid composition affect potency in bioassays, but all insulins exert some typical hormone actions in very different as well as in closely related species. (Guinea pigs and coyotes exhibit some obvious genetic peculiarities; insulins from these animals differ from most mammalian insulins, and these animals exhibit poor responses to hormones derived from other species.)

Immunological differences are of practical as well as theoretical interest. Patients on long term hormone therapy sometimes develop high antibody titers; in some cases it becomes advantageous to switch to insulin from different sources.

Some species synthesize more than one insulin.^{1c} Two have been identified in the rat, the mouse, and some of the fishes. It has been proposed that some patients with maturity-onset diabetes associated with high concentrations of hormone in the plasma are producing abnormal insulins. The finding that insulins from some juvenile diabetics exhibit deviations in susceptibility to insulinase has spurred interest in the problem. Unfortunately, the technical difficulties involved in the investigation are formidable.

Insulin is not directly synthesized as such; a large linear peptide, *proinsulin* is formed on the ribosomes and is later folded for formation of the disulfide bonds. Converting enzymes (identified in Golgi vesicles and also in immature secretion granules) then split proinsulin at two points yielding the active hormone and a connecting peptide or *C chain* (Fig. 5-2).

Proinsulin, partially cleaved proinsulin, insulin, and the C peptide are all present in secretion granules, and all are released during normal processes of hormone secretion.

Questions have been raised about possible metabolic functions of proinsulins, since they exhibit weak insulin-like activity and are found in high concentrations in the blood plasma during fasting. (But prohormone accumulation could result from a slow rate of degradation.)

Immunological procedures can distinguish between proinsulins and insulin. Diabetic patients receiving purified insulin preparations develop antibodies which interfere with immunoassay of native insulin. Methods specific

INSULIN

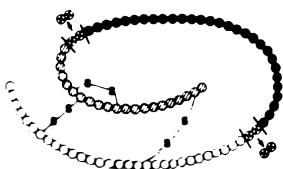


FIG. 5.2. Relationships between proinsulin and the A and B chains of insulin. Each circle represents one amino acid residue. The black circles belong to the C chain or connecting peptide; the striped circles show the A chain, and the B chain is represented by clear circles. Circles with x represent dipeptides split off during formation of active hormone.

for determinants on the C segment have proven useful for measurement of endogenous secretion in such patients. Certain differences between immunological and bioassay measurements are not readily explainable. Blood plasma contains substances which have *in vitro insulin-like activity (ILA)*.¹³ Some effects can be abolished by specific insulin antibodies, but most (the NSILA or nonsuppressible insulin-like activity) cannot. The origin and importance of these substances are being debated. Evidence favors the concept that these materials do not contribute to insulin function. They have been identified by some investigators with the somatomedins (Chapter 10). A soluble fraction (NSILA_s) with limited influence on metabolism but high growth-promoting potency competes with both insulin and somatomedins for receptors in some cell types.²²

Aggregation of insulin molecules has been studied under a variety of conditions. It is believed that hexamer forms stabilized by zinc are present in the islet cell.⁹ This permits compact storage and probably protects the molecules against degradation. Dimers may form in physiological solutions, but the monomer seems to be the active form of the hormone, and only monomers have been found when insulin concentrations are low. Aggregation influences antigenicity as well as activity.

Insulin is secreted into vessels which lead into the hepatic portal system. The liver is believed to take up and destroy about half the insulin it receives, and *insulinases* have been studied by several investigators. Excessive insulinase activity has been proposed as a possible cause of diabetes mellitus, but there is no laboratory support for the suggestion.

Insulin combines with plasma proteins, and the combination must be broken before insulin can act on target organs.

The kidneys remove and degrade about one-third of the plasma insulin. Usually only traces of the hormone appear in the urine, but larger quantities are found after injection of exogenous insulin.

REGULATION OF INSULIN SECRETION^{10, 11, 12, 13}

Packaging, storage, and release processes resemble those described for other endocrine glands (Chapter 1).

Hormone release is associated with depolarization of the β -cell membrane, formation of microvilli at the cell surface, and inward movement of sodium and calcium ions. Calcium is an essential requirement in the plasma (or in the medium bathing *in vitro* preparations). A contractile process for migration of the secretory granules toward the plasma membrane mediated by the microtubule system and triggered by calcium has been proposed.¹⁰ High extracellular concentrations of magnesium block hormone secretion.^{11, 14} ATP seems to be required for several aspects of hormone secretion. Anoxia and agents which impair oxidative phosphorylation block islet cell responses to the usual stimuli.

Importance of Plasma Glucose Concentrations

Glucose provides the major stimulus for insulin secretion in mammals that regularly consume carbohydrates and absorb glucose from the small intestine. Unlike other stimuli, it also promotes insulin synthesis. Influences on quality and quantity of RNA synthesized by islet cells and on post-transcriptional events have been demonstrated.

Glucose also plays a role in induction of its own receptor. Starvation and low carbohydrate diets decrease sensitivity to the glucose stimulus; responses can be restored within 24 hr by feeding a high carbohydrate diet, but restoration is blocked by simultaneous administration of inhibitors of protein synthesis.

Studies of electrical activity of the islet cells indicate that a small number respond to low glucose concentrations, and that elevation of the concentrations leads to recruitment of additional cells. By contrast, low concentrations of galactose affect a few insulin-secreting cells but higher concentrations of this sugar elicit no

greater response. Pancreatic receptors have an even higher affinity for mannose than for glucose but relatively low affinity for fructose. Data are consistent with stimulation of insulin secretion following ingestion of any of the sugars tested.¹¹

When β -cells are exposed *in vitro* to concentrations of glucose within the "physiological" range, 2-5% of the insulin content is discharged within 2 min. A "refractory period" then ensues, in which responses to otherwise potent stimuli are dampened. Continued exposure to glucose then promotes a second, *sustained* secretion at a rate intermediary between the initial rapid phase and the basal output. Up to 20% of insulin content may be depleted within an hour. When very high glucose concentrations are administered (300-500 mg/100 ml), a progressive further rise in secretion rate can be achieved, and a plateau several times higher than the basal rate is maintained for many hours.¹²

The early rapid response could result from discharge of insulin from granules close to the cell periphery, while the second response may require prior movement of more centrally located granules. (Morphological studies indicate that severe degranulation does not occur even after prolonged high rates of secretion.)

An alternate suggestion is that the early response sets up an internal feedback inhibition which suppresses further rapid discharge. This could explain the refractory period. Presumably, internal adjustments then "reset" the cells for sustained secretion at a lower rate. The delayed response could involve different receptors. A mechanism of this type might protect against a hyperglycemic surge during the first minutes of absorption of a carbohydrate-rich meal without danger of hypoglycemic overshoot; then, with onset of insulin actions on skeletal muscle, liver, and adipose tissue, a slower discharge rate could maintain metabolic function.

Inhibitors of protein synthesis do not diminish the early response but may reduce or abolish the slow (second) phase of insulin release. This has been interpreted as evidence for either an association between the late response and the synthesis of new insulin or alternatively as a need for synthesis of some specific protein involved in the prolonged response. Tolbutamide (an orally effective agent which has been used without insulin in treatment of mild maturity-onset diabetes) stimulates insulin release but not synthesis. It promotes the rapid insulin response consistently but may also induce a more sustained secretion. Most authors seem to favor the concept that insulin released for at least the first hour is predominately preformed insulin, and that additional time must elapse before appreciable quantities of new insulin can

be secreted. The belief is supported by information on the time required for radioactively labeled amino acids to appear in the secretory product, and because there is no increase in the relative quantities of proinsulin secreted. On the other hand, the late (third) response to sustained administration of high glucose concentrations seems to depend on secretion of insulin newly synthesized after the first hour.

There is an unsettled question about whether glucose itself or some metabolite of glucose actually provides the trigger for insulin release.¹³ The question is raised because certain inhibitors of hexose phosphorylation abolish the islet cell response to sugars. There are also interesting questions about why glucose preferentially stimulates insulin synthesis without exerting obvious influences on synthesis of other proteins.

Importance of Foods Other Than Glucose

Amino Acids. Arginine is a potent stimulator of insulin release. Lysine is almost as potent, leucine and phenylalanine somewhat less so, and valine and methionine only moderately effective. Other amino acids tested, e.g., histidine, do not seem to affect insulin secretion.

As with glucose, amino acids promote depolarization of the islet cell membranes, and also calcium and sodium uptake. Low to moderate concentrations of amino acids synergize with submaximal glucose concentrations, but amino acids are also effective in the absence of glucose and also in the presence of agents (such as mannoheptose) which block responsiveness to glucose. They do not exert further stimulatory actions when maximal concentrations of glucose are administered.

Studies with specific inhibitors suggest that there is more than one type of amino acid receptor, and that the receptors are different from those sensitive to glucose. Leucine stimulation can be potentiated by agents which do not influence arginine stimulation. Arginine affects mostly the second phase of insulin release; it must be transported across the cell membrane to act, but it need not require metabolic transformation.

Amino acid stimulation is not impaired by long term administration of a low carbohydrate diet, and it is possible that amino acid regulation is especially important under such conditions. This type of regulation could function regularly in carnivores, since they consume little carbohydrate.

Lipids. Ruminants (cows, sheep, etc.) derive most of their energy from short-

chain fatty acids. Propionate and butyrate (formed in large quantities by gastrointestinal bacteria) are potent stimulators of insulin release in these animals and also in dogs.

Many obese humans with high circulating insulin and fatty acid concentrations fail to reduce insulin output in response to low carbohydrate diets. It is possible that such individuals have hormone receptors resembling those of ruminants.

CAMP and Insulin Secretion^{10, 11}

Insulin secretion is stimulated by many agents known to activate adenylate cyclase (including glucagon, adrenocorticotropic hormone, thyroid stimulating hormone, and isoproterenol) and also by N⁶O^{3'}-dibutyryl cAMP (D-cAMP) and agents which retard cAMP degradation.

The "oral hypoglycemic agents," tolbutamide and chlorpropamide, are believed to promote insulin release through inhibition of phosphodiesterases. Theophylline and caffeine act similarly, but they may also directly alter β -cell plasma membranes.

Agents elevating cAMP synergize with submaximal concentrations of glucose or amino acids but are ineffective in the absence of glucose. It has therefore been proposed that cAMP functions to modulate influences of other stimuli and especially of glucose. The nucleotide may promote phosphorylation of membrane proteins and thereby favor fusion of secretory granule and plasma membranes. Another possibility is that influences on calcium distribution induce changes in the microtubules.

Although epinephrine stimulates adenylate cyclase in many cell types, it is a potent inhibitor of insulin release (see below).

Nervous Control of Insulin Secretion^{12, 13}

Administration of parasympathomimetic agents or stimulation of vagus nerves to the beta cells promotes insulin secretion; the responses can be blocked with atropine (which interferes with acetylcholine actions).

Nervous stimulation of insulin release during feeding can provide for "anticipatory" secretion which need not await elevation of plasma glucose or amino acid concentrations. Conditioned release of insulin

has been developed in experimental animals. A similar mechanism may underlie clinical hyperinsulinism which some individuals exhibit in response to stress. High insulin secretion rates in animals with hypothalamic lesions probably result from parasympathetic overactivity.

Importance of Other Hormones

Gastrointestinal Peptides.^{14, 15, 16}

Food stimuli (glucose, amino acids, etc.) are far more potent insulin releasers when given orally than when injected into the bloodstream. Orally administered glucose is also more rapidly utilized. Parasympathetic nerves are stimulated by food ingestion; but release of gastrointestinal hormones probably has far greater influence on insulin release. An old concept that gastrointestinal and islet-cell functions are closely interrelated in mammals (as they are in some "lower" vertebrates) was replaced for a time with the belief that gut hormones act only on the gut. But the original concept is now being revived.

Release of insulin in response to gastrointestinal hormones could be important for efficient utilization of food materials during the period preceding an actual rise in postfeeding blood glucose, especially when the meal is rich in protein and lipid foods. Since the effects of the gastrointestinal peptides seem to be of long duration, they could also ensure continued insulin secretion when food materials are still abundant but effects of insulin on muscle, adipose tissue, and liver tend to lower blood glucose concentrations below levels providing adequate direct stimulation to the β -cells.

A gastrointestinal peptide *enteroglucagon* is released during feeding and is a potent stimulant for insulin release. Two fractions with immunological properties in common with glucagon (described in Chapter 6) and therefore referred to as having glucagon-like immunoreactivity (GLI) have been identified; one is devoid of glucagon type actions on the liver.

Secretin promotes insulin secretion in the intact animal and is also effective *in vitro* when tested on pieces of pancreatic tissue. But it does not stimulate insulin secretion in isolated islets and has no measurable influence on pancreatic glucagon output. Presumably the effect on in-

sulin is indirect and requires exocrine cells.

Intravenous injection of *gastrin* preparations can lead to increased β -cell release of insulin; it has therefore been suggested that gastrin plays a role in β -cell responses to feeding. But *in vitro* exposure of islet cells to gastrin has not uniformly resulted in stimulation of insulin release. In fact, even inhibitory influences have been observed. Some authors believe, therefore, that gastrin effects on insulin secretion may depend on indirect mechanisms, especially on increased *secretin* release resulting from gastrin stimulation of hydrochloric acid output. The effects of gastrin are difficult to assess because a small percentage of the islet cells (the D, Δ , or α_1) cells synthesize gastrin (or something similar to it) and local effects of pancreatic gastrin have not been sufficiently studied.

Cholecystokinin-pancreozymin stimulates insulin release both *in vivo* and *in vitro*. It also promotes secretion of pancreatic glucagon but probably affects the β -cells in an independent manner, since it is effective in the complete absence of glucose, while glucagon is effective only when glucose is present.

The gastrointestinal hormones stimulate β -cell adenylate cyclase, and their actions may result from elevation of intracellular cAMP. They affect release but not *de novo* synthesis of insulin.

Pancreatic Glucagon. Pancreatic glucagon secretion is stimulated by feeding of amino acids and proteins. Glucagon may act both locally and systemically to promote insulin release and also biosynthesis, but this occurs only when glucose levels are adequate. Although the two islet hormones exert opposing metabolic actions, there are conditions under which the simultaneous presence of both may be required (Chapter 6). Glucagon does not stimulate insulin release during starvation.

Growth Hormone (Somatotrophin, STH). Interrelationships between insulin and STH are complex (Chapter 10). STH stimulates insulin secretion; it has been implicated in directly promoting hormone biosynthesis and also in exerting a "permissive" role for responses to other stimuli. In addition it tends to elevate plasma glucose and fatty acid concentrations and may enhance glucagon release. Some actions of STH increase the need for insulin.

Chronic administration of large doses of STH can induce exhaustion of the β -cells. There may be some connection between this phenomenon and clinical observations that onset of juvenile-type diabetes char-

acteristically follows a growth spurt in children with genetically defective β -cells. Plasma STH levels are not consistently elevated in such children.

Miscellaneous Hormone Effects. Several hormones exert indirect stimulation of insulin secretion. These include *thyroxine*, *glucocorticoids*, and *sex steroids*. Prolonged use of oral contraceptives is associated with modest elevation of plasma insulin concentrations in some individuals, and may rarely promote islet cell exhaustion.¹⁷

Inhibition of Insulin Secretion. Adrenomedullary release of *epinephrine* occurs in response to stress, during exercise when blood glucose concentrations are declining, and at other times when it would be physiologically advantageous to suppress insulin release (Chapter 9). Epinephrine inhibits insulin secretion. (Epinephrine interaction with adrenergic α -receptors leading to inhibition of adenylate cyclase are described in Chapter 9.) *Angiotensin II* (Chapter 11) can elevate plasma glucose concentrations and induce glycosuria. A combination of direct inhibition of insulin release, stimulation of epinephrine secretion, and direct actions on the kidney have been implicated. Severe parathyroid hormone deficiency may indirectly inhibit insulin secretion through reduction of plasma calcium ion concentrations.

Insulin may also exert *localized inhibitory control* over its own secretion. The concept is based on effects of *in vitro* exposure of islet cells to high hormone concentrations.

Recently it has been shown that *somatostatin*, a hypothalamic peptide which inhibits adenohypophyseal secretion of somatotrophin, inhibits insulin secretion in a dose-related manner.⁸ The action seems to be exerted directly on the islet cells of the pancreas. Both basal insulin secretion and the responses to arginine are affected. (Somatostatin also inhibits pancreatic release of glucagon.) The presence of somatostatin in the peripheral circulation remains to be demonstrated. The hormone has been identified in the gastro-intestinal tract and in pancreatic islets, and has been shown to influence secretion of gastrointestinal hormones.²² Factors reported to influence insulin secretion are summarized in Table 5-4.

TABLE 5-4
Factors Influencing Insulin Secretion

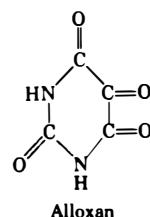
A.	Physiological factors believed to stimulate insulin secretion
1.	Elevation of blood glucose concentrations
2.	Ingestion of food containing carbohydrates, certain amino acids
3.	Ingestion of fatty acids and high levels of plasma free fatty acids in some individuals
4.	Gastrointestinal hormones
5.	Stimulation of vagus nerves to the islet cells
6.	Glucagon
B.	Physiological factors associated with indirect stimulation of insulin secretion
1.	Secretion of increased quantities of <u>STH</u> , thyroid hormones, glucocorticoids, sex hormones
C.	Pharmacological agents stimulating insulin secretion
1.	Dibutyryl cAMP
2.	Theophylline, caffeine, and other agents which elevate or maintain intracellular cAMP
3.	Alpha-adrenergic blocking agents
4.	Parasympathomimetic agents
5.	Tolbutamide
6.	Some vasodilators: histamine, etc.
7.	Ethyl alcohol
D.	Physiological inhibitors of insulin secretion
1.	Epinephrine (and to a lesser extent, norepinephrine)
2.	Possibly insulin itself, acting locally
3.	Starvation and prolonged low carbohydrate diet
4.	Somatostatin
E.	Pharmacological agents and other conditions reducing insulin secretion
1.	Atropine
2.	Beta-adrenergic blocking agents
3.	Calcium-lowering agents (and severe hypocalcemia)
4.	Some vasoconstrictors, e.g. angiotensin II.

prolonged manipulation under deep anesthesia and excision of the *exocrine* pancreas. Damage to neighboring structures is usually unavoidable. The exocrine pancreas secretes enzymes needed for digestion of proteins, carbohydrates, and lipids, and possibly additional agents which function in lipid mobilization. Animals deprived of this organ are delicate and require specialized nutrition.

Removal of insulin-secreting cells is simpler in some of the fish in which endocrine and exocrine organs are anatomically separated. But functions of insulin in fishes seem to be very different from those in mammals, and fish studies provide relatively little information on metabolic regulation of mammalian metabolism.

Experimental insulin deficiency is more commonly achieved in warm-blooded animals by administration of agents which selectively destroy the β -cells of the pancreas (although all pharmacological agents may be expected to exert actions on structures other than those under investigation).

Alloxan has proven useful for selectively destroying insulin-secreting cells while leaving glucagon-secreting cells of the islets intact. A single dose usually produces the desired effects (but it may also injure bone marrow cells). In mammals, alloxan administration and pancreatectomy produce qualitatively similar effects. In some birds and reptiles, however, pancreatectomy leads to *hypoglycemia* because loss of glucagon (Chapter 6) is of greater importance than loss of insulin.



It is often useful to induce less severe insulin deficiency states than those imposed in mammals by either removal of the pancreas or administration of alloxan. *Streptozotocin* has been satisfactorily used for this purpose.

Anti-insulin sera induce insulin deficiency free of complications imposed by

EXPERIMENTAL INSULIN DEFICIENCY

The most obvious procedure for preparation of experimental animals with specific hormonal deficiencies is removal of the associated endocrine gland. However, surgical ablation of the pancreas is a difficult and hazardous procedure. In most species the pancreas is an extensive, diffuse structure; complete removal usually requires

surgery or administration of pharmacological agents. (But antibodies can interfere only with those molecules with which they come in contact.) Agents (*e.g.*, mannoheptose) are available which suppress secretory responses of β -cells to the usual stimuli. β -cell exhaustion can be achieved by prolonged administration of somatotrophin ("metahypophysial diabetes") or glucocorticoids or by chronic force-feeding of large quantities of glucose. There are marked species (and strain) differences in susceptibility to agents acting via exhaustion.

DIABETES MELLITUS^{10, 11}

As noted, diabetes is a *clinical* condition in which metabolic disorders resembling those induced by insulin deficiency are seen and in which correction of such disorders can be achieved by administration of exogenous hormone. But the clinical disease cannot be equated with insulin deficiency. Many patients have high circulating titers of insulin; and most that require insulin therapy need more hormone than is secreted by the β -cells in normal individuals. Diabetes mellitus patients usually suffer from a variety of pathological problems (especially *microangiopathy*) which can be detected before metabolic symptoms become prominent. Such problems are not precipitated in experimental animals following insulin deprivation; and they cannot be prevented or ameliorated in patients by administration of insulin in sufficient dosage to maintain normal blood glucose concentrations.

Some of the possible causative factors were mentioned above (pp. 44-47). There are clinicians who believe that *long term* insulin deficiency (and especially effects of hormone deficiency on *mucopolysaccharide* and protein metabolism) lead ultimately to *thickening of capillary basement membranes*. Poor responses of insulin target organs to actions of insulin ("insulin resistance") and inadequate responses of islet cells to the usual physiological stimuli are therefore regarded as secondary manifestations of the capillary defect. A related concept is that genetic disorders lead to formation of an abnormal fragment of somatotrophin (or to excessive accumula-

tion of or hypersensitivity to a normally formed fragment) and that this in turn promotes defective collagen synthesis and thickening of the capillary membranes.¹⁰ Others believe that the genetic defect which impairs islet cell function also affects capillary structure.

It is likely that etiology varies from patient to patient. The possibility of infectious origin of the disease was mentioned above; a genetic component may increase islet-cell susceptibility to viral damage. *Autoimmune disorders and hypersecretion of hormones antagonizing insulin actions* probably play a role in some individuals.

Treatment of the clinical disease usually involves regulation of the diet. *Restriction of carbohydrate and calorie content* may constitute adequate treatment in mild cases; but most patients require some measure to counteract absolute or relative insulin deficiency. Several types of insulin preparations are available which differ from each other in absorbability. Use of suitable combinations can minimize the number of injections needed to maintain metabolic control.

Repeated insulin injection is inconvenient and painful and often provokes localized reactions. There has been considerable use of "oral hypoglycemic agents" in patients with mild, maturity-onset disease. *Tolbutamide* (orinase) is one of the sulfonylurea group of agents which has been widely used; but recent data have raised the possibility that such drugs affect the heart and shorten survival. Tolbutamide stimulates insulin secretion when the islet cells are sluggish but have a measure of reserve, and it enhances insulin secretion in previously normal experimental animals. It also exerts limited insulin-like actions, *e.g.*, reduction of hepatic release of glucose; but it cannot maintain life of pancreatectomized animals.

Phenformin is an example of biguanide type agents which have been used to exert insulin-like actions in patients with poor pancreatic reserve; it is effective in pancreatectomized animals. Early hopes that it would reduce obesity of patients with maturity-onset disease have not been realized. Use is limited by occurrence in many patients of unpleasant "side effects" including gastrointestinal upsets and nausea.

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6. Glucagon

HORMONAL PROTECTION AGAINST HYPOGLYCEMIA

Although actions of insulin are indispensable for survival of most mammals, they could, if unopposed, lead rapidly to death from hypoglycemia. Several hormones are required to ensure that adequate glucose concentrations will be maintained.

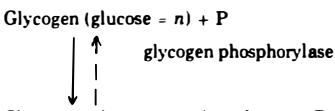
Glucagon secretion is triggered by a fall in glucose concentrations; most of its actions lead to increased sugar availability. The hormone assumes greatest importance at times when glycogen reserves have been built up from the previous meal, and completion of carbohydrate absorption leads to a shift from rising to falling blood sugar concentrations. It is secreted by the α_2 -cells of pancreatic islets¹ and is transported via hepatic portal vessels to the liver.

Glucagon and Hepatic

Phosphorylase ^{IC, 1d, 2, 8, 10}

Enzymes influence the rates at which equilibria are attained in reversible reactions; they do not affect equilibrium point concentrations of substrates and reaction products. It is evident, therefore, that metabolic control requires use of one pathway and set of enzymes for synthesis and a different pathway for degradation. The *glycogen synthetase* pathway favoring glycogen formation was described in detail in the previous chapter. Glycogenolysis is catalyzed by the *phosphorylase system*. Although the latter has been studied most extensively in skeletal muscle (and enzymes involved differ in certain properties such as molecular weight), control mechanisms in liver are in many ways similar.

Glycogen phosphorylase catalyzes the following reaction between glycogen and inorganic phosphate (P):



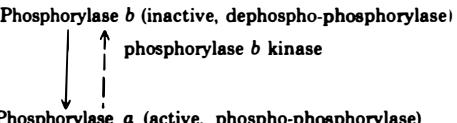
The equilibrium point of the reaction strongly favors glycogenolysis.

Since glucose may be suddenly needed,

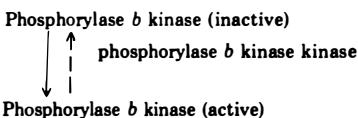
it is essential that the phosphorylase enzyme be continuously available. (Emergency mechanisms never depend upon synthesis of new enzymes.) Controls are needed to suppress phosphorylase activity when the blood glucose concentration rises and when conditions favor glycogen synthesis.

Phosphorylase is usually present in an inactive or *b* form, ready to be converted to the active or *a* form at any moment. The conversion is catalyzed by the *phosphorylase b kinase enzyme*.

In common with other kinase reactions, ATP is utilized. Both magnesium and calcium are required as cofactors:



Phosphorylase *b* kinase is also usually present in inactive form. Its conversion is catalyzed by *phosphorylase b kinase kinase* (also known as kinase II):



In addition to ATP and magnesium, the kinase II enzyme requires cAMP as an activator; this provides a mechanism for efficient metabolic control: (1) glucagon activates liver adenylate cyclase; this leads to elevation of intracellular cAMP concentrations and calcium ion uptake; (2) cAMP serves as a cofactor for action of phosphorylase *b* kinase kinase; (3) phosphorylase *b* kinase kinase catalyzes activation of phosphorylase *b* kinase; (4) in the presence of increased Ca^{++} concentrations, the phosphorylase *b* kinase catalyzes activation of phosphorylase *b* (transformation of phosphorylase *b* to phosphorylase *a*); and (5) phosphorylase *a* catalyzes glycogenolysis.

The cascade of reactions is summarized in Figure 6-1.

Control is accomplished in several ways: (1) The signal for glucose need almost instantaneously triggers release of a hormone which travels directly to the liver and rapidly promotes activation of the phos-

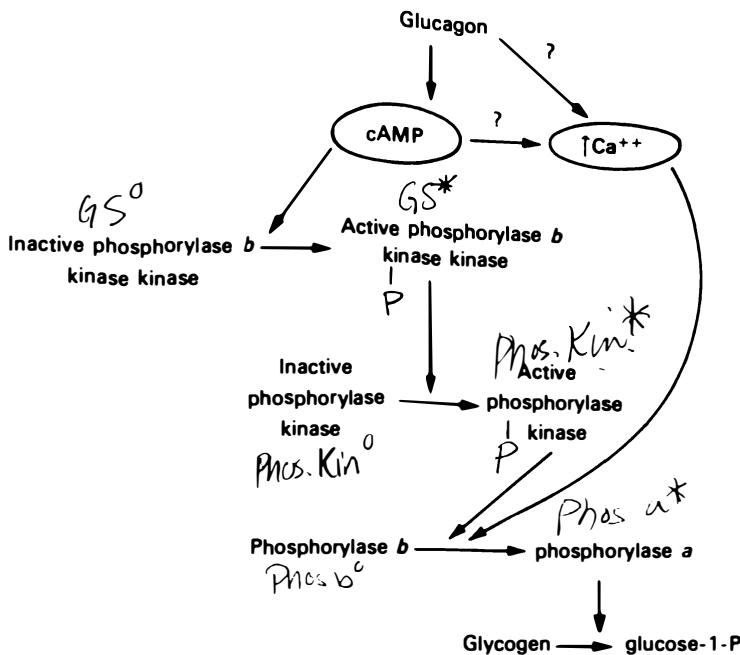


FIG. 6-1. Glucagon stimulation of glycogenolysis.

phorylase system through formation of cAMP and calcium uptake. Activation continues as long as the glucose need persists. But a marked rise in plasma glucose concentration shuts off the hormone signal, while cytosol phosphodiesterases destroy cAMP and provide for rapid shutdown. (2) Several successive steps are involved in the activation of the final enzyme of the pathway. This provides for *amplification*: a very small quantity of cAMP activates kinase II; the latter activates relatively large amounts of phosphorylase b kinase; this leads, in turn, to formation of still larger amounts of phosphorylase a. Therefore it is possible to achieve formation of substantial quantities of glucose-phosphate within moments after the need has arisen.

There are times when rapid shutoff is important, e.g., soon after drinking the breakfast fruit juice. Additional controls assist in rapid termination of glycogen breakdown:

Phosphorylase a spontaneously reverts slowly to phosphorylase b when phosphorylase b kinase is inactive. The reaction is speeded by the

phosphorylase phosphatase enzyme:

Phosphorylase a

phosphorylase phosphatase

← → Phosphorylase b

Phosphorylase b kinase reverts to the inactive form when there is reduction of activity of the kinase kinase enzyme. The latter, in turn, becomes nonfunctional when cAMP levels fall; moreover, a protein which interferes with cAMP interaction with the kinase kinase has recently been identified.

Interaction of Glucagon and Insulin in Regulation of Hepatic Glycogenolysis^{1d}

Activation of the glycogenolytic system involves *phosphorylation of enzymes*, while inactivation requires *dephosphorylation*.

Phosphorylase b kinase kinase is the same enzyme as glycogen I synthetase kinase (Chapter 5). Therefore, when glucagon activates the glycogen degrading system, it simultaneously inhibits the glyco-gen-synthesizing apparatus. Similarly, as

insulin promotes glycogen formation, it simultaneously inhibits glycogen breakdown. The interactions are accomplished through influences of both hormones on both phosphorylases and phosphatases. (Glucagon may also impair insulin interaction with its receptors.)

Insulin reduction of cAMP effectiveness has been variously attributed to any or a combination of the following: inhibition of adenylate cyclase, stimulation of phosphodiesterases, interference with actions of existing cAMP, and conversion of cAMP to some (unidentified) less active form.

The hormonal influences are summarized in Table 6-1.

Three enzymes are involved in regulation of glycogenolysis but only two in glycogenesis. It has been suggested that this relates to the more urgent need for amplification of events which protect against hypoglycemia.

Although the phosphorylase system of skeletal muscle resembles that of liver (and also requires cAMP), glucagon does not control muscle phosphorylase. This is because glucagon travels directly from the pancreas to the liver, and very little of the hormone ever reaches muscle cells; more-

over, muscle cell membranes lack high affinity receptors for glucagon. Muscle and liver glycogens subserve different functions. Major control over the muscle system is exerted by epinephrine (Chapter 9).

Although glucagon and insulin exert opposing actions on most metabolic processes, glucagon stimulates insulin secretion. There are two conditions under which this is advantageous: (1) Glucagon is needed when insulin secretion is released in response to intestinal absorption of amino acids. (2) Since the sensitivity of muscle cells to insulin is greater than the sensitivity of hepatic cells, minute quantities of insulin in combination with glucagon provide optimal conditions for muscle use of glucose derived from hepatic glycogen reserves.

Glucose 6-Phosphatase Activity

Glucose 1-phosphate derived from hepatic glycogenolysis is rapidly converted first to glucose 6-phosphate and then to free glucose. The final step is catalyzed by the enzyme glucose-6-phosphatase:

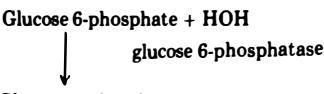


TABLE 6-1

Reciprocal Control of Glycogenesis and Glycogenolysis in Liver by Glucagon and Insulin

	Glucagon	Insulin
cAMP concentration and effectiveness	↑	↓
Phosphorylase b kinase kinase activity	↑	↓
Phosphorylase b kinase activity	↑	↓
Phosphorylase phosphatase activity	↓	↑
Phosphorylase b → a	↑	↓
Glycogen → glucose-P	↑	↓
Glycogen synthetase kinase activity	↑	↓
Glycogen synthetase phosphatase activity	↓	↑
Glycogen synthetase I → D	↑	↓
Glucose-P → glycogen	↓	↑

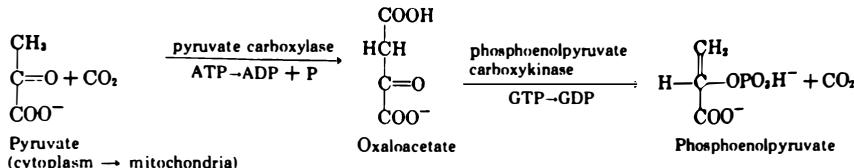
Activity of the glucose-6-phosphatase is increased by glucagon (and cAMP); insulin reduces activity (and synthesis) of the enzyme, possibly through its influence on reduction of cAMP concentrations. Thus, reciprocal control by insulin and glucagon are exerted on the product as well as the process of glycogenolysis. Glucose-6-phosphatase activity is also enhanced by other hormones which elevate blood glucose concentrations.

Other Influences of Glucagon on Carbohydrate Metabolism

Glucagon enhances synthesis of glucose from pyruvate and lactate.^{1, 9}

Proposed sites include (1) transport of pyruvate from its site of formation in the cytoplasm across mitochondrial membranes; (2) the pyruvate carboxylase enzyme; and (3) the phosphoenolpyruvate carboxykinase:

GLUCAGON



Glucagon (and cAMP) also stimulate glucose formation from amino acids and especially from alanine and arginine.

Proposed sites of action include increased rates of hepatic uptake of glucogenic amino acids, depression of amino acid incorporation into proteins, increased proteolysis (possibly through release of lysosomal proteolytic enzymes), and both activation and synthesis of tyrosine transaminase and other enzymes catalyzing amino acid deamination. Urea formation is simultaneously increased. The gluconeogenic action of glucagon is interrelated with functions of glucocorticoid hormones; it is not seen in adrenocortical insufficiency.⁷ Insulin opposes these actions.

In addition, both glucagon and cAMP stimulate activity of hepatic lipases and thereby accelerate fatty acid oxidation. Ketone formation is increased this way, and probably also through inhibition of short chain fatty acid utilization for synthesis of other lipids. These actions indirectly contribute to blood glucose elevation, since the supply of noncarbohydrate fuels to tissues with flexible requirements is increased. During the first few days of food deprivation, high glucagon levels help maintain the glucose supply to neurons.

Actions of Glucagon on Other Tissues^{1c, 8}

Pharmacological doses of glucagon affect a wide variety of cell types; but only a few observations have any relationship to physiological functions. (Supraphysiological doses stimulate the heart; attempts have been made to use them in treatment of myocardial insufficiency.)

Glucagon stimulation of lipolysis can be readily demonstrated in epididymal fat pads of rodents. The action is enhanced by inhibitors of phosphodiesterases and antagonized by some of the prostaglandins.

While lipolytic actions seem to have physiological significance in birds,¹ there is no compelling evidence for such function in

mammals. But glucagon administration has been useful for reduction of concentrations of certain plasma lipids in patients with hyperlipemic conditions. Pharmacological doses have been given to obese patients⁸ not only in the hope of accelerating breakdown of depot fat but also because glucagon depresses appetite and gastrointestinal motility. The treatment may be dangerous as well as ineffective, since large doses stimulate the heart, induce ulcer formation (although gastric acidity is diminished), and promote hypersecretion of insulin.

Influences of glucagon on calcium metabolism are described in Section IV and on excretion of water and electrolytes in Section III. Glucagon also promotes somatotrophin (STH) secretion.

Since large doses of glucagon are anti-inflammatory, they have been tested for treatment of rheumatoid arthritis. It is likely that effects are related to cAMP influences on lysosomal membranes.

REGULATION OF GLUCAGON SECRETION^{1b, 1e, 9}

The Role of Plasma Glucose Concentrations

It was previously noted that pancreatic β -cells secrete very small amounts of insulin until stimulated by rising glucose levels. By contrast, α -cells seem to secrete rather large amounts of glucagon until inhibited by rising glucose concentrations. It has been suggested that glucose provides energy to shut off the secretory processes. (But inhibitors of glycolysis do not affect release by islet cells *in vitro*.)

It has been stated that patients with diabetes mellitus maintain glucagon levels within the normal range but ability to shut off secretion of the hormone in response to hyperglycemia is defective. Others looking at the same data state that "normal" levels for individuals in hormonal balance are

inappropriately high in diabetics. High glucagon levels during meal absorption contribute to problems of relative insulin deficiency.

Calcium^{6, 8}

Calcium in the extracellular fluids is an absolute requirement for secretion of a very large number of hormones (including insulin). Glucagon is one of two hormones whose secretion is increased when plasma calcium ion concentrations are reduced. (The other is parathyroid hormone;⁹ interactions are described in Section IV.)

Amino Acids

Glucogenic amino acids promote glucagon release when glucose concentrations are low. It will be recalled that dietary amino acids also stimulate insulin secretion. Glucagon probably protects against the hypoglycemia which might otherwise occur during absorption of a low carbohydrate, protein-rich meal; it also enhances the glucose supply needed for conversion of dietary amino acids and fatty acids into proteins and certain lipids.

While arginine stimulates both insulin and glucagon release, potency of other amino acids for stimulation of the two cell types differs. For example, leucine acts on β - (but not α -) cells, while alanine and some other glucogenic amino acids promote glucagon but not insulin release.

Fatty Acids

High free fatty acid concentrations in the blood plasma inhibit release of glucagon in some carnivores. Since glucagon is lipolytic, this could provide a negative feedback mechanism for regulation of glucagon secretion; however, attempts to demonstrate the existence of such a mechanism in primates have yielded negative data.

During the early stages of food deprivation, concentrations of both glucagon and fatty acids are elevated. But during actual starvation, fatty acid concentrations can rise still higher as glucagon secretion diminishes.

Glucagon secretion can be maintained when plasma insulin concentrations are low; but animals totally deprived of insulin fail to secrete glucagon in response to an arginine stimulus. This raises the possibil-

ity that insulin exerts a "permissive action" on α -cells, and that loss of glucagon after prolonged food deprivation is partially attributable to insulin deficiency.

Gastrointestinal Hormones

Proteins and amino acids are more effective regulators of glucagon secretion when ingested than when taken parenterally, and this has been related to their strong stimulation of *pancreozymin* secretion. Pancreozymin augments amino acid influences on glucagon-secreting cells *in vitro*; it may also directly stimulate the cells. Secretin seems to reduce glucagon secretion, while gastrin has no direct influence.

Nervous Control of Glucagon Secretion³

It has been reported that sympathetic stimulation promotes glucagon secretion and alpha cell degranulation, while vagotomy may cause α -cell hypertrophy.

A six-fold increase in blood glucagon levels in response to strenuous exercise has been described in dogs and rats. (Effects were blocked by administration of phentolamine, an α -adrenergic blocking agent, but not with propanolol.)

Glucagon could effectively contribute to the metabolic roles of epinephrine during stress and exercise (Chapter 9); it may also promote epinephrine release.

Hypothalamic stimulation can release glucagon as well as insulin. Much of the earlier data is being reevaluated in the face of evidence that hypothalamic peptides directly affect pancreatic islet cells (see below).

Other Factors Influencing Glucagon Secretion⁴

Influences of somatostatin and of somatotrophin are described in Chapter 10. The role of cAMP has not been adequately investigated. Glucagon release after administration of adrenocorticotrophic hormone and some of the reproductive hormones may be secondary to activation of adenylate cyclase. Hypocalcemia probably removes calcium inhibition of the enzyme.

Glucagon may inhibit its own secretion when plasma levels rise excessively. Defects in glucagon suppression by glucose in

diabetes mellitus which improve with insulin therapy raise the possibility that insulin is needed to promote glucose entry into the alpha cell.

CHEMISTRY, BIOSYNTHESIS, AND METABOLISM OF GLUCAGON^{1a, 1f}

Glucagon is a linear peptide which in all species thus far studied contains 29 amino acid residues but no cysteine, isoleucine, or proline. It has the following amino acid sequence:

His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr

A peptide with twice the molecular weight which shares immunological properties with glucagon, but not hormonal actions (*large glucagon immunoreactivity, LGI*) seems to be a *proglucagon* released with glucagon from the α -cell.

Approximately 50% of the glucagon which reaches the liver is usually degraded there. Enzymes catalyzing the degradation may be identical with insulinases.

Very small quantities of the hormone circulate in the blood plasma. Most of the

circulating hormone is taken up by and destroyed in the kidney. Little or no glucagon can be recovered from the urine.

GLUCAGON DEFICIENCY

Although inadequate glucagon secretion has been implicated in the etiology of some hypoglycemic conditions, no clinical disorder has been firmly linked with glucagon deficiency.

Experimental hormone deficiency can be produced. As previously noted, pancreatectomy is usually avoided because of technical problems; but a substantial fraction of glucagon-secreting cells can be removed without disturbing other pancreatic functions in a few species such as the chicken. Studies in birds require careful interpretation however, since glucagon may perform functions not seen in mammals, and quantities secreted are often very large. (Some fishes and amphibians may secrete little or no glucagon, while amount products by others are substantial.)

Pharmacological agents with fairly selective toxicity for glucagon-secreting cells include *cobalt chloride*, *neutral red*, and *synthalin A* (decamethylenediguaniidine). Antisera are also available.

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7. Glucocorticoids

THE NEED FOR GLUCOCORTICOIDS^{1, 3, 27}

Glucagon-insulin interactions preside effectively over short range fluctuations of blood sugar levels when the diet is adequate. Insulin provides for storage of nutrients, while glucagon governs disbursement of reserves.

But liver glycogen stores are limited. Amounts deposited after a late lunch might not last until dinner time, and glycogen synthesized after dinner would surely be totally depleted before breakfast if replacement mechanisms were unavailable.

Amino acids provide the major replacement source. The small quantities stored in the liver can be used first; but later amino acids must be recruited from peripheral tissues.

Glucocorticoids are essential for *mobilization of tissue proteins* and for transport of the derived amino acids to the liver. In addition, they promote synthesis of enzymes catalyzing *deamination* reactions and of others utilized in gluconeogenesis. They step up *lipid mobilization* and oxidation, reduce *glucose catabolism* by tissues with flexible requirements, and favor glycogen synthesis.

As long as palatable food is continuously available, animals deprived of glucocorticoid hormones can (under suitable conditions) survive in seemingly good health. Blood glucose concentrations are maintained, and some glycogen is stored in the liver. But if the animals are deprived of food for just a few hours, they rapidly succumb to hypoglycemic convulsions. Life can be preserved by prompt administration of glucose. Glucocorticoids alone, given earlier, can protect against the hypoglycemia; increased nitrogen excretion following hormone administration indicates that amino acids have been utilized for maintaining the blood sugar.

Maintenance doses vary with prevailing conditions. Larger amounts are needed by animals subjected to a variety of stressful conditions or made to increase activity of skeletal muscles. However, similar doses given to animals at rest can be damaging.^{1b}

Patients suffering from Addison's disease (adrenocortical insufficiency) must guard against hypoglycemia through proper use of both diet and glucocorticoid replacement therapy; they, too, require more hormone to cope with higher levels of activity or with even mildly stressing stimuli.

Patients with Cushing's syndrome (hyperadrenocorticalism) and animals injected with excessive dosages of glucocorticoids exhibit, among other things, a mild hyperglycemia and glycosuria. Chronic exposure to high levels leads to widespread *destruction of tissue proteins* and a negative nitrogen balance even when sufficient food is taken to maintain body weight. Muscular weakness, thinning and striation of the skin, osteoporosis (thinning of the bones), impaired wound healing, and reduced resistance to infection are among the prominent symptoms.

Tissue destruction results at least in part from disruption of normal processes of protein synthesis. Although extensive, it is also selective. Up to 95% of the weight of the thymus gland may be lost in a few days; lymph nodes diminish markedly in size, the blood lymphocyte count declines, cutaneous and bone collagen are depleted and skeletal muscle is wasted. But protein synthesis is actually *increased* in the liver and there is accelerated production of red blood cells, while kidneys and nervous tissue maintain their structure.

Since amino acids are converted to glucose, and carbohydrate metabolism in muscle and other tissues is diminished, it is not surprising to find increased dependence upon lipids for fuel. Glucocorticoids promote extensive *mobilization of depot fat* reserves through facilitation of actions of lipolytic hormones. This influence is also selective. For reasons which have not been adequately defined (but which must certainly depend upon distribution of glucocorticoid receptors, sympathetic innervation, and prevailing levels of other hormones) there is preferential depletion of fat from appendicular structures (arms and legs), while excess fat tends to be deposited on structures supported by the axial skeleton.

Glucocorticoids are administered in pharmacological dosage for treatment of nonhormonal disorders. Symptoms of overdosage include accumulation of fat on the cheeks ("moon face"), on the back of the neck ("buffalo hump"), and on the abdomen. Although glucocorticoids often stimulate the appetite and promote fluid retention, fat accumulation at these sites can occur even when food intake is limited to the point where there is reduction of body weight.

The free fatty acid content of the blood is elevated. Ketosis does not pose the threat associated with the ketosis of insulin deficiency because other actions of glucocorticoids promote retention of sodium and water; moreover, compensatory elevation of insulin secretion directs some of the short chain fatty acids into pathways of new fat synthesis.

ACTIONS OF GLUCOCORTICOIDS ON THE LIVER^{1, 3, 10, 23, 28}

Conversion of Amino Acids to Glucose Precursors

Glucocorticoids markedly increase the synthesis of enzymes catalyzing metabolic degradation of amino acids. *Tyrosine transaminase* (TAT) and *tryptophan pyrolase* (TP) have been studied the most, since large increases can be demonstrated within a few hours after hormone administration.

Trpysine transaminase (TA, tyrosine- α -ketoglutarate transaminase, TKT, tyrosine aminotransferase) is an enzyme of low specificity which catalyzes transfer of amino groups from tyrosine, tryptophan and phenylalanine to glutamate, thereby increasing synthesis of glutamine. *Tryptophan pyrolase* (TP, TPO) catalyzes oxidation of tryptophan to formylkynurenone.

Serine deaminase and ornithine decarboxylase activity are also increased, and there is a slowly developing influence on alanine transaminase. As with other glucocorticoid influences, the actions are selective; histidine transaminase and arginase activity do not seem to be directly affected in mature animals.

Much information has been gathered on mechanisms whereby glucocorticoids induce synthesis of new enzymes; but there are many unanswered questions. In com-

mon with other steroid hormones, glucocorticoids are known to combine with cytoplasmic "receptor" molecules, and a steroid-peptide complex later attaches to components of the nucleus. Most actions of the hormone can be blocked by administration of actinomycin D.^{12c, 28}

It is easy to visualize a sequence of events in which the hormone complex binds to repressors of specific deoxyribonucleic acid (DNA) sequences and thereby "unmasks" sites for translation of specific messenger ribonucleic acid (RNA) molecules that code for synthesis of the enzymes.

The model is attractive for its simplicity. But there are no compelling reasons for believing that all glucocorticoid actions depend upon a single mechanism; and no adequate explanation has been advanced for discrepancies between the very large increases in RNA promoted and the very small amounts of RNA that seem to be needed to provide for synthesis of a few specific proteins. Moreover, there are indications that glucocorticoids affect some post-translational events.

Four binding proteins with varying affinities for glucocorticoids and their metabolites have been identified.^{12c, 28} Probably, one functions as the specific receptor; others have been implicated in metabolic transformation of the steroids and in limitation of duration of action. (Some glucocorticoid actions may require modifications of the steroid molecule.)

Binding to cytoplasmic macromolecules is "specific"; steroids which effectively mimic or antagonize glucocorticoid actions compete for binding sites, whereas molecules chemically related but devoid of hormone action do not. Glucocorticoids induce changes in the "receptors" which either facilitate their transport to the nucleus or promote their stabilization. Receptor peptides accumulate in the nucleus after (but not without) previous exposure to the steroids. Steroids can enter the nucleus without the peptides, but they do not seem to bind to chromatin when present alone.

Exactly what happens after the complex attaches to the nucleus is not known. Aside from the general problems of trying to identify small quantities of messenger RNA when hormones stimulate formation of large amounts of ribosomal RNA, difficulties distinguishing between new messenger synthesis and selective protection or transport of the messenger to active ribosomes, and the greater sensitivity of ribosomal as compared with messenger RNAs to

actinomycin D inhibition, there are problems which apply more specifically to glucocorticoid actions on the liver.

It has been shown, for example,¹⁰ that glucocorticoids can promote synthesis of TAT in livers of *infant* rats without prior induction of RNA synthesis, while in livers of older rats TAT synthesis is blocked by actinomycin D. This leaves open questions such as (1) can the hormone induce enzyme synthesis by two very different mechanisms and (2) is RNA synthesis in livers of older rats needed for something other than formation of messengers for the enzyme?

In rat hepatoma cells (which resemble normal liver cells in many respects but are easier to study), withdrawal of the hormone leads to *prompt* reduction of TAT; but administration of actinomycin D after enzyme induction *maintains* the enzyme levels. Therefore RNA synthesis may be involved in *deinduction* as well as in messenger formation.

While low doses of actinomycin D given early prevent enzyme induction, *very high* doses increase the amount of enzyme synthesis; "super-induction" of TO following actinomycin D has been demonstrated in normal liver.

A further complication arises from observations that *tryptophan* is a highly efficient inducer of TO in adrenalectomized rats, while several of the amino acids increase both TO and TAT in the presence of glucocorticoids. (Glucocorticoids accelerate entry of the amino acids into the liver through influences on peripheral protein metabolism; therefore it becomes difficult to determine which mechanism assumes greatest physiological importance.)

It has also been observed that agents which increase ammonia concentrations in liver (including ammonia itself, glutamine and D amino acids which cannot be used for glucose synthesis) stimulate protein synthesis in livers of adrenalectomized animals.¹¹ Glucocorticoids may therefore be promoting RNA synthesis indirectly, since they provide the liver with ammonia sources.

Another concept is that ammonia-producing reactions (*preferential* deamination of certain amino acids) serve the purpose of increasing delivery of amino acid building blocks to the liver through creation of an amino acid imbalance in the plasma which impairs peripheral protein synthesis. (Tissue proteins will not be synthesized if key amino acids are unavailable in sufficient quantity; and glucocorticoids reduce availability of tryptophan, tyrosine, and phenylalanine.)

Soon after glucocorticoid administration, morphological changes in the endoplasmic reticulum of liver cells can be demonstrated by electron microscopy, and this is followed within hours by increased aggregation of ribosomes.

This raises the possibility of direct hormonal influences on *translation* mechanisms; however, similar changes follow nonhormonal as well as hormonal stimulation of protein synthesis.

Glucocorticoids also attach in small quantities to mitochondria, and ATP synthesis is characteristically increased. While ATP is not rate-limiting for glucocorticoid actions, it probably contributes to effectiveness of hormone action.

Whatever the mechanism, glucocorticoids clearly promote amino acid degradation in the liver and conversion of the derived products to carbohydrate. They also are essential for gluconeogenic actions of glucagon (Chapter 6). The actions are essential for maintaining blood glucose concentrations and glycogen formation during periods of fasting.

Influences on Other Enzymes Needed for Gluconeogenesis

Glucocorticoids increase synthesis of *glucose-6-phosphatase* and are required for glucagon stimulation of activity of this enzyme.

Fructose 1,6-diphosphatase synthesis is also increased. (This enzyme is rate-limiting for conversion of phosphoglycerate to glucose phosphate (and therefore is essential for synthesis of glucose from pyruvate and lactate). In addition, glucocorticoids stimulate production of glucose from pyruvate by increasing activity of *pyruvate carboxylase*, *phosphoenol pyruvate carboxykinase*, and *glyceraldehyde phosphate dehydrogenase* (Fig. 5-1).

Influences of Glucocorticoids on Glycogen Synthesis

Glucocorticoids promote liver glycogen synthesis through stimulation of conversion of glycogen synthetase D to glycogen synthetase I, as well as by provision of glucose phosphate. Activity of the glycogen synthetase D phosphatase is increased with no increase in enzyme synthesis. (A bioassay procedure for glucocorticoids measures glycogen deposition in livers of fasted adrenalectomized rats.)

Concentrations of glucagon too small to activate the phosphorylase system (Chapter 6) can antagonize glycogen-enhancing actions of the glucocorticoids. Therefore

when glucose and its precursors are in short supply, glycogen synthesis can be shut down without extensive destruction of existing stores. Glucocorticoids may also promote formation of an as yet unidentified protein required for insulin stimulation of glycogenesis, since glycogen deposition in adrenalectomized animals given glucose plus insulin is subnormal.

ACTIONS ON THE THYMUS GLAND^{1, 14, 19, 39}

Thymus gland function is described in Chapter 25. As noted above, the thymus responds dramatically to glucocorticoid administration; involution is marked within 24 hr after administration of a large dose, and up to 95% of thymus weight can be lost within 2 days. Similar involution follows exposure of intact animals to a variety of "stress" stimuli which promote glucocorticoid secretion (food or water deprivation, extreme environmental temperatures, forced muscular work, trauma, infection, loud noises, electric shocks, hemorrhage, immobilization, and others). Stimuli of this nature are lethal to adrenalectomized animals, but the latter can be effectively protected by prior administration of glucocorticoids.

When the stressing stimuli or the hormone injections are stopped, the thymus gland very rapidly regenerates and becomes responsive to a new assault. Interestingly, much more attention has been directed at the biochemical mechanisms involved in the thymus gland responses than to the physiological significance of these impressive phenomena.

The thymus gland is especially rich in nucleic acids and proteins. It has been suggested¹⁴ that the thymus gland provides important nutrients and regulators to other tissues which are essential for normal growth and repair, effective responses to trauma and infection, and early adaptation to stress. (But thymectomized animals beyond the weaning stage exhibit normal growth patterns and can withstand conditions not tolerated by adrenalectomized animals.)

The thymus contains specific receptors for glucocorticoid hormones,³⁹ and some steroids undergo metabolic conversions

within the thymus gland. Within 2 hr after exposure of the gland to steroid hormones, RNA and protein synthesis are severely disrupted. DNA synthesis and mitosis are later impaired, so that new cells do not replace older ones that degenerate.

Since glucose and ATP are needed for synthesis of macromolecules, much attention has been directed to the observation that uptake of glucose (and related molecules) is inhibited within 15 min after exposure of thymocytes to glucocorticoids, and glucose phosphorylation is secondarily impaired. But the fact that prior administration of actinomycin D prevents changes in glucose uptake suggests that small amounts of specific RNAs must be first synthesized.¹⁹ Glucocorticoids impair uptake of RNA and protein precursors by normal thymus glands.

In one study of thymus-derived lymphosarcoma cells, it was shown that glucocorticoids inhibited uptake and incorporation of uridine (with no effect on 2-deoxyglucose uptake) in steroid-sensitive but not in steroid-insensitive cells.³⁹

Localization of glucocorticoid receptors to the nuclei of thymus cells³⁹ raises the possibility of a primary site of action there. But the steroids also bind to intracellular membranes,³⁹ and early morphologically demonstrable influences on ribosomal aggregation are consistent with inhibition of translation. The hormones also affect lysosomal membranes, and it has been suggested that such action releases cyto-destructive hydrolases and secondarily impairs mitotic processes. Influences of glucocorticoids on lymphatic tissues resemble those described for the thymus gland.

GLUCOCORTICOID ACTIONS ON ADIPOSE TISSUE

Glucocorticoids promote *degradation of fats and release of fatty acids* into the blood plasma. They also *inhibit synthesis of new fat molecules*. High circulating concentrations of fatty acids affect plasma membranes of many cell types and decrease their ability to take up glucose. An increased rate of fatty acid delivery to the liver leads to ketogenesis and accumula-

tion of acetyl coenzyme A. The latter is an activator of hepatic pyruvate carboxylase and therefore contributes to gluconeogenesis.

Lipolytic actions of epinephrine and other hormones are mediated via stimulation of adenylate cyclase. Animals deficient in glucocorticoids exhibit elevation of cAMP content in response to such hormones, but effects of the nucleotide on hydrolysis of fat molecules are impaired. Since glucocorticoids restore responses (via an actinomycin D-sensitive mechanism), it has been proposed that the steroids induce formation of small quantities of a labile protein on which cAMP exerts its actions. Inhibition of fatty acid incorporation into triglycerides is attributed to effects on membranes which reduce glucose uptake from the plasma.

INFLUENCES ON SKELETAL MUSCLE

Profound influences of large doses of glucocorticoids on skeletal muscle lead to debilitation and extensive loss of muscle mass. This tissue supplies the major part of the protein utilized for gluconeogenesis.

Impaired protein synthesis results from reduced ability to take up glucose and amino acids, and possibly also from the amino acid imbalance associated with deamination reactions in the liver.

GLUCOCORTICOID ACTIONS ON SKIN AND BONE

Patients suffering from hyperadrenocorticism develop thinning of the skin, and purple striations are commonly seen over the abdomen. *Synthesis* of skin and bone collagen and ground substance are impaired, and fibroblasts exhibit altered morphological appearance and reduced mitotic rate. Wound healing becomes defective. *Bone demineralization* is reflected in the rise in urinary calcium; and osteoblasts are reduced in number (Section V).

GLUCOCORTICOIDS AND INFLAMMATION^{1c, 8, 21}

While little information is available on possible roles of *physiological* concentrations of glucocorticoids in regulation of inflammatory processes, extensive therapeutic application has been made of the finding that *pharmacological* doses can

alleviate symptoms associated with rheumatoid arthritis and related diseases, and can reduce undesirable vascular reactions in infectious conditions of the eye and other structures which can be otherwise controlled with antibiotics.

Large doses of glucocorticoids (and of highly potent synthetic analogs) diminish the capillary dilation and endothelial changes (increased permeability) which characterize the inflammatory process. Fewer leukocytes accumulate, and proliferation of fibroblasts is inhibited. By contrast with glucocorticoid actions on normal tissue, the steroids can protect against widespread destruction of collagen. Stabilization of lysosomal membranes of cells involved in mediation of the inflammatory process also contrasts with actions on lysosomes of thymocytes and other cells. When relative potencies of different steroids are compared, hepatic and thymolytic actions seem to run parallel, while anti-inflammatory actions cannot be readily predicted from effects on liver and thymus.

INFLUENCES OF GLUCOCORTICOIDS ON THE CIRCULATORY SYSTEM^{1b}

Glucocorticoids contribute to maintenance of circulating blood volume because they influence transfer of water from cells to extracellular fluids (Section III) and maintain the integrity of capillary membranes. They exert what is sometimes described as a "permissive action" on functions of catecholamines; in adrenocortical insufficiency, vasoconstrictor responses to epinephrine and norepinephrine are diminished or abolished. Glucocorticoids tend to increase cardiac output; while evidence for a direct action on cardiac muscle is still controversial, specific glucocorticoid receptors have been identified in the heart.²⁴ Glucocorticoids also exert some mineralocorticoid activity (Section III); plasma potassium concentrations are reduced, while sodium concentrations may be elevated. (But glucocorticoids can also antagonize effects of excessive mineralocorticoids.)

GLUCOCORTICOIDS AND RESPONSES TO NONSPECIFIC STRESS¹

Unprotected adrenalectomized animals succumb rapidly to procedures which are

well tolerated by intact or glucocorticoid-treated animals. Death usually results from cardiovascular collapse, sometimes accompanied by hypoglycemia. Interestingly, the animals can be gradually acclimated to increasing levels of many of the stimuli which prove lethal if suddenly administered in full strength;^{1b} they can also withstand conditions imposed before performance of the adrenalectomy.

Intact animals release catecholamines from sympathetic nerves and from the adrenal medulla when exposed to stress; one consequence is prolonged peripheral vasoconstriction. Adrenalectomized animals also discharge catecholamines from sympathetic nerves and may suffer adversely from this. They exhibit transient vasoconstriction, but this is soon followed by a sharp fall in systemic blood pressure and pooling of blood in small vessels (arterioles and venules).

The concept that adrenalectomized stressed animals succumb to their endogenous catecholamines is supported by observations that (1) adrenalectomized animals are exquisitely sensitive to stimuli which promote catecholamine release, (2) some protection is afforded by catecholamine antagonists, and (3) animals subjected to repeated epinephrine injection prior to adrenalectomy exhibit greater resistance to stress.

OTHER INFLUENCES OF ADRENOCORTICAL HORMONES

The Nervous System ^{1a, 1b, 3, 4}

Glucocorticoids influence nervous tissue in several ways. Changes in electroencephalograms following adrenalectomy or administration of glucocorticoids have been observed, and the hormones are known to influence the threshold to convulsion-inducing electric shocks.

Patients suffering from Addison's disease characteristically exhibit depression and apathy which seems to be in excess of that which might be anticipated from the weakness and debilitation resulting from hormone deficiency effects on skeletal muscle, blood glucose concentrations, and on blood vessels. The condition responds to hormone treatment.

Pharmacological doses induce euphoria which is followed by depression. Early elevation of mood in patients effectively treated for diseases such as rheumatoid arthritis has been attributed by some clinicians to relief of painful and distressing symptoms, while later depression has been related to refusal to obtain adequate rest. But most attribute the mental effects to direct actions of glucocorticoids on brain cells.

There have been numerous cases of psychotic manifestations during glucocorticoid therapy, some of which were reversible when the hormone was discontinued. Again, some clinicians ascribe such findings to the emergence of preexisting pathology, while others believe it represents a direct effect of the hormone overdosage which is elicited in previously normal individuals. Patients with untreated Cushing's disease have a high incidence of emotional disturbances and even psychotic symptoms, and depression is especially prevalent. Many such patients respond well to removal of the source of hormone hypersecretion.

Glucocorticoids have been reported to affect learning processes (and time required to establish and extinguish conditioned reflexes), and to influence catecholamine metabolism in the brain. They also affect water balance in the nervous system and have been used in treatment of brain edema.

Gastrointestinal Functions

Maintenance of tone and motility of the gastrointestinal tract requires both glucocorticoids and mineralocorticoids secreted by the adrenal gland. Glucocorticoids also promote secretion of hydrochloric acid. The high incidence of ulcers in patients receiving large doses has been attributed to increased gastric acidity, possibly aggravated by reduced or altered mucin formation.

Body Temperature

The many metabolic influences of glucocorticoids contribute to elevation of body temperature. But glucocorticoids are also known to be antipyretic, and this action has been attributed, at least in part, to

inhibition of release of pyrogens from leukocytes.

Blood

Patients with Cushing's disease have a ruddy appearance often associated with polycythemia, while patients with Addison's disease suffer from anemia. A direct action of glucocorticoids on bone marrow has been proposed. The eosinophil count is reduced after glucocorticoid administration; this has been attributed to sequestration of the cells in nonvascular sites. The lymphocyte count is reduced for reasons cited above. Glucocorticoids also accelerate blood coagulation.

ADRENOCORTICAL HORMONES

Anatomical Organization of the Adrenal Gland^{1a, 2, 3}

The mammalian adrenal gland derives its name from its position on the anterior surface of the kidney. The outer portion or cortex secretes glucocorticoids and related steroids; it surrounds the inner medulla which secretes catecholamines. Although the two parts of the gland are anatomically distinct, glucocorticoids influence synthesis of epinephrine.

The term *adrenal gland* is loosely applied to structures in other vertebrates which secrete steroid hormones and catecholamines. In birds this is appropriate; but in other forms steroid-secreting tissue can occur anterior to, embedded within, or between the kidneys, and may consist of separate bits of unconnected tissue or even of scattered cells. In some nonmammalian vertebrates, cells secreting steroids surround those secreting catecholamines; but in others the two cell types are anatomically separated. It is therefore more appropriate to speak of *steroidogenic* and *chromaffin* tissues than of adrenal cortex and medulla. (The term chromaffin refers to staining of catecholamine-producing cells with chromate dyes. In mammals chromaffin tissue is found outside as well as within the adrenal medulla, Chapter 9.)

The corpuscles of Stannius are structures in teleost fishes which seem to participate in steroid hormone functions; they exert influences on calcium metabolism

and other physiological processes not directly linked with mammalian glucocorticoid or mineralocorticoid actions.

Functional Zonation²

The outermost region of the mammalian adrenal (just below the gland capsule) is called the *zona glomerulosa* because the cells are arranged in whorls (glomeruli). It is the site for synthesis of aldosterone (Chapter 11).

Just below the glomerulosa is the *zona fasciculata* in which cells form cords or fascicles. It is usually the largest part of the adrenal cortex and is the principle site for synthesis and secretion of glucocorticoids. In some species it contains large "clear" cells; the appearance has been attributed to abundant content of cholestrylo esters which serve as precursors for hormone synthesis.

The innermost part (closest to the adrenal medulla) is the *zona reticularis* in which cells are arranged in a network (reticulum). In common with the fasciculata, it secretes glucocorticoids and sex steroids.

Zonation is more apparent in some species than in others. Complete separation of the inner cell groups is virtually impossible, and this has made it difficult to fully define functional differences. It has been suggested that the reticularis region contains degenerating cells derived from the fasciculata; but reticularis cells are stimulated by adrenocorticotrophic hormone and are known to release steroid hormones.

It has also been considered that adrenocortical cells originate in the reticularis region and migrate outward. This is difficult to reconcile with the knowledge that removal of all adrenocortical cells except a few from the glomerulosa adhering to the capsule is soon followed by regeneration of a complete adrenal cortex, while implants of only fasciculata or reticularis cells fail to "take" and soon degenerate. It has also been shown that glomerulosa cells in tissue culture respond to adrenocorticotrophic hormone and become transformed into cells morphologically resembling those of the inner zones.^{3a}

The two principle glucocorticoids are *corticosterone* and *cortisol* (described below). Most species produce at least some of each type, but there are marked differences

in relative quantities secreted; some animals also secrete small amounts of the related cortisone.^{1a, b}

Humans, monkeys, hamsters, birds, and most fishes secrete predominantly cortisol, while rats, mice, rabbits, and snakes secrete mostly corticosterone. The dog secretes more cortisol than corticosterone, but cats, ferrets, and sea lions regularly secrete both in varying proportions.

Chemical Nature of Steroid Hormones^{1, e}

The steroid hormones all contain a 5-carbon ring structure (cyclopentane, Fig. 7-1A) fused to a saturated (perhydro) phenanthrene (Fig. 7-1, B and C). They are therefore said to be cyclopentanoperhydrophenanthrene derivatives (Fig. 7-1D). The names *sterane* and *gonane* are sometimes used to designate this basic structure which the hormones share with cholesterol, bile acids, vitamin D precursors, some clinically useful agents such as digi-

talis, and the African arrow poison, ouabain.

Numbering of the carbon atoms, and letters assigned to the four rings are indicated.

The sterane structure contains asymmetric carbon atoms at positions 5, 8, 9, 10, 13, and 14. For purposes of nomenclature, it is convenient to think of the four rings as lying in a flat plane parallel to the surface of the paper (although it is recognized that this does not represent the true spatial configuration). Atoms or chemical groups attached to the asymmetric carbons may then be regarded as projecting above or below the plane. Those projecting upward are said to be in the *beta* or *cis* position and attachments are represented as solid lines; broken lines represent attachments of *alpha* or *trans* groups. Additional asymmetry is introduced by substitutions for hydrogen atoms on other carbon atoms. A 3 α -OH, 5 β -methyl-substituted sterane is shown in Figure 7-1E.

The adrenocortical hormones, progesterone and male sex hormones (androgens)

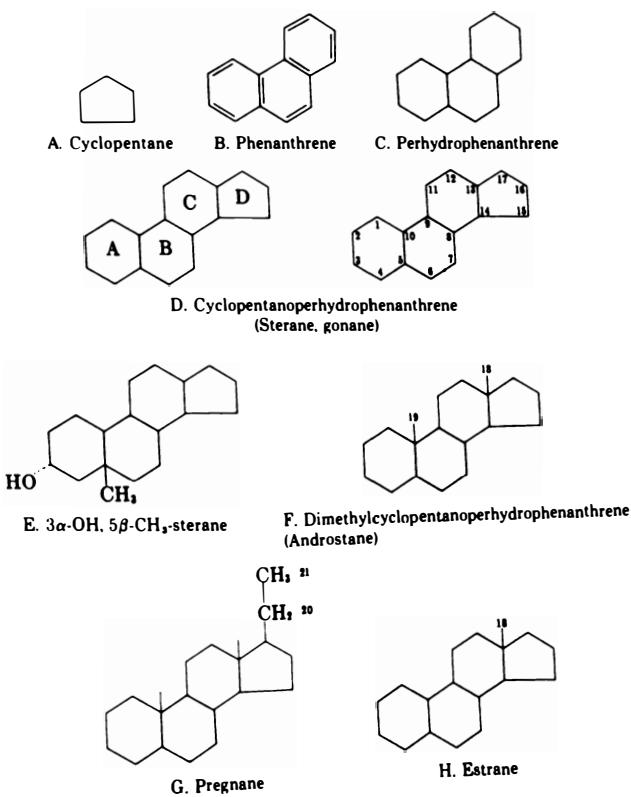


FIG. 7-1. Cyclic hydrocarbon structures related to the steroid hormones.

have methyl groups attached to carbon atoms 13 and 10 and are therefore derivatives of *dimethylcyclopentanoperhydrophenanthrene*, which is also known as *androstane* (Fig. 7-1F).

Androgens have a total of 19 carbons. But glucocorticoids, mineralocorticoids, and progesterone have two additional ones attached at the 17 position and are therefore derivatives of *pregnane*, Figure 7-1G. They have a total of 21 carbon atoms.

Estrogens lack carbon additions numbers 19, 20, and 21 and are derivatives of *estrane* (Fig. 7-1H).

The structures of the two major glucocorticoids, corticosterone and cortisol are compared with that of progesterone in Figure 7-2. In common with many other steroid hormones, all have a *double bond* between carbons 4 and 5. The location of the double bond is usually indicated by the carbon having the lower number, but sometimes the symbol Δ is used (e.g., all steroids shown in Figure 7-2 are $\Delta 4$ - or $\Delta 4,5$ steroids. The suffix *ene* (as in pregnene) denotes unsaturation. The suffix *one* signifies the presence of a ketone group. Alcoholic groups are indicated by the suffix *ol* or as *hydroxy*.

It may already be apparent from the preceding that very small changes in chemical structures of steroids profoundly influ-

ence biological properties. Many examples will be cited throughout the chapter.

BIOSYNTHESIS OF ADRENOCORTICAL HORMONES^{3, 27}

Fifty or more steroids have been identified in adrenal cortices of vertebrates; but only a few are believed to be secreted in measurable quantities. Structures of some, along with letters assigned to them early in the course of investigations (before chemical structures had been established) are shown in Figures 7-3 and 7-4. Others are described below. Corticosterone and cortisol (hydrocortisone) shown in Figure 7-2 were originally known as compounds B and F, respectively, while aldosterone (which regulates electrolyte metabolism) was called *electrocortin*.

Since all steroids are synthesized from *acetyl coenzyme A*, the building blocks are never in short supply. Most (or all) pathways involve prior formation of *cholesterol* which can be directly synthesized by the steroidogenic tissues or produced in the liver and transported to the glands by the blood plasma. Cholesterol has 27 carbons, a fully saturated A ring, and a double bond between carbons 5 and 6 (Fig. 7-5A). It is devoid of hormonal potency.

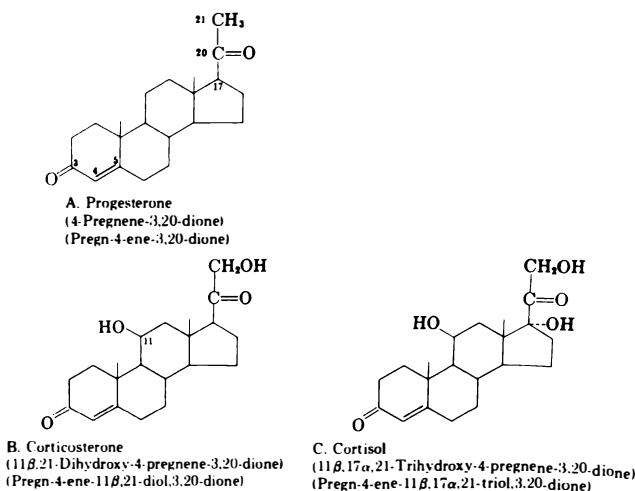


FIG. 7-2. Structures of progesterone, corticosterone, and cortisol.

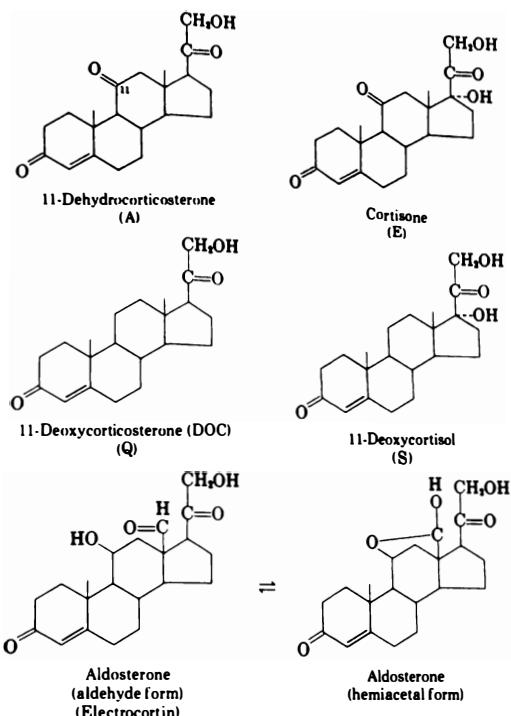


FIG. 7-3. Glucocorticoid and mineralocorticoid type hormones.

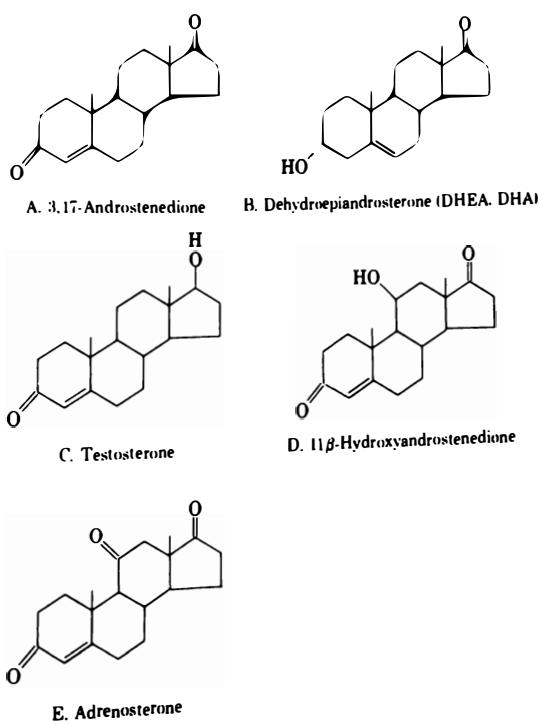


FIG. 7-4. Adrenocortical androgens.

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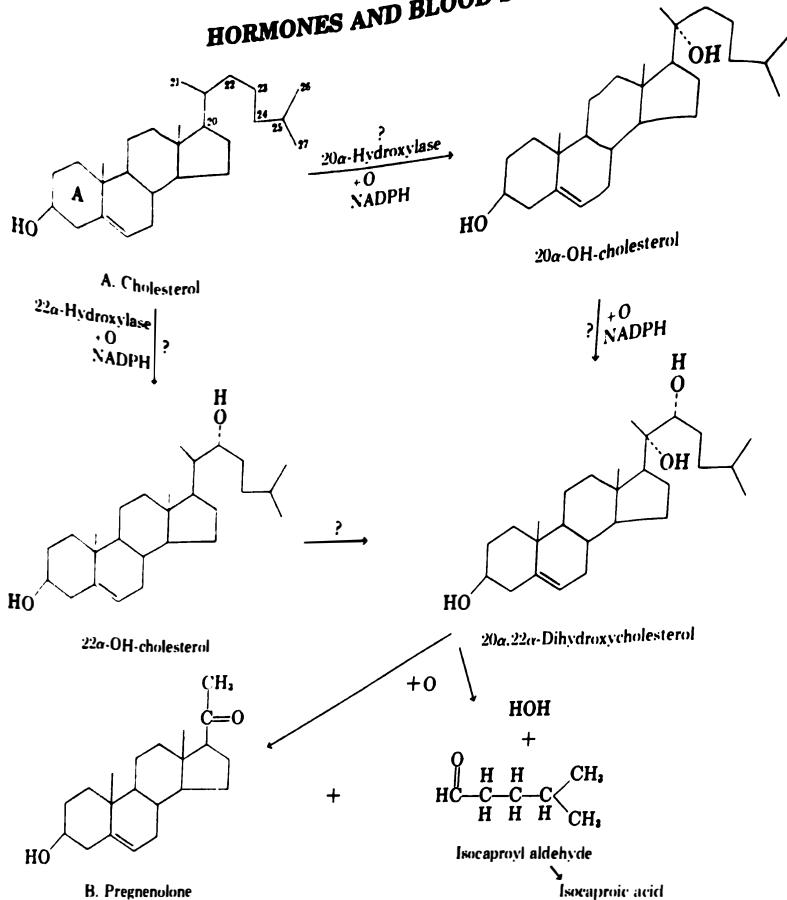


FIG. 7-5. Hypothetical pathway for conversion of cholesterol to pregnenolone.

The adrenal gland can take up and store large quantities of cholesterol esters. Differences in structures of glandular and plasma esters suggest that plasma derivatives are hydrolyzed and later reesterified.

Conversion of Cholesterol to Pregnenolone

Cholesterol is taken up by mitochondria and converted to *pregnenolone* (Fig. 7-5B) by a process which requires removal of a 6-carbon chain. The terms *desmolase system* and *pregnenolone synthetase* have been applied to the associated enzymes. The reaction may be *rate limiting* for steroid hormone synthesis and provides the most important site for *regulation* by ACTH in the adrenal and by gonadotrophins in the testes and ovaries. Pregnenolone is then sent back to the cytoplasm for further conversion.

NADPH, an NADPH-specific flavoprotein, cytochrome P-450 and a nonheme iron contain-

ing protein are required along with molecular oxygen. It has been proposed that cholesterol is hydroxylated twice (at carbons 20 and 22) before the side chain is cleaved, and that a single enzyme catalyzes both hydroxylations. A 20-hydroxylated (but not 22-hydroxylated) intermediate has been identified in the gland; however the intermediate formed under physiological conditions may be a short-lived free radical. A quantitatively less important pathway may involve hydroxylations at carbons 17 and 20 with formation of 17α-OH-pregnenolone, Fig. 7-7).

Conversion of Pregnenolone to Progesterone

This step proceeds rapidly and is catalyzed by microsomal enzymes. Formation of progesterone involves shifting of the position of the double bond from the B to the A ring and removal of hydrogen from the 3 position (Fig. 7-6C).

An NAD-dependent 3-β-steroid dehydrogenase catalyzes oxidation of the alcoholic group to

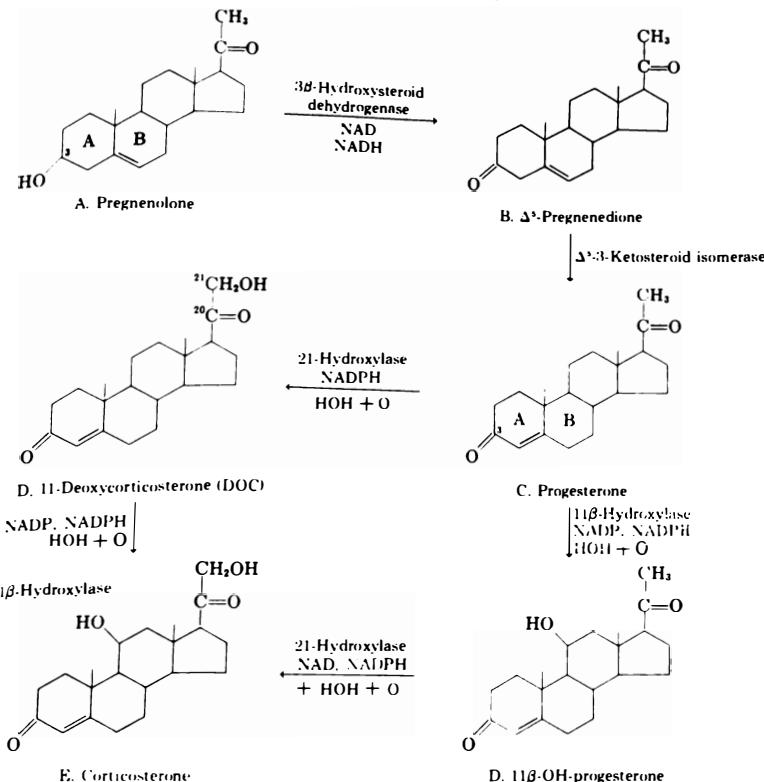


FIG. 7-6. Biosynthesis of progesterone, 11-deoxycorticosterone and corticosterone from pregnenolone.

a ketone. A Δ^5 -3-ketosteroid isomerase promotes migration of the double bond. Pregnenedione may be formed as an intermediate.

Some progesterone is secreted by the adrenal.³³ It exerts weak influences on salt and water metabolism (Section III) and affects reproductive structures (Section VI).

Biosynthesis of 11-Deoxycorticosterone (DOC) and Corticosterone

Conversion of progesterone to DOC (Fig. 7-6C) is accomplished in one step, catalyzed by a cytoplasmic (microsomal) enzyme, and involves addition of an OH group to carbon 21. Mineralocorticoid actions of DOC are described in Section III.

DOC must be returned to the mitochondrion for formation of the glucocorticoid corticosterone (Fig. 7-6E), since the enzyme catalyzing addition of an OH group is located there.

An alternate pathway for corticosterone synthesis bypasses DOC; pregnenolone (or an intermediate) can be hydroxylated at the 11 position while still in the mitochondrion; 11-OH-progesterone (Fig. 7-6F) then travels to the cytoplasm for 21 hydroxylation.

Corticosterone is the major glucocorticoid secreted by rats and some other animals; but humans and many others convert most of this steroid to the glucocorticoid cortisol.

Biosynthesis of Cortisol (Hydrocortisone)

Cortisol (Figs. 7-2, 7-7) is formed from corticosterone by addition of an OH group at position 17. Cortisol differs from corticosterone in that large doses exert anti-inflammatory actions. It is also a more potent glucocorticoid and exerts less influence on electrolyte metabolism. For these reasons and also because it is cheaper

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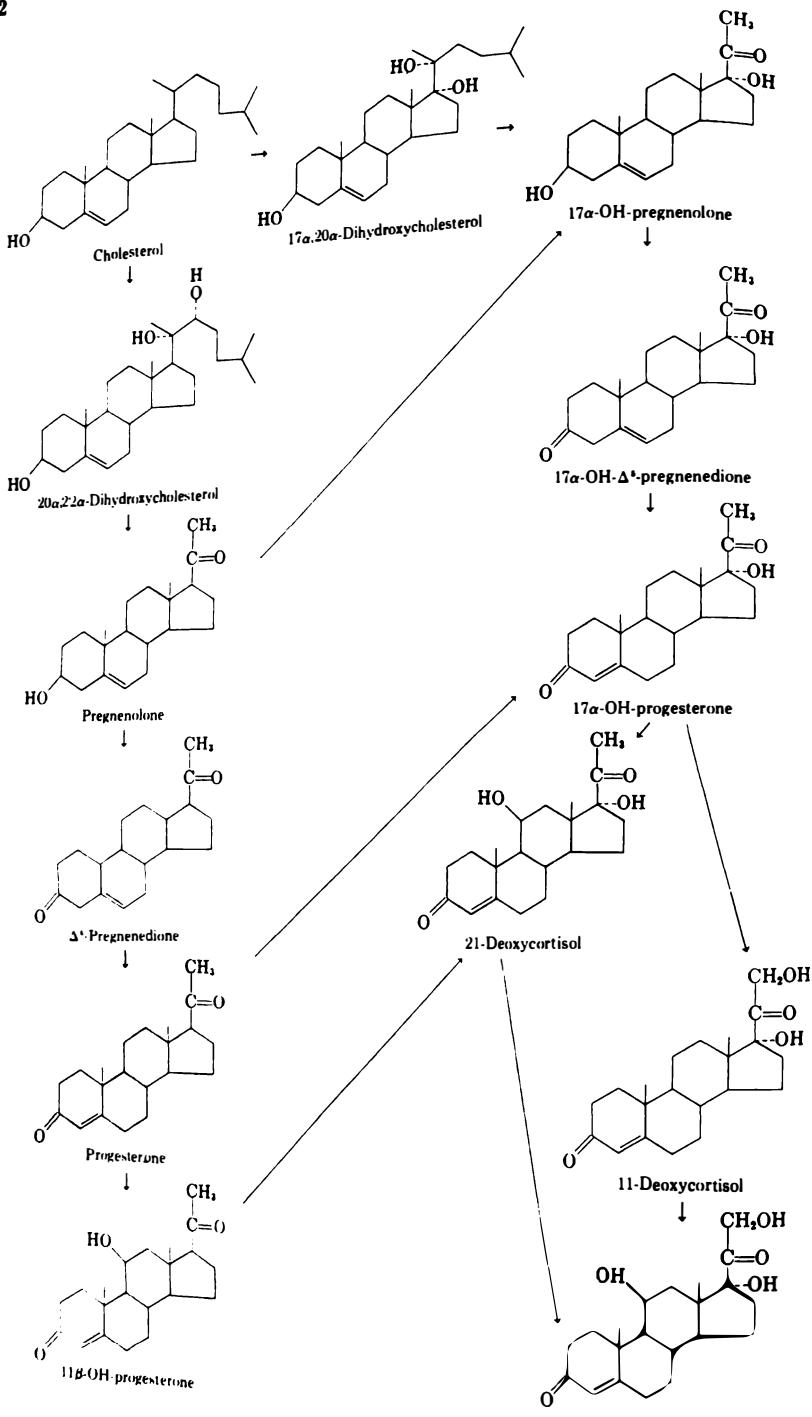


FIG. 7-7. Biosynthesis of cortisol.

nd available, cortisol finds wide clinical application whereas corticosterone does not.

In some species (e.g. rats, mice and rabbits) little cortisol is formed, although the cytoplasm contains a 17 α -hydroxylase enzyme.

Cortisol can be formed by several alternate pathways (Fig. 7-7), since cholesterol, pregnenolone, progesterone, and other steroids are apparently directly acted upon by the 17-hydroxylase. In humans and some others, considerable quantities of 17 α -OH-pregnenolone and of 17 α -OH-progesterone are formed.

most aldosterone is synthesized directly from corticosterone.

The presence of an OH group at position 11 confers glucocorticoid potency. Very large doses of aldosterone do show some glucocorticoid activity, but physiological amounts do not. This may result from presence of most of the hormone in hemiacetal form, as well as from the low concentrations.

Establishment of biosynthetic pathways for synthesis of aldosterone presents technical difficulties, one of which is the small quantity of hormone secreted. There is no clear evidence that a single pathway predominates. Probably much is synthesized by hydroxylation of corticosterone to yield 18-OH-corticosterone followed by oxidation of the OH group. This and an alternate pathway involving 18-hydroxylation of DOC are shown in Figure 7-8.

Biosynthesis of Aldosterone

Aldosterone, the major mineralocorticoid, receives its name from the presence of an aldehyde group attached to carbon 18 (Figure 7-3). The aldehyde interacts reversibly with the OH group attached to carbon 11 to form a hemiacetal. Probably

Biosynthesis of Adrenal Androgens

A large number of 19-carbon steroids possessing varying male sex hormone po-

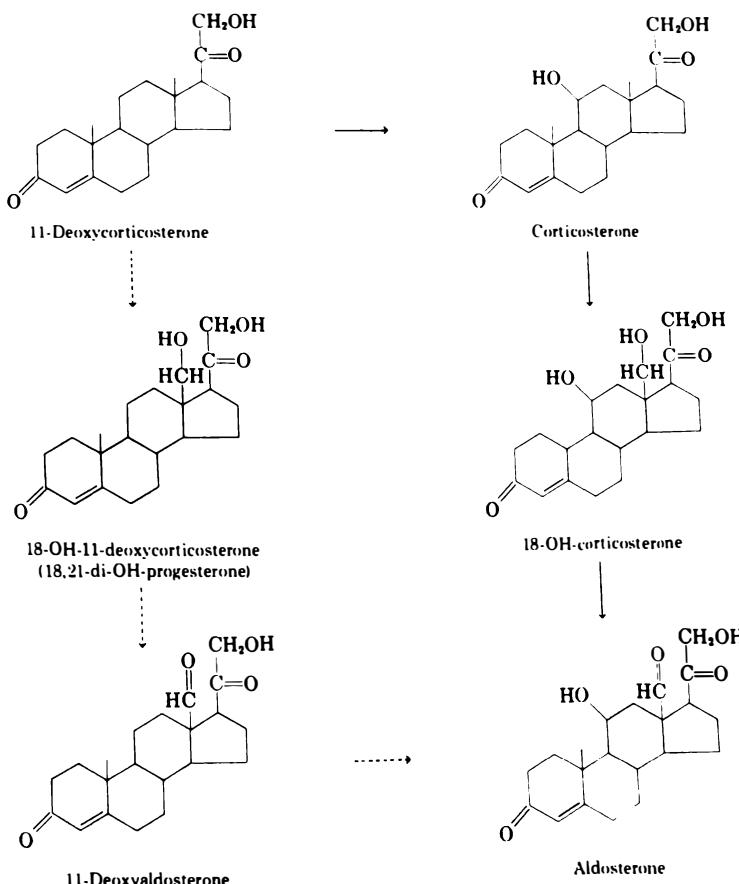


FIG. 7-8. Biosynthesis of aldosterone.

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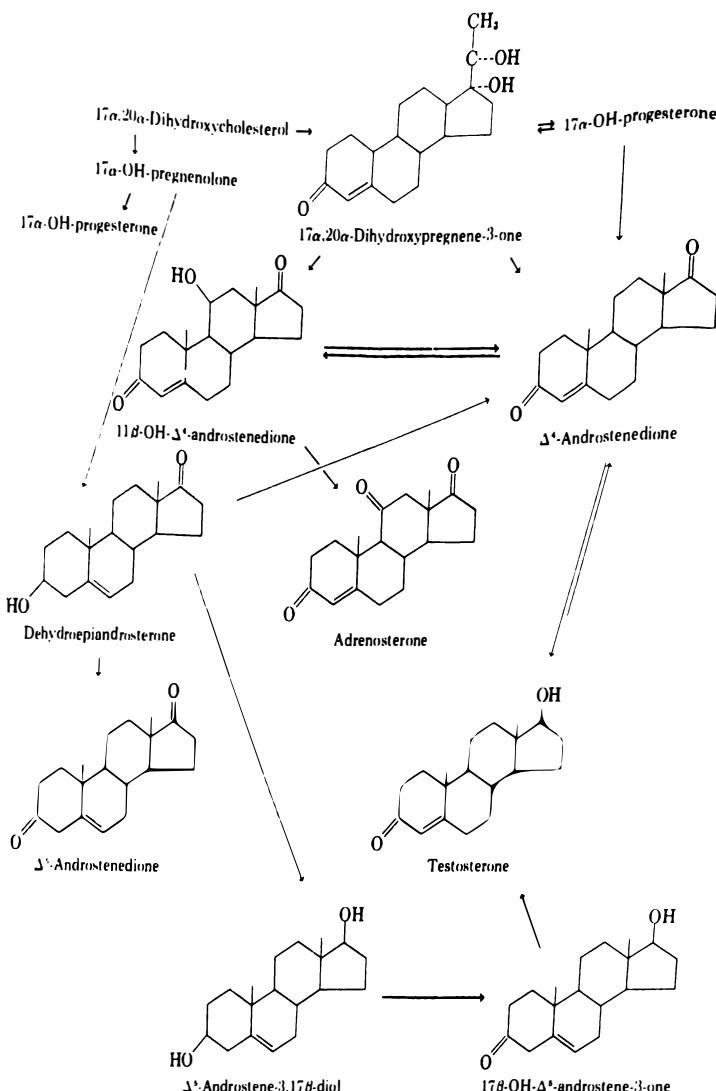


FIG. 7-9. Pathways for biosynthesis of adrenocortical androgens (see Fig. 7-7 for structures not shown).

tencies are synthesized. A different hormonal balance in the testis leads to production of similar steroids but much greater quantities of testosterone.

Androstenedione and its 11- β -hydroxy derivative (Figs. 7-4 and 7-9) are secreted in fairly large quantities by some species; others put out greater amounts of the less potent dehydroepiandrosterone (DHA, DHEA). Little of the highly potent *testosterone* is secreted by most adrenal glands because of the high activity of the 11-hydroxylase enzyme.

It is likely that several alternate routes (Fig. 7-9) are utilized. Under pathological

conditions (or after administration of certain enzyme inhibitors), androgen production is greatly increased.

Adrenal Estrogens

The normal adrenal cortex secretes minute quantities of *estrone* and the more potent *estradiol* (Fig. 7-10). Testosterone is an important precursor in adrenals, testis, and ovary.

Sulfation of Adrenocortical Steroids

The adrenal cortex contains one or more *sulfokinases* which catalyze transfer of sulfate

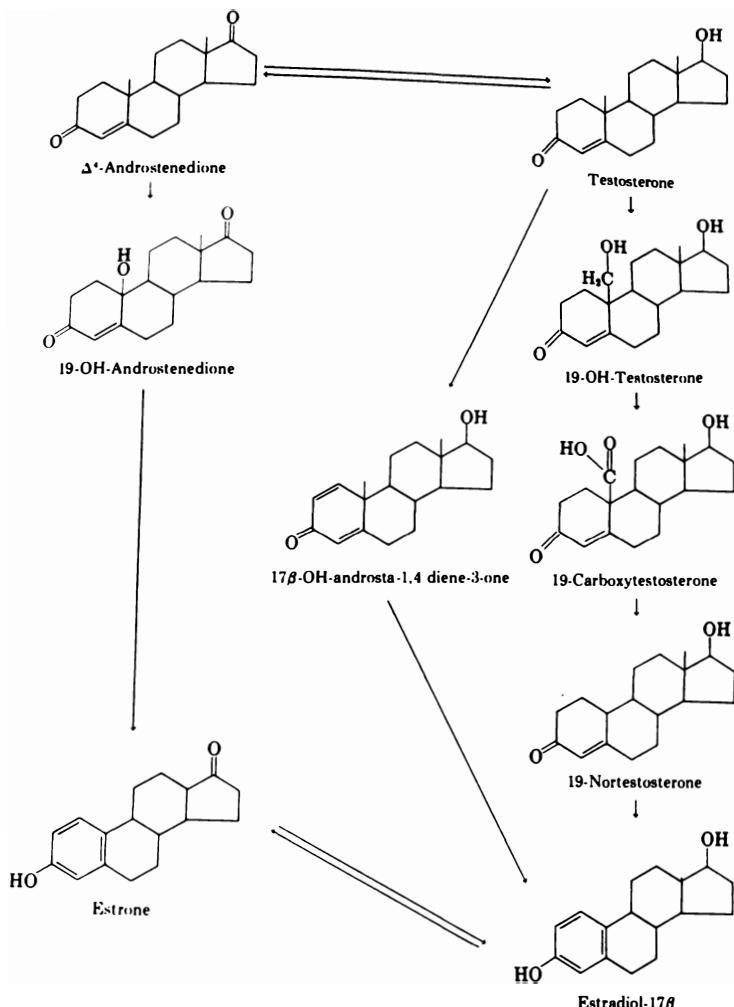


FIG. 7-10. Biosynthesis of adrenocortical estrogens.

groups to the steroids. DHA-sulfate is found in relatively large quantities. Other 11-deoxycorticoids (deoxycorticosterone, testosterone, and estrone) may also be sulfated to some extent. There are suggestions that adrenocortical cells can directly utilize cholesterol-sulfate for formation of sulfated hormones.

Enzymatic Disorders of the Adrenal Cortex^{a, b, 27}

Congenital deficiency or absence of hydroxylase enzymes and pathological processes associated with tumor growth can markedly alter the secretory products of the adrenal cortex. The consequences of

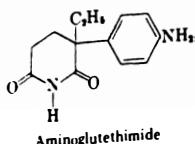
such disorders may be especially devastating if the defects are present during the prenatal period, but very serious problems can also arise during childhood and in adult life. Rather specific inhibitors of adrenocortical enzymes have been useful in elucidation of the nature of the problems.

A deficiency of C-20 hydroxylating activity prevents formation of all adrenocortical hormones. Survival depends on prompt administration of appropriate substitution therapy. Cases of infants with the disorder have been described.

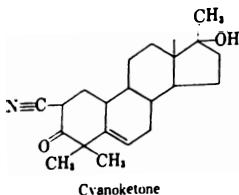
Aminoglutethimide has been used experimentally to study the disorder and for treatment

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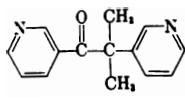
of special cases of overproduction of adrenocortical steroids in patients.



3- β -OH-steroid dehydrogenase deficiency impairs conversion of pregnenolone to progesterone and results in glucocorticoid and mineralocorticoid insufficiency, while androgen and estrogen synthesis may be increased. Cyanoketone blocks the enzyme and has been used to study the defect.



Glucocorticoid synthesis is impaired by *11- β -hydroxylase* deficiency; aldosterone is also directly affected, but large quantities of the mineralocorticoid DOC and also of 11-deoxycortisol and 18-OH-DOC may be secreted. Metyrapone (metopirone, mepyrapone, Su-4885) inhibits the enzyme.



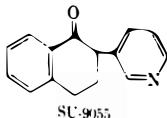
Metyrapone

The most widely occurring congenital defect in human infants involves the 21-hydroxylase. Complete block permits formation of progesterone but not of either glucocorticoids or mineralocorticoids; most victims produce small amounts of the enzyme.

C-17 hydroxylase deficiency permits formation of corticosterone, DOC, and progesterone, but not of cortisol, or the sex hormones.

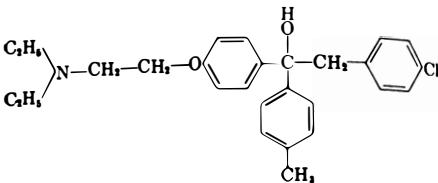
A deficiency of the *C-18 hydroxylation* system has also been described in which aldosterone formation is impaired.

SU-9055 impairs both 17-and 18-hydroxylation.



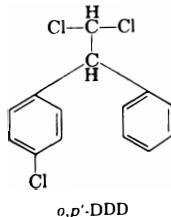
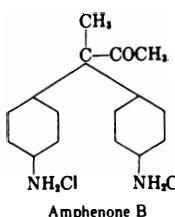
C-19 hydroxylation is necessary for synthesis of estrogens. While a deficiency of the associated enzyme system has been described, it is the defect least likely to be noticed since adrenal estrogen production is very limited.

In addition to the agents described above, triparanol (MER-29) has been used because it inhibits synthesis of cholesterol.



Amphenone B blocks several of the adrenocortical enzymes. In recent years, it has been largely replaced by DDD (dichlorodiphenyl-dichlorethane) and its more active isomer, *o,p'DDD*. Large doses may induce selective necrosis of the zona fasciculata and reticularis, and they affect metabolism as well as formation of the steroid hormones. Small doses affect mostly cholesterol side chain cleavage.

MER-29



An interesting congenital defect leads to excessive synthesis of *etiocholanolone* (Figure 7-12), a reduction product of the adrenal androgens. This steroid has little or no potency as a hormone but may cause high fevers. Suppression of androgen production by administration of glucocorticoids reduces etiocholanolone synthesis.

When enzymatic deficiencies impair glucocorticoid production, they also remove the physiological inhibitor of ACTH secretion (Chapter 8). Hypersecretion of this pituitary hormone promotes excessive growth of the adrenal cortex and overproduction of those steroids that can be synthesized. Treatment with glucocorticoid hormones not only corrects the deficiency, but it also suppresses excessive ACTH output.

GLUCOCORTICOIDS

77

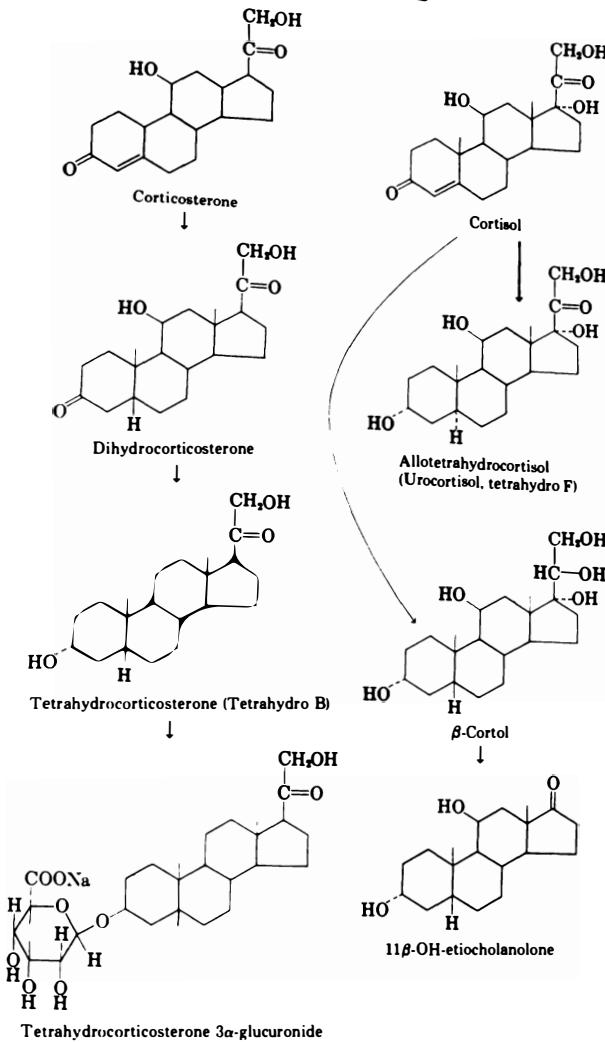


FIG. 7-11. Some metabolic degradation products of glucocorticoids.

METABOLISM OF ADRENOCORTICAL HORMONES

Binding to Plasma Proteins

Corticosteroid binding globulin (CBG, transcortin) binds glucocorticoids with high affinity; however, quantities present in the plasma are usually sufficient for combination with only one-half to two-thirds of the circulating hormone. Plasma albumin binds with lower affinity but is

present in much greater amounts; some aldosterone also binds to albumin.

Sex hormone binding globulin (SHBG) combines with adrenocortical as well as gonadal androgens and estrogens.

Metabolism and Excretion

The liver is the major site. Most reactions result in formation of steroids with little or no hormone potency or conjugates that are more readily excreted than the

HORMONES AND BLOOD SUGAR

original hormone. The outstanding exception is conversion of cortisone to the biologically active cortisol (and the related oxidation of the C-11 ketone of 11-dehydrocorticosterone to form corticosterone).

Conjugates of steroid hormones (and their metabolites) with glucuronate and to a lesser extent with sulfate and other small ions are excreted by the kidney. (Only traces of free hormones normally appear in the urine.) There is little biliary excretion of adrenocortical hormones. Most fecal steroids originate in the gonads or liver or are formed by intestinal microorganisms.

Glucocorticoids, mineralocorticoids, and

progesterone undergo saturation of the A ring, reduction of the C-20 ketone, oxidation of the C-21 alcoholic group, or loss of carbons 20 and 21. Androgens are reduced while estrogens are commonly hydroxylated. Some steroid metabolites are shown in Figures 7-11 and 7-12.

SYNTHETIC STEROIDS^{1a, 2, 1a_d}

Synthetic analogs of adrenocortical steroids which differ from the natural hormones in properties such as potency, solubility, and resistance to degradation have been developed for therapeutic and inves-

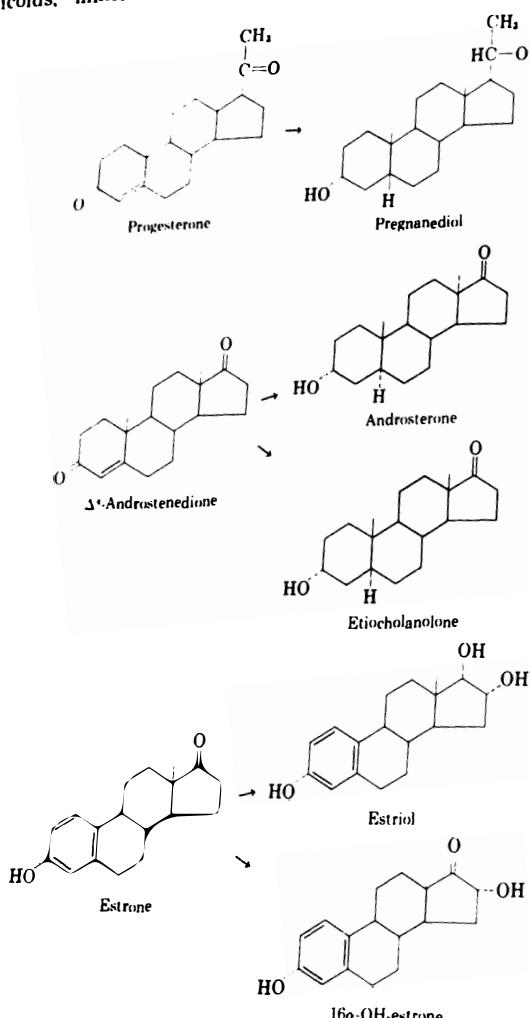


FIG. 7-12. Some metabolites of progesterone, androgens, and estrogens.

tigative purposes; and information has accumulated on relationships between molecular structure and biological activity. Greatest interest has been directed at synthesis of agents which have high anti-inflammatory potency and can be taken by mouth, but do not produce symptoms of hormone overdosage. Considerable success has been achieved in separation of anti-inflammatory from salt-retaining actions; but glucocorticoid potency of most anti-inflammatory agents is high.

Steroids with a double bond between carbons 1 and 2, including prednisone and prednisol (Fig. 7-13, A and B) have far greater anti-inflammatory and glucocorti-

coid potency than cortisol but relatively less salt-retaining activity; further reduction of salt-retaining action is achieved by addition of an α -methyl group (Fig. 7-13C).

Addition of a fluorine atom at position 9 greatly enhances potency. 9 α -Fluorocortisol (Fig. 7-13D) (and an orally active related compound in which the 21-hydroxy group is replaced by acetate) are highly effective for treatment of aldosterone deficiency. Addition of the 1, 2 double bond to fluorinated derivatives yields compounds with especially great anti-inflammatory potency but almost totally abolishes effects on sodium retention (Fig. 7-13, E, F, and G).

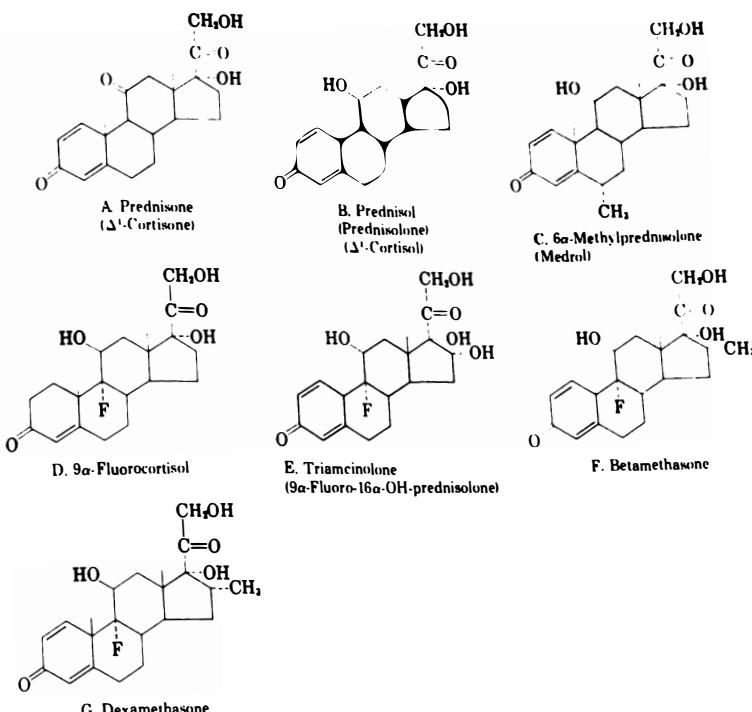


FIG. 7-13. Synthetic corticoids.

8. Adrenocorticotrophin, Corticotrophin Release Factor, Prostaglandins and Other Regulators of Glucocorticoid Secretion

Mechanisms controlling secretion of glucocorticoid hormones were described briefly in Chapter 2. The physiology and chemistry of ACTH, corticotrophin release factor (CRF), and other regulators are considered in this chapter. Structure and functions of the hypothalamus and pituitary gland are presented in Section VII. Additional relevant material will be found in Chapters 9 and 25.

GENERAL NATURE OF ACTH ACTIONS ON THE ADRENAL CORTEX^{10, 13a, 31}

ACTH maintains both structure and functions of the adrenal cortex and especially of the cells which secrete glucocorticoids.

Steroid hormone output can be augmented several fold within minutes after administration of ACTH, and the high secretion rate can be maintained for several hours if stimulation is continued. Since the adrenal cortex stores very little glucocorticoid, the secretion arises from new synthesis.

Increased protein and RNA content, and morphological evidence of growth and heightened activity can be demonstrated several hours after initiation of chronic treatment. Gains in gland weight and cell number are detectable within 24 hr, and extensive growth occurs within 4–5 days.

The effects are associated with enhanced glucose uptake and utilization, and accelerated blood flow. Activity of existing enzymes is increased, new enzyme formation is induced, and greater sensitivity to the pituitary hormone reflects formation of new receptors.

Glucocorticoid secretion diminishes within minutes after hypophysectomy and soon declines to low levels. Activity can be readily restored (in a dose-related manner)

by early administration of ACTH; however, sensitivity to stimulation otherwise declines within hours. Atrophy becomes apparent within a day. The zona fasciculata and zona reticularis gradually shrink to a fraction of preoperative size.

CHEMISTRY^{13b, 42}

ACTH is a peptide with a molecular weight of 4,500. Amino acid sequences have been determined for several mammalian species. All contain 39 amino acids, including long identical sequences. Structures of human, bovine, and porcine hormones are compared in Figure 8-1B.

Synthetic ACTHs containing the common N-terminal residues (Fig. 8-1A) are as potent as the native molecules. Amino acids 25–33 (Fig. 8-1B) are believed to participate in binding to receptors and exhibit species specificity.

STORAGE, SECRETION, METABOLISM

Very little ACTH is normally stored in the pituitary gland. Secretory rates are low under most conditions (not more than a few units per day in humans), and blood levels are measured in microunits. Synthetic, release, and plasma concentrations can be rapidly augmented several fold following exposure to stress.

ACTH is taken up by many cell types and is soon degraded by proteolytic enzymes of plasma, liver, and other tissues. Half-life in humans has been estimated at 25 min.

MECHANISM OF ACTION OF ACTH ON THE ADRENAL CORTEX^{13a, 31}

Binding to the Hormone Receptor

In common with other peptide hormones, ACTH is believed to exert its

REGULATORS OF GLUCOCORTICOID SECRETION

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
 Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Try-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg.
 18 19 20 21 22 23 24
 Arg-Pro-Val-Lys-Val-Tyr-Pro-

A. Common sequence of first 24 amino acid residues

25 26 27 28 29 30 31 32 33 34 35 36 37 38 39

Human: Asp-Ala-Gly-Glu-Asp-Gln-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe
Bovine: Asp-Gly-Glu-Ala-Glu-Asp-Ser-Ala-Gln-Ala-Phe-Pro-Leu-Glu-Phe
Porcine: Asp-Gly-Ala-Glu-Asp-Gln-Leu-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe

B. Amino acid sequences of ACTHs of three species

FIG. 8-1. Structure of ACTH.

actions on the plasma membrane. The concept is supported by several kinds of evidence including demonstration of effectiveness of the hormone when linked to macromolecular particles that cannot enter the cell (Chapter 2).

Hormone action is initiated by binding to a specific receptor, and a protein with high affinity for ACTH has been identified. Although resting potentials and influences on them of extracellular ions have been observed, attempts to demonstrate that ACTH depolarizes membranes of adrenocortical cells have yielded negative data.

Activation of Adenylate Cyclase

cAMP mimics most ACTH actions (including stimulation of protein synthesis, steroid secretion, and growth of the gland) and has been implicated as the mediator. Large doses of ACTH activate adenylate cyclase. Unlike the situation for many other cell types, the activation is dependent upon the presence of extracellular calcium. As yet unidentified steps may be interposed between hormone binding and cAMP generation.

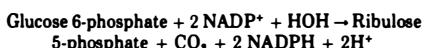
There are controversies concerning interactions between cAMP and ACTH. Small doses of the latter markedly stimulate steroid synthesis without significant elevation of cAMP concentrations. It has therefore been proposed that the nucleotide mediates only those responses to strong stimulation such as might occur during times of stress; but *localized* cAMP levels have not been determined. Low doses of ACTH

increase concentrations of cGMP, and the latter has been implicated in mediation of responses; but it is far less potent than cAMP for stimulation of steroidogenesis.

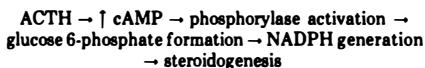
Role of cAMP²⁴

A cAMP-dependent *protein kinase* which catalyzes phosphorylation of ribosomal and other proteins (and possibly also lipids) has been identified, and the concept that cAMP is directly involved in promoting formation of one or more *highly specific proteins* (see below) has gained favor.

The role of cAMP in activation of phosphorylases in liver, skeletal muscle, and other tissues is well established. Glucose 6-phosphate derived from phosphorylase-catalyzed glycogenolysis can be utilized via the *hexose monophosphate pathway* (hexose monophosphate shunt, pentose pathway) in the adrenal cortex; substantial quantities of *NADPH required for steroid hormone synthesis* can then be generated:



The following mechanism of action of ACTH in mediation of steroidogenesis has been proposed:



The concept is supported by (1) rise in cytosol cAMP after strong ACTH stimulation; (2) presence of glycogen in adrenal cortices of most species, and its depletion following gland stimulation; (3) existence of the necessary enzymes

for the pentose pathway, evidence for increased NADPH generation and utilization, and absolute requirement for the coenzyme in steroidogenesis; and (4) stimulation of steroidogenesis in broken cell preparations by addition of glucose 6-phosphate and NADPH.

While the described processes undoubtedly contribute to physiological functions, they cannot be invoked as the mechanism of action for many reasons including the following: (1) glycogen depletion after ACTH does not seem to occur in some species such as the rat; and steroidogenesis can proceed if glycogen is deliberately depleted before application of the ACTH stimulus; (2) effects of NADPH and ACTH are not identical in broken cell preparations; after maximal effects of NADPH addition have been achieved, steroidogenesis can be further enhanced by addition of ACTH; (3) in some studies a quantitative relationship between utilization of the pentose pathway and steroidogenesis could not be demonstrated. (Moreover, activation of adrenocortical phosphorylase differs from activation of the related enzyme in liver and muscle; in the adrenal gland, cAMP inhibits phosphorylase *a* phosphatase but does not seem to stimulate activity of the phosphorylase *b* kinase kinase.)

Protein Synthesis^{13a}

Actions of both ACTH and cAMP are blocked by inhibitors of protein synthesis administered just prior to or along with the former. However, the inhibitors exert little or no early influence on *basal* levels of steroid output and are ineffective if given even minutes after the hormone.

It has been proposed that ACTH actions are mediated via *formation of a specific protein* (sometimes called the X protein) which is rapidly formed and can continue to stimulate steroidogenesis for a short time when additional protein synthesis is blocked.

Since actions of ACTH are not blocked by administration of actinomycin D, and also because hormone actions appear very early, ACTH may be acting on *translation* utilizing a relatively stable template. The protein formed may be highly labile (or readily sequestered), since effects of ACTH withdrawal can be detected within minutes.

As yet unsuccessful attempts have been made to isolate the protein which may be present in minute amounts. Questions have been raised about whether a *new* protein is actually needed or whether the inhibitors interfere with processes of protein synthesis that go

on in the absence of ACTH but are required to continue if hormone action is to be expressed. Additional questions have been raised concerning mechanisms whereby puromycin blocks steroidogenesis, since the antibiotic can influence glycogenolysis and phosphodiesterase activity.

A different ("Y") protein may mediate ACTH and cAMP influences on growth of the adrenal gland.

Influences of ACTH on Adrenocortical Desmolase

In common with other peptides which stimulate steroidogenesis and activate adenylate cyclase (e.g., luteinizing hormone influences on the testis and ovary), ACTH seems to accelerate conversion of cholesterol to progesterone (Fig. 7-5); increased rate of formation of radioactively labeled progesterone following administration of labeled cholesterol has been repeatedly demonstrated. Studies in *some* species (e.g., the rat) have shown no influence on rates of formation of glucocorticoid hormones from labelled progesterone.

The concept of a *direct* influence on activity of some enzyme of the desmolase system (the complex involved in conversion of cholesterol to pregnenolone) is difficult to reconcile with the observation that, whereas glucocorticoid secretion diminishes within minutes after ACTH withdrawal, activity of the desmolase system of hypophysectomized rats does not fall off appreciably for at least 24 hr. (Steroidogenesis could, however, be affected in many ways; and conditions for measuring desmolase activity may differ in crucial respects from physiological ones.)

Effects on Mitochondrial Membranes

Transport of cholesterol into the mitochondria could be rate-limiting for steroidogenesis. ACTH accelerates adrenal uptake of plasma cholesterol (even in the presence of glutethimide which blocks cholesterol utilization) and also promotes synthesis of cholesterol from acetate. Moreover, ACTH (indirectly) and cAMP (directly) increase activity of plasma and adrenal cholesterol esterases. In addition to such influences which increase cholesterol availability, ACTH may affect properties of the mitochondrial membrane.

Pregnenolone can exert negative feed-

back control of its own synthesis by decreasing the rate of hydroxylation of cholesterol (Chapter 7). It has been proposed that *ACTH promotes egress of pregnenolone* and thereby *removes* otherwise present *inhibition of the desmolase system*. (Pregnenolone entering the cytoplasm is rapidly converted to progesterone.) A protein formed under the influence of ACTH could function in pregnenolone transport. ACTH may also promote synthesis of something which facilitates release of the finished hormone; steroid secretion is accelerated almost immediately after cells are exposed to ACTH, and it has been reported that corticosterone buildup inhibits adrenocortical RNA and ATP synthesis.

An objection to the pregnenolone egress hypothesis comes from the observation that pregnenolone accumulates when cyanoketone is used to block its conversion to progesterone. But *intramitochondrial* concentrations of pregnenolone have not been determined in such studies, and cyanoketone could also exert other actions.

There are good reasons for suspecting that a "physiological brake" is present in resting adrenal cells, and that the brake is removed by ACTH stimulation. It has long been known that excessive handling of adrenal glands during processing for *in vitro* studies yields preparations with "excessively high basal steroid outputs" and low sensitivity to ACTH. (Indeed, it is the practice in some laboratories to routinely discard such preparations in the belief that they are defective.) Freezing and thawing, and treatment with detergents, increases mitochondrial permeability and pregnenolone formation. The basal output by adrenal glands of intact animals is very low, and diurnal secretion rhythms are closely linked with fluctuations in ACTH output.

Influences of ACTH on development of adrenocortical cells and on restoration of glands atrophied following hypophysectomy are associated with morphologically visible changes in mitochondria.

Mitochondrial Enzymes

There is no reason for assuming that ACTH acts at a single site. Early influences on activity and delayed influences on content of several enzymes have been observed.

Very soon after hypophysectomy there is *increased activity of adrenal 5-OH-reductases* and release of biologically ineffective steroids preceding decreases in rate of cholesterol utili-

zation. ACTH could be influencing the enzyme itself or the availability of the glucocorticoid substrate.

Developmental influences of ACTH on mitochondria of fetal adrenocortical cells maintained in tissue culture are accompanied by increased *11-β-hydroxylase* activity (with no influence on 21-hydroxylation which is catalyzed by microsomal enzyme). Inhibition of the processes by chloramphenicol raises the possibility that ACTH promotes mitochondrial synthesis of proteins. ACTH also increases activities of adrenodoxin and cytochrome P-450 but not of respiratory chain cytochromes in adrenal tumor cells.

Influences of ACTH on mitochondria that are associated with growth of the adrenal must be at least partially mediated via stimulation of nucleic acid synthesis; they are associated with early, morphologically demonstrable influences on the *nucleolus*.

Glucose Uptake

ACTH promotes glucose uptake and glycolysis and thereby provides ATP for hormone synthesis. There is no evidence that the effects are *primary*; ACTH can stimulate steroidogenesis when no glucose is added to extracellular fluids *in vitro*.

Cytosol Calcium

Calcium ions have been implicated in *activation of mitochondrial hydroxylase enzymes* and also in steroid hormone release. As noted above, they are essential for ACTH activation of adenylate cyclase, but neither ACTH nor cAMP can accelerate steroidogenesis in cells exposed to calcium-free media. Very high concentrations of calcium ion *inhibit* adenylate cyclase and also reduce the rate of conversion of pregnenolone to progesterone. ACTH could be involved in direction of calcium ions away from inhibitory sites to those associated with steroid hormone synthesis and release.

Ascorbic Acid

Concentrations of ascorbic acid are higher in adrenocortical than in all other cells investigated, and amounts found in ACTH-sensitive fasciculata-reticularis regions are greater than those of the zona glomerulosa. Some investigators have implicated the vitamin in regulation of mito-

HORMONES AND BLOOD SUGAR

chondrial hydroxylation reactions; but functions remain unknown.

ACTH promotes adrenal ascorbic acid depletion in a dose-related manner that is utilized in hormone bioassay. The action is mediated via cAMP¹² and can be blocked with doses of cycloheximide which prevent stimulation of steroidogenesis. ACTH inhibits ascorbic acid uptake from the plasma; but this action does not account for the rapid depletion seen after hormone administration.

Most of the data is consistent with an inhibitory influence of ascorbic acid on steroid hormone release. Steroid synthesis occurs *in vitro* in preparations very low in ascorbic acid content, and NADPH can promote steroid release.

The fact that scurvy animals tend to put out higher than normal quantities of glucocorticoids has been cited as evidence for inhibitory influences of the vitamin. But others have suggested that the high glucocorticoid output indicates that the vitamin C deficiency has imposed a severe stress. It has also been proposed that vitamin C-deficient animals can utilize other substances to replace normal functions of ascorbic acid in steroid synthesis.

Extra-adrenal Actions of ACTH

High doses of ACTH promote cAMP-mediated lipolysis in adipose tissue cells, leading to elevation of the fatty acid content of the plasma and accelerated delivery of fatty acids to the liver. They also affect neurons involved in regulation of ACTH secretion and others affecting establishment and extinction of conditioned reflexes.*

ACTH secretion lead to increased pigmentation of the skin.

SOURCES OF ACTH

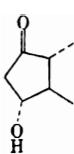
Corticotrophs of the pituitary gland which synthesize and secrete ACTH are described in Section VII. Cells located in the *pars distalis* have been most directly associated with secretion of ACTH under normal conditions; but others have been identified in the *pars intermedia* and in the neurohypophysis.

PROSTAGLANDINS AND ADRENOCORTICAL FUNCTION^{7, 12}

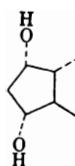
Chemical Nature and Sources

The prostaglandins are a specialized group of *fatty acids* containing 20 carbons, a five-membered ring, oxygen atoms, and strategically located double bonds. They are synthesized by a very large number of cell types and are widely distributed in animal tissues. Precursors for prostaglandin biosynthesis are the 20-carbon *essential ("polyunsaturated") fatty acids—arachidonic and di-homo- γ -linolenic acids*. Nomenclature of the prostaglandins is based on a hypothetical unsaturated molecule, *prostanoic acid*. The structures of prostanoic acid and of the precursor fatty acids are shown in Figure 8-2.

The nomenclature most widely used employs Roman capital letters, numbers, and Greek letters. The *capital letter* designates the nature of the five-membered ring. All prostaglandins of the *E* series have a ketone group attached to carbon 9 of the unsaturated ring, while prostaglandins of the *F* series have an OH group at the 9 position:



Ring structure of *E* prostaglandins



Ring structure of *F* prostaglandins

The *number* indicates presence of *double bonds* between carbons outside the ring structure. Thus prostaglandin E₁ (PGE₁) has a single, and PGE₂ two double bonds.

Attachment of the OH group to the 9-carbon of the F prostaglandins can have one of two

ACTH shares a common 13-amino acid sequence with α -melanocyte-stimulating hormone (Sections VII and VIII). Quantities released when adrenocortical insufficiency removes inhibitory influences on

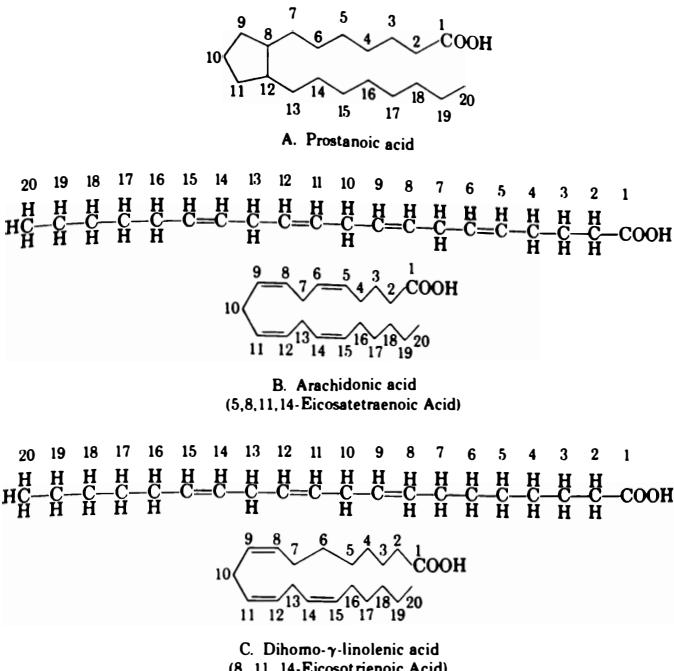


FIG. 8-2. Structures of Prostanoic acid and of the precursors for biosynthesis of prostaglandins.

possible spatial arrangements; the *spatial configuration* is designated by Greek letters. (Thus PGF, α and PGF, β are stereoisomers.)

At least 14 different prostaglandins have been found in mammalian tissues (13 of them in seminal vesicles). But the number of potential stereoisomers is far greater. Chemists have developed a more precise system for naming these compounds, but it has not yet been widely adopted by biologists.

Di-homo- γ -linolenic acid is the precursor for PGE₁ and PGF₁, while arachidonic acid is the precursor for PGE₂ and PGF_{2\alpha}. It has been stated that the letter designations refer to similarities between the ketone group of the E series and glucocorticoid E (Chapter 7) and between the alcoholic group F series and glucocorticoid F; but the letters were originally assigned on the basis of greater ether (E) solubility of the first group and greater solubility of the F group in phosphate buffers (Fosfat in Swedish).

Structures of PGE₁, PGF₁ α , PGE₂, PGF₂ α , and of the rings of the F β , A and B series are shown in Figure 8-3. The A series is derived (by dehydration) from the E series; isomerization of the A series yields the B group.

The naturally occurring prostaglandins have an α -OH group attached to carbon 15, and those of the E and F series have an additional OH

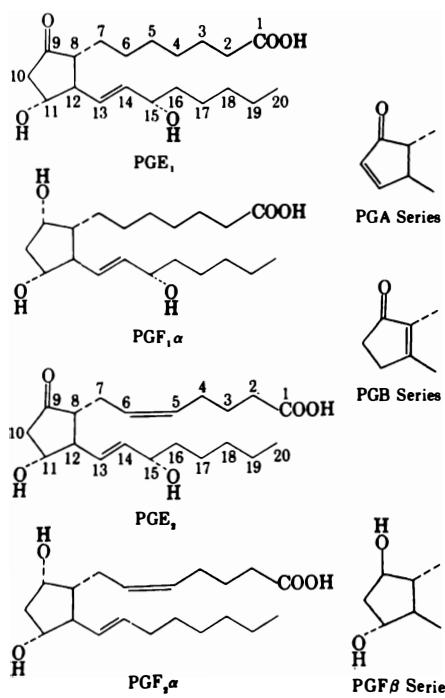


FIG. 8-3. Structures of PGE₁, PGF₄ α , PGE₂, PGF₂ α , and ring structures for the A, B, and F β series.

HORMONES AND BLOOD SUGAR

group attached to carbon 11. Those with triple bonds (PGE₂, PGF_α, etc.) have the extra double bond between carbons 17 and 18.

Biosynthesis of prostaglandins is catalyzed by a multienzyme microsomal complex known collectively as the *prostaglandin synthetase system*. The *rate-limiting* factor seems to be the *availability* of the *precursor* fatty acids which are present mainly as components of membrane phospholipids and as esters contained in lipid droplets of the cytoplasm. It is believed that cell perturbation (by hormones and other means, including neurotransmitters and mild mechanical stimuli) leads to increased activity of phospholipases (especially of phospholipase A) and of other acid hydrolases which catalyze release of the fatty acids and thereby provide the necessary substrate.

The prostaglandins are very rapidly inactivated within the cells that produce them, but small quantities may leave the cell and enter the circulating blood. Plasma prostaglandins are quickly taken up by a variety of tissues. In a single passage of blood through the lung, approximately 90% of the activity of most injected prostaglandins is lost, while 80% can be destroyed by a single passage through the liver. The prostaglandins are subjected to dehydrogenation, oxygenation, and side-chain cleavage. The degradation products are excreted mostly by the kidneys, but small amounts appear in the bile.

The prostaglandins play a role in *inflammation*, *fever*, and perception of *pain* and have been implicated in numerous pathological conditions. Clinically useful drugs such as *aspirin* and *indomethacin* are potent inhibitors of *prostaglandin synthesis*; other agents which inhibit synthesis (and have been used in research) include certain fatty acids (linoleic and α-linoleic). *Glucocorticoids* with anti-inflammatory potency interfere with actions of prostaglandins. 7-Oxa-14-prostynoic acid seems to compete with PGEs for receptor sites.

Role in Regulation of Secretion of Adrenocortical Hormones^{7, 12, 20}

The adrenal cortex is rich in cholesteryl esters of unsaturated fatty acids including a high proportion of arachidonic and lesser amounts of eicosatrienoic acids, and con-

tains enzymes which catalyze their hydrolysis.

ACTH promotes rapid depletion of adrenocortical lipids and enhances rates of prostaglandin release. Inhibitors of prostaglandin synthesis can block steroidogenic actions of ACTH.

Problems of defining the role of prostaglandins are complicated by the use of different experimental conditions in the various laboratories and the fact that prostaglandin production and prostaglandin influences on the adrenal are strongly affected by the experimental conditions.

An 18-fold increase in production of PGE₂ and PGF_α, and a 4-fold increase in PGF_α have been reported after exposure of adrenal gland homogenates to ACTH. Since such influences have not been found after administration of the hormone to intact animals, it has been considered that ACTH may act differently *in vivo*; however, animals very rapidly degrade prostaglandins.

Species variations and differences in pre-treatment of test animals may account for apparent discrepancies in reports of relative potencies of the prostaglandins and in the quantitative nature of the secretory products obtained after their administration.

Some investigators have found that PGE₂ and PGF_α are especially effective in *in vitro* situations (incubations, perfusions, superusions), while others have reported that PGE₁ is the most potent.

An influence of prostaglandins on intracellular cAMP concentrations has been observed in many different kinds of studies on a wide variety of tissues. Where prostaglandins seem to antagonize hormone actions, it has been proposed that cell stimulation promotes a rise in both cAMP and prostaglandins, and that the prostaglandins provide an *internal inhibitory mechanism* for protection against excessive stimulation; in other cell types, prostaglandins have been reported to *mimic hormone action* by elevating cAMP concentrations.

In the adrenal cortex, evidence *against* an action of prostaglandins on steroidogenesis mediated via cAMP is stronger than evidence in favor of such a mechanism. The *time course* for prostaglandin stimulation seems to be very different from that of

ACTH or cAMP stimulation, and prostaglandins have been reported to stimulate steroid hormone secretion without elevating cAMP levels.

Prostaglandins *enhance blood flow* to the adrenal cortex and affect the condition of small blood vessels. This may contribute to physiological function; but there is no consistent relationship between blood flow and hormone release when the former is varied in other ways.

Prostaglandins may promote *displacement of membrane calcium* and therefore influence steroid hormone secretion in several different ways. They have also been implicated in *promoting release of ACTH*. Most studies measuring direct influences on the pituitary gland have yielded inconclusive data, but evidence for an effect on *hypothalamic release of CRF* (see below) is stronger.

CORTICOTROPHIN RELEASE FACTOR^{5, 8, 9, 18, 40, 41, 42}

General Nature of CRF Function

ACTH-secreting cells of the adenohypophysis require regular stimulation by corticotrophin release factor (CRF) for maintaining structure and function. Evidence for this and discussion of cells involved in CRF synthesis are presented in Section VII.

The hypothalamic hormone travels a short distance through a specialized set of blood vessels, and most of it reaches the pituitary; little CRF enters the major systemic circulation under most conditions.

Since substances other than CRF are capable of directly or indirectly promoting ACTH secretion, some authors prefer to use the term *corticotrophin release hormone (CRH)* for the specific hypothalamic stimulant, and to describe the less specific agents as corticotrophin releasing factors.

Chemical Nature of CRF^{5, 18, 40}

Although the active principle has not been chemically identified, it has been separated from other hypothalamic hormones and gives evidence of being a *small peptide*.

Chemical identification has been hindered by the unexplained instability of the hormone when hypothalamic extracts are subjected to

conventional purification processes, by the problems of bioassaying a regulator that is part of a highly complex system with numerous hormonal and neuronal controls (both stimulatory and inhibitory) in which other physiological agents exert some CRF-like activities, and by uncertainties regarding the specificity of inhibitors used in preparation of the test animals.

It was recently reported that two or more hypothalamic factors *act together* to promote ACTH release.⁴³ Separation of the factors during purification procedures could account for the apparent instability of CRF.

Peptides chemically related to (but distinguishable on basis of amino acid composition from) MSH and vasopressin have been isolated from hypothalamic extracts, characterized, synthesized, and shown to have highly potent CRF-like activity. Whether either of the peptides is the "true" CRF, or whether a family of CRF peptides exists which may exhibit species specificity remains to be established.

Effects of CRF

CRF promotes *depolarization* of the corticotroph plasma membrane, and ACTH release is evident within 2 min. Actions seem to be mediated via *adenylate cyclase*, and *calcium ions* are required in the extracellular fluids.

The existence of *separable processes of ACTH synthesis and release*, each controlled by its own *hypothalamic regulatory factor*, has been proposed. A brief period of ACTH secretion can be invoked in the presence of inhibitors of protein synthesis.

The quite early increase in bioassayable ACTH content of pituitary cells (which can be blocked with actinomycin D, puromycin, or ethionine) has been cited as evidence that CRF *directly* promotes hormone synthesis. But it is also possible that CRF-stimulated ACTH release *secondarily* leads to activation of synthetic mechanisms. It has also been proposed that CRF promotes conversion of a biologically inactive *precorticotrophin* to active ACTH.

Since influences of actinomycin D on ACTH secretion can be detected very early, it was suggested that a specific species of RNA is required for *release*; but substantial increases in RNA content of corticotrophs can be demonstrated after CRF stimulation.

Site of CRF Synthesis

Hypothalamic. The *median basal hypothalamus* of all species tested contains quite high

HORMONES AND BLOOD SUGAR

concentrations of CRF activity, but it is not certain whether this is because the hormone is largely synthesized there or whether hormone synthesized in other parts of the hypothalamus is sent there for storage and release. In the dog, signs of CRF deficiency can be invoked by making relatively discrete lesions in the hypothalamus; but the rat seems to be affected only by lesions involving a very extensive area. This could mean that either the CRF-secreting cells of the rat are widely distributed or that the rat more readily recruits accessory secretion sites when the major CRF-secreting cells are damaged.

Extrahypothalamic^{39, 41}. The ability of animals with damaged hypothalamus and ectopic pituitary glands to respond to surgical stress by exhibiting a delayed but quantitatively impressive increase in secretion of adrenocortical hormones has led to the suggestion that "wound hormones" may be produced at some peripheral site. More recently it was demonstrated that unstressed hypophysectomized rats with extensive hypothalamic lesions did not have detectable quantities of hypothalamic CRF in their circulating blood. But after they were exposed to laparotomy, a potent substance was found in the blood which promoted an intense and sustained elevation of glucocorticoid secretion when injected into intact recipient animals. It has been proposed that "*tissue CRF*" may supplement hypothalamic CRF at times when there is a need for *sustained massive output* of adrenocortical hormones.

Regulation of CRF Secretion^{8, 9, 11, 42}

CRF-secreting cells possess some autonomy, but they are subject to a wide range of regulatory influences, both stimulatory and inhibitory, mediated by neurotransmitters, hormones, and humoral agents which do not fit neatly into either classification.

There is understandable confusion regarding the control of CRF secretion. Each of the techniques used to obtain information suffers from obvious limitations. The problem is compounded by species variations, individual differences within a species, the fact that subtle changes in the condition of the animals at the time of testing can affect the results, and the release of a variety of other agents affecting ACTH secretion at times when CRF release is stimulated.

CRF-secreting cells are located in a region which receives extensive and varied neuronal inputs with efferent and afferent connections to all parts of the central nervous system; cells sensitive to one or more humoral influences are

intermingled with cells affected by different influences. It is difficult to determine precisely how far implanted agents penetrate, whether they are taken up by small blood vessels or components of the ventricular system, and how much the injury resulting from the implantation process affects the results; moreover, cells may be influenced not only by the *presence* of the agent but may show different responses to high and low concentrations and may even be sensitive to the *rate of change of the concentration*. The concentrations of humoral agents in highly localized areas are not necessarily reflected in measurements of quantities in somewhat larger areas, and the specificities of pharmacological agents are not always known. Destruction of specific neuron groups is usually accompanied by disruption of nerve pathways, and deliberate sectioning of nerve fibers may involve destruction of important cell bodies.

In the intact, unstressed animal, a *diurnal pattern* of CRF secretion obtains, but hormone concentrations at a specified time can be expected to be maintained within narrow limits. Many different kinds of *stress stimuli* rapidly and markedly *elevate CRF secretion*, and the response depends on the nature, intensity, and duration of the stimulus and the time of day. Most conditions promoting increased CRF secretion also affect release of other hormones.

If afferent nerves from the anterior hypothalamic region to the medial basal hypothalamus are severed, basal secretion of CRF is maintained; but the *circadian rhythm* is abolished, and response to some kinds of stress are impaired. Neurons directly involved in maintaining the circadian rhythm are believed by some investigators to reside in the *hippocampus* and *amygdala*, and there is some evidence that they are *serotonergic*. A diurnal rhythm of serotonin concentration in these structures has been described.

Electrical stimuli to parts of the amygdaloid complex increase, and to parts of the hippocampus decrease ACTH secretion. Serotonin has direct stimulatory actions when applied to the medial (but not lateral) parts of the hypothalamus and when injected into the lateral ventricles of intact rats.

Other investigators believe that diurnal rhythms are controlled by the suprachiasmatic nuclei of the hypothalamus.

Tonic inhibitory influences, both cholinergic and adrenergic, may reach the CRF cells via the

posterior hypothalamus. Total deafferentation of the medial basal hypothalamus enhances CRF output, while electrical stimulation of parts of the pons and midbrain leads to decreased ACTH secretion.

Carbachol implantation into the *anterior* hypothalamus is stimulatory, and effects are blocked by atropine. Adrenergic stimulatory influences have also been described; these and epinephrine-associated stress stimuli are abolished by midbrain section.

Attempts to demonstrate *dopaminergic* control of CRF secretion have yielded largely negative data. This has been attributed by some to the poor penetration of dopamine into active sites; some positive effects following administration of dopa (which penetrates more readily) have been described.

There are contradictory findings which are difficult to reconcile with some of the observations noted above. A complete understanding of control of CRF secretion must await clarification of the numerous pathways impinging on the hypothalamus, the role of steroid-sensitive and steroid-insensitive neurons, and a fuller understanding of the participation of peripheral mechanisms and of interactions between CRF and vasopressin.

CATECHOLAMINES AND ACTH SECRETION^{9, 11, 17, 18}

Interactions between catecholamines and the pituitary-adrenocortical system are numerous and complex. When animals are stressed, they release large quantities of adrenomedullary and adrenocortical hormones and exhibit activation of sympathetic neurons and accelerated metabolism of norepinephrine within the brain.

Systemic injection of epinephrine promotes ACTH release, and injection of epinephrine into the posterior (but not anterior) hypothalamus leads to increased release of adrenocortical hormones. Glucocorticoids participate in regulation of brain metabolism of catecholamines.

Lesions of the midbrain abolish ACTH responses to some forms of stress, effects of injection of epinephrine into the posterior hypothalamus, and some effects of systemic injection of epinephrine.

It has been proposed that catecholamines in the hypothalamus activate a pathway leading via the brainstem to the spinal cord, and that this pathway activates *peripheral* mechanisms which are relayed to neurons stimulating CRF release. Catecholamines may also override tonic cholinergic inhibition of CRF release. Attempts

to demonstrate a *direct relationship* between catecholamine concentrations within the vicinity of CRF-secreting neurons (or those implicated in stimulation of the CRF cells) have been unsuccessful.

A direct action of epinephrine on the pituitary corticotrophs can be demonstrated, but fairly high concentrations are required, and the physiological significance (if any) of this finding has not been ascertained. Lower concentrations can synergize with effects of hypothalamic extracts; it is possible that this contributes to the rapid release of ACTH under some conditions.

Since the direct effects of epinephrine on the pituitary gland can be blocked with pharmacological agents which do not abolish responses to median eminence extracts, the mechanisms of action of epinephrine and CRF may be different; but the possibility that only the receptors differ cannot be ruled out by such studies. It is likely that both hormones influence the *adenylate cyclase* activity of the corticotrophs. The fact that a simple dose-response relationship between CRF concentrations and ACTH release does not seem to pertain (as it does for other hypothalamic releasing hormones) points up the possibility of some physiological importance of the synergism with epinephrine.

Catecholamines do not act directly on the adrenal cortex. But glucocorticoid hormones induce formation of the enzyme needed for conversion of norepinephrine to epinephrine. Numerous references have been made in the previous chapter to "permissive" effects of glucocorticoids needed for expression of the actions of catecholamines.

INFLUENCES OF VASOPRESSIN (ANTIDIURETIC HORMONE, ADH) ON SECRETION OF ADRENOCORTICAL HORMONES^{26, 40}

Large doses of vasopressin can promote release of ACTH, and this action is influenced by feedback mechanisms involved in regulation of ACTH and CRF secretion. It was believed at one time that vasopressin is, in fact, the hormone directly responsible for stimulation of the corticotrophs; but the belief is no longer held.

The concept of *neurohypophysial* control of ACTH secretion is interesting from several standpoints. Vasopressin is released in response to many of the "stress" conditions

which are known to promote augmented secretion of ACTH. There has been much speculation about the role in fresh water fishes of neurohypophysial hormones which are chemically similar to those promoting water retention in land animals (Section III). The close anatomical association between the neurohypophysis and the adenohypophysis in some of the fishes is consistent with neurohypophysial control over functions of corticotrophs in those animals. The influence of vasopressin on the corticotrophs of land animals may represent an evolutionary carry-over from the time before more specific regulators assumed control over adenohypophysial functions.

Although it is not the CRF, vasopressin may play an important role in regulation of adrenocortical secretion in mammals which complements or supplements that of CRF. Small amounts of vasopressin can synergize with small amounts of CRF to promote ACTH release. Vasopressin has also been implicated in the release of CRF. In addition, vasopressin can directly affect secretory functions of adrenocortical cells.

While stimuli such as histamine and ether promote release of both CRF and ADH, and release of ACTH in response to both hypothalamic hormones is diminished by high levels of glucocorticoids, neural pathways controlling release of CRF and of ADH seem to be quite different. Although both sets of pathways have been said to be cholinergic, it was shown in one study that atropine implanted into the hypothalamus in the midline just rostral to the paraventricular nuclei inhibited elevation of CRF secretion in response to surgical stress but did not diminish the ADH response. Possibly, each pathway subserves specific functions, and activation of both under certain conditions provides for enhanced release of CRF when needed.

INFLUENCES OF THE PINEAL GLAND^{15, 16, 32}

It has been reported that removal of the pineal gland leads to sustained reduction of adrenal ascorbic acid content, increased secretion of corticosterone, and heightened activity of adrenocortical 5-reductase activity. It has also been reported that intraventricular injection of melatonin (a pineal hormone) leads within an hour to reduction of plasma corticosterone levels of intact rats, while pineal activation inhibits ACTH secretion.

The findings are consistent with an inhibitory influence of the pineal on ACTH secretion; however other investigators have found that melatonin administration increases adrenal gland weights and corticosterone production (see also Section VIII).

ADRENOCORTICAL RHYTHMS^{30, 42}

In nocturnal animals (*e.g.*, laboratory rats), glucocorticoid concentrations in the blood rise shortly before the onset of darkness and decline to lowest levels in the early morning. In animals active during the daylight hours, adrenocortical hormone secretion tends to reach a peak in the early morning and declines to lowest levels during the night (although recent studies indicate that the secretion pattern for individual subjects may be more complex than has been surmised from examination of data based on group averages).

Lesions of the *suprachiasmatic nuclei* of the *hypothalamus* have been reported to abolish not only adrenocortical but also a variety of other recurrent patterns. Stimuli for synchronization of the rhythms with environmental lighting changes probably feed into the nuclei via the *retino-hypothalamic* tracts now known to exist in a variety of mammalian species. When animals are deprived of light-dark cues (by being maintained in continuous darkness or through destruction of the eyes or optic tracts), they continue to exhibit rhythmic secretory patterns; but peaks and troughs can occur at different hours, and the periodicity may be somewhat less than 24 hr (see also Section VIII). On the other hand, continuous light can disrupt the rhythmic patterns.

Although rhythms are no longer believed to be initiated in the temporal lobes of the brain, these areas probably exert important influences. Time courses for periodic changes in serotonin content of the hypothalamus and amygdala coincide with those for CRF release; and administration of pharmacological agents which alter serotonin synthesis and release can disrupt adrenocortical rhythms. Moreover, patients with several kinds of brain lesions and altered states of consciousness exhibit altered (or no clear-cut) adrenocortical rhythms, and the condition is often corrected following clinical recovery.

Brain catecholamine rhythms have been demonstrated, but agents affecting catecholamine synthesis and release do not disrupt the adrenocortical patterns, and administration of

epinephrine seems to have little influence on a long range basis.

While CRF secretion probably directs changes in the pituitary and adrenal glands, it is known that adrenocortical cells maintained in tissue culture exhibit *intrinsic* rhythmic secretion patterns (which could have been entrained during previous exposure to intermittent ACTH stimulation). Since glucocorticoids affect ACTH release (see below), peripheral mechanisms probably modify influences of hypothalamic control.

A regulatory hierarchy is found in many parts of the body e.g., in control of cardiac function by sinuatrial and atrioventricular nodal tissues. But there is no evidence for reestablishment of adrenocortical rhythms following abolition of CRF control.

High levels of glucocorticoids just before awakening and during the hours of activity are useful for assuring the maintenance of blood glucose levels, and may be especially important for the process of acquiring breakfast after carbohydrate reserves have been partially depleted. Excessive glucocorticoid function can be antagonized during and soon after meal ingestion by the metabolic actions of insulin. The fall in glucocorticoid levels during the periods of inactivity is conducive to sleep and anabolic repair processes. Excessive tissue destruction seen in patients with Cushing's disease seems to result from inability to appropriately reduce glucocorticoid secretion at night; maximal secretion rates during daylight periods may be no greater than those of normal individuals. The widespread occurrence of adrenocortical rhythms among the vertebrates probably results from genetic selection since synchronization of the rhythms with environmental changes contributes to survival.

Secretion rhythms can be shifted in response to a change in light-dark patterns, but a lag period is usually seen. Animals subjected in the laboratory to sudden reversal of lighting patterns may require several days to make the adjustment. Humans traveling from northern to southern hemispheres may take a week or more to adapt. Those switching from day to night work in the same locality exhibit wide variations in adjustment time; this could result in part from individual differences but is probably also related to the presence

of light, noise, and other stimuli during the sleeping hours and to irregularity of activity patterns especially in those who sleep the nights they do not work.

The lag in shift of adrenocortical rhythms has been blamed for at least some of the discomfort and fatigue experienced by persons taking long distance west-east airplane flights.

It has been suggested that "morning people" (those who feel alert immediately upon arising but tend to feel tired in the late afternoon or evening) differ from "evening people" in that their glucocorticoid hormone levels rise earlier; and that mothers who give birth during the very early morning hours have different rhythms from those who give birth during the late morning or afternoon.

Growth hormone secretion is maximal at times when glucocorticoid levels are low. It is probable that synchronization of a large number of endocrine rhythms contributes substantially to optimal function. Perhaps future investigations will reveal some of the interactions that are needed to maintain alignment of functions not obviously influenced by environmental lighting or sleep-activity patterns. Interestingly, although glucocorticoids can rapidly induce synthesis of hepatic tyrosine transaminase, and levels of this enzyme undergo diurnal variations, no influence of adrenalectomy on transaminase rhythms has been found.

FEEDBACK REGULATION OF GLUCOCORTICOID SECRETION

The more obvious aspects of feedback regulation of the hypothalamo-hypophyseal-adrenocortical system were described in Chapter 2. High levels of glucocorticoid hormones lead to inhibition of glucocorticoid secretion. Controls are exerted over both release and synthesis of the various hormones; there are indications that release may be affected first.

Glucocorticoids can directly inhibit adenohypophyseal secretion of ACTH by a mechanism that seems to require synthesis of new protein. Soon after administration of high doses of glucocorticoids, the corticotrophs exhibit increased granulation (consistent with impaired ACTH release); later ACTH synthesis declines, and chronic administration of potent glucocorticoids leads to atrophy of the adrenocortical cells.

Removal of one adrenal gland soon leads to compensatory hypertrophy of the one that remains, and this is associated with increased secretion of ACTH. The response is blocked by hypothalamic damage; this suggests that corticotrophs may not be directly sensitive to a fall in plasma glucocorticoid levels.

CRF-secreting cells may be affected by (1) excessively *high*, (2) very *low*, and (3) *rate of change*, of glucocorticoid concentrations of the plasma or cerebrospinal fluid. Some of the neurons which affect CRF cells bind glucocorticoid hormones and may respond to steroids directly; but others are glucocorticoid-insensitive.

Glucocorticoids inhibit *responses* of pituitary cells to CRF, vasopressin, and to other stimuli. They also seem to be capable of exerting a direct inhibitory influence on their *own secretion* in the adrenal cortex. (And they affect the conversion of norepinephrine to epinephrine.)

ACTH seems to exert inhibitory influences at several levels. The target sites include the CRF-secreting neurons, other neurons in the brain, and probably also the ACTH-secreting cells of the adenohypophysis.

CRF can inhibit its own secretion and may also influence other neurons. The existence of a separate hypothalamic corticotrophin *inhibitory* factor has been proposed, but there is no clear evidence for its existence.

During times of stress very large quantities of CRF and of ACTH are released even though glucocorticoid concentrations in the blood plasma are far in excess of those capable of inducing inhibition under the more usual conditions. The most obvious explanation lies in the recruitment of numerous *stimulatory influences* on CRF-secreting cells, suppression of neuron-mediated *inhibitory* influences, and the ability of large amounts of CRF to overcome steroid inhibition of the corticotrophs. (When sufficient glucocorticoid has been given to completely suppress ACTH secretion, it is still possible for the pituitary gland to respond to very high doses of CRF.) But it is likely that accessory mechanisms are needed when adaptations to stress demand sustained high levels of glucocorticoids. At such times, the actions of vasopressin, epinephrine, and of the

proposed extrahypothalamic ("peripheral") CRF may assume importance.

OTHER FACTORS AFFECTING SECRETION OF ADRENOCORTICAL HORMONES

Histamine is found in high concentrations in some parts of the brain (including the hypothalamus) and is synthesized locally from histidine. It is a very potent releaser of ACTH both *in vivo* and *in vitro*. Vasodilator actions may contribute to effects in the whole animal.

Other agents capable of directly promoting ACTH release include *angiotensin II* which is released in response to some forms of stress (Section III). *Spermidine* promotes ACTH release when present in very low concentrations; but no physiological significance has as yet been attached to this influence of a naturally occurring polyamine.

Many *pharmacological* agents have been used to study ACTH release. Some seem to exert multiple actions, e.g., directly on CRF cells, on blood pressure changes which affect neuroendocrine mechanisms, and on histamine release.

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Additional references on ACTH are included in Section VII, and on the thymus and pineal glands in Section VIII.

9. Catecholamines

INTRODUCTION

Catecholamines are dihydroxyphenylamines; the biologically important ones are *epinephrine* (adrenalin), *norepinephrine* (noradrenalin), and *dopamine*. The first is

a "proper" hormone, since it is secreted into the circulation by the adrenal medulla. (Small quantities are synthesized locally in brain tissue, but it is unlikely that brain contributes measurably to plasma epinephrine.) Epinephrine is taken

up by sympathetic nerves, and it exerts some actions which are indistinguishable from those of neurotransmitters.

Norepinephrine is synthesized in the adrenal medulla and released into the circulation. It is also synthesized within the central nervous system where it may act locally, and by postganglionic neurons of the sympathetic system from which it is released to function as a neurotransmitter. The adrenomedullary "hormone" and the neurotransmitter are both released in response to some stimuli, and they interact; it is impossible to completely separate certain functions of the amines arising from different sources.

Dopamine behaves primarily as a neurotransmitter; however, small amounts are secreted by the adrenal medulla, and large amounts injected into the blood plasma can exert hormone-like actions.

This chapter describes metabolic and related physiological functions of epinephrine and norepinephrine. The term catecholamine is used loosely to designate both, except where discussion of dopamine is indicated.

THE NEED FOR EPINEPHRINE

Resting skeletal muscle derives most of its energy from short chain fatty acids supplied by the blood plasma. Metabolic needs are satisfied as long as fuel requirements are limited and the oxygen supply is adequate for aerobic processes. At times when both insulin and glucose are abundant, the latter is taken up and stored as glycogen; this ensures that fuel for glycolysis will be recruitable to support activity during periods of fasting.

Epinephrine sets in motion a large number of physiological mechanisms required to sustain vigorous activity.^{1, 5, 7, 12} It promotes conversion of muscle glycogen to glucose phosphate, and contributes to glucagon stimulation of glycogenolysis and gluconeogenesis in the liver. Activation of lipases enhances the supply of fatty acid fuels to cells not dependent upon carbohydrate and furnishes small amounts of glycerol for use in glucose synthesis. Elevation of plasma fatty acid concentrations also reduces sugar uptake and utilization in cells with flexible metabolic requirements. Further reduction of nonessential glucose

metabolism results from inhibition of insulin release. Although insulin facilitation of glucose uptake by muscle cells involved in contraction is thereby lost, this presents no problem since the mild anoxia, increased ATP use and some as yet unidentified factor associated with exercise enhance entry of the necessary fuel.

Epinephrine stimulates the heart, increases cardiac output, and promotes redistribution of the blood supply. Vessels supplying structures not involved in muscle activity (skin, mucous membranes, viscera) are constricted, while increased blood flow through muscle cells brings in food and oxygen and carries away carbon dioxide and lactic acid. Removal of the end product of glycolysis serves a dual purpose; muscle fatigue is delayed, and a substrate is supplied to the liver for new glucose synthesis. Accelerated blood flow through the liver enhances both lactic acid uptake and glucose secretion.

Pulmonary ventilation is increased through stimulation of the respiratory system, constriction of pulmonary vessels, and dilation of the bronchioles. More direct actions on skeletal muscle include facilitation of neuromuscular transmission and stimulation of contractile processes in white or "fast" type fibers.⁷

While the entire spectrum of epinephrine actions prepares the organism for sustained physical activity, the hormone performs much broader functions. A colorful concept introduced years ago is that epinephrine prepares the organism for "fight or flight" when threatened; and, indeed, epinephrine is rapidly released in response to awareness of potential danger.

Fight or flight requires all of the adjustments described above but also additional actions exerted by adrenalin. It is appropriate to suspend nonessential visceral activities; in addition to diversion of the blood supply away from such structures, catecholamines directly inhibit tone and motility of the gastrointestinal tract and reduce secretion of some digestive juices. Muscle tone of the urinary bladder is diminished, while both gut and bladder sphincters are stimulated. Reduced blood supply to the kidney diminishes urine formation.

Accelerated blood coagulation can be useful in event of injury, while contraction

of the spleen releases blood cell reserves and contributes to oxygen-carrying capacity and carbon dioxide removal. Increased blood flow through the brain increases awareness. Direct influences on sweat glands aid in dissipation of extra heat generated by muscle contraction. Piloerection makes furry animals appear larger and more fierce. The relaxing effect on red or "slow" striated muscle is useful in situations where immobilization ("freezing") constitutes an appropriate response.

Contraction of the radial muscles of the eye permits entry of more light, while relaxation of the ciliary muscles confers accommodation for vision of a broader field at times when ability to read fine print becomes irrelevant.

The few known actions of epinephrine which do not obviously apply here include increased activity of the exocrine pancreas and reduction of the number of circulating eosinophilic leucocytes.

While the concept is useful for appreciation of epinephrine actions, it portrays a narrow view of the function of this hormone and of the related catecholamine norepinephrine. Both are released in response to a wide variety of stimuli not directly associated with body movement, e.g., strong emotions other than fear; and they protect against bronchiolar constriction, hemorrhage, hypoglycemia, and cold exposure.

Smaller amounts are needed to maintain autonomic balance and provide for the minute adjustments that are continually needed. Unopposed actions of acetylcholine released by the parasympathetic neurons could dangerously slow the heart and reduce systemic blood pressure; tonic influences of catecholamines keep this under control, while small increments provide for such things as change from recumbent to upright posture.

ADRENAL DEMEDULLATION

It is relatively easy to remove adrenomedullary cells from laboratory animals without seriously compromising adrenocortical functions. The capsule of the adrenal gland is split, and the contents scooped out and removed. The few cortical cells adhering to the capsule soon grow and divide, and a functioning gland is reestablished. If some adrenomedullary cells are

inadvertently left behind in the animal, catecholamine function is not reestablished since the cells of the medulla (which are actually modified sympathetic ganglia cells derived from the neural crest) do not regenerate.

Animals subjected to such adrenal "enucleation" can survive for long periods of time under suitable conditions, and may appear healthy; but they are unable to perform sustained muscular work (e.g., forced swimming), exhibit impaired responses to some forms of stress, and may succumb to situations well tolerated by intact animals (e.g., sudden exposure to cold environments, hemorrhages, or injections of moderate doses of insulin).

SYMPATHECTOMY

It is not possible to study animals totally deprived of sympathetic nerves. But the importance of norepinephrine under non-stress conditions can be observed in animals with partial surgical sympathectomies, with selective destruction of sympathetic neurons, or following administration of agents described below which interfere with development or secretory function of the nerves or antagonize actions of secreted norepinephrine.

Animals treated in this way have difficulty maintaining normal blood pressure, have impaired ability to utilize reserve food materials, and are unable to make many adjustments that are part of ordinary living patterns.

METABOLIC FUNCTIONS OF EPINEPHRINE COMPARED WITH THOSE OF GLUCAGON

Since most actions of epinephrine are mediated via cAMP, some similar influences are exerted on metabolic processes. Differences are related in part to distribution of the two hormones and to the nature of tissue receptors.

Epinephrine exerts numerous glucagon-like actions on liver, including activation of the glycogen phosphorylase system and promotion of gluconeogenesis. However, only relatively large amounts of epinephrine (e.g. those released in response to hypoglycemia, stress, and strenuous exercise) contribute substantially in this way

to elevation of blood glucose concentrations.

Glucagon regulation of hepatic phosphorylase was described in detail in Chapter 6. The chemical structure of muscle phosphorylase and certain molecular details of its activation differ from those described in the liver, but basic control mechanisms exerted by epinephrine are similar. (Glucagon does not ordinarily affect the muscle system directly because little of the peptide reaches the cells and because the latter are relatively insensitive to glucagon; however, it can promote epinephrine release.)

It was pointed out that the phosphorylase control system provides for very rapid amplification of a small signal so that large quantities of glucose-phosphate can be formed within minutes, and that rapid shutdown is accomplished after withdrawal of the hormonal stimulus.

The shutdown system assumes even greater importance in muscle and can be readily effected because of the short duration of action of epinephrine (p. 105). Considering the fact that muscle glycogen is synthesized only by resting muscle and only at times when glucose and insulin are abundant, it is constructive to ponder the situation in which a small hungry animal suddenly becomes aware of the presence of a predator. Rapid activation of the muscle phosphorylase system required for flight is facilitated by release of epinephrine. If the animal manages to escape, it is appropriate to immediately halt depletion of the limited remaining glycogen reserves. The predator utilizing glycogen in pursuit of his dinner has a similar need to terminate glycogenolysis if the prey escapes.

Epinephrine activation of the muscle system leads to production of substantial quantities of lactic acid (since this is the end product of muscle glycolysis). Glucagon activation of the liver system leads instead to production of free glucose for release into the circulation (but glucagon can indirectly increase lactic acid production through stimulation of adrenomedullary secretion).

While pharmacological doses of glucagon affect adipose tissue, physiological amounts of catecholamines activate adipose lipase enzymes. Some consequences were mentioned above (p. 94); others are described in Section V.

An important difference between glucagon and epinephrine is that the former stimulates insulin release while the latter inhibits it; this has been explained on the basis of epinephrine activation of α -adrenergic receptors of the islet cells (see below).

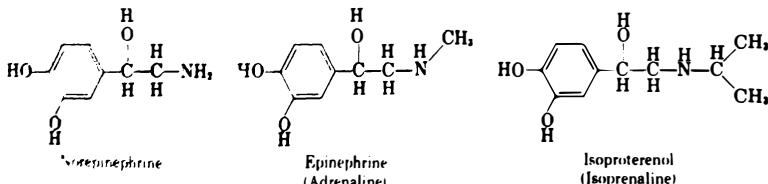
EXCITATORY VS. INHIBITORY ACTIONS OF CATECHOLAMINES

Hormones of the adrenal medulla (and sympathetic nerve stimulation) promote contraction of smooth muscle in some parts of the body (radial fibers of the eye, arterioles supplying the skin and mucous membranes) and relaxation elsewhere (e.g., the bronchioles, intestine, and some skeletal muscle arterioles). Lactic acid has been implicated as the agent which promotes dilation of muscle blood vessels during exercise.

An explanation was offered in the 1930's: two different mediators are involved, sympathin E which excites, and sympathin I which inhibits.¹³ As information on comparative actions of norepinephrine and epinephrine accumulated, attempts were made to equate the former with sympathin E and the latter with sympathin I, but it soon became apparent that each of the catecholamines exerts some actions which can be classified as inhibitory and others as excitatory (see below and also Table 9-3).

A more recent concept (useful in many ways but not free of drawbacks) is that cells responding to catecholamines have two different kinds of receptors, α and β types.^{1, 2, 4, 5, 7, 13}

The classification was based on the effects of administration of the naturally occurring norepinephrine and of the synthetic analog of epinephrine, isoproterenol:



All responses to norepinephrine were designated α and those to isoproterenol β , but not every epinephrine response could be classified one way or the other.

The concept of the existence of two types of receptors was strengthened when pharmacological agents became available which could selectively block one kind of response without substantially affecting the other. But it has been pointed out that the use of such agents and development of new ones frequently involves a kind of circular reasoning. For example, an agent could be classified as a β -blocker because it affected certain responses to isoproterenol; then, a previously unclassified effect of epinephrine could be assigned to the β group because it is blocked by the agent. After this, a new agent might be called a β -blocker because it affects the newly classified response.

One classification of adrenergic responses is presented in Table 9-1. The table might be regarded as a kind of generalized summary of majority opinions, rather than an absolute classification which agrees with all kinds of experiments under all conditions in all species. It is now felt that norepinephrine is more accurately described as possessing strong α (and weak β) type activity, while isoproterenol exerts strong β (and weak α) influences. Epinephrine has both activities, but the β actions predominate.

Some of the agents used to study adrenergic responses are shown in Table 9-2.^{1, 2, 3, 4}

Difficulties with the α - β concept arose when it was found that some pharmacological agents are highly potent for blocking certain responses of one type but are relatively ineffective on other responses within the same classification. Some authors suggested that there are more than two types of adrenergic receptors and have introduced additional Greek letters to distinguish them. But others prefer the interpretation that there are classes or families of α -type and β -type receptors.

Some of the difficulties of interpretation arise from the presence and apparent interaction of both types of receptors within the same tissues. (For example, inhibition of intestinal muscle may depend on both types, and a combination of α - and β -blockers is needed to abolish the responses to catecholamines.) Others are related to species differences; there are reasons for classifying the receptors involved in lipolysis

as β for most species but as α -type in rats and humans.

The use of pharmacological agents always poses problems of yet another nature. Inhibition of a response to catecholamines could result from interference with combination of the hormone with the receptor (or destruction of the receptor), from antagonism at some point beyond hormone-receptor interaction, or because the agent elicits an effect which is actually unrelated to hormone action but whose end result is opposite in direction to that of the hormone. Theoretically, drugs should be considered blocking agents only when they interfere with the action of the hormone. But this does not exclude the possibility that in addition they may exert more direct influences on the response, or they may alter metabolism of the hormone.

Despite the problems, the concept remains useful, at least for the present. There is a close relationship between the ability of a catecholamine to induce a rise in cAMP concentrations and the ability to evoke a β -type response; and cAMP (or its dibutyryl derivative) can usually be shown to produce the same effect. A similar relationship between the ability to lower cAMP concentrations and to evoke α -type responses also pertains; but the consistency with which this can be demonstrated is less convincing than is the case with the β group. There is still some question about whether all α -type responses can be related to lowering of cAMP levels, and part of this stems from inability to accurately determine nucleotide concentrations in localized portions of the cells. Epinephrine has been shown to increase incorporation of radioactive phosphate into phospholipids, and this does not seem to be associated with cAMP generation.

Attention has been directed at the close chemical similarity between agents which mimic and those which block β -type responses. It has been proposed that both types interact with β receptors, and some investigators believe that adenylate cyclase is the receptor. On the other hand, α -blocking agents are quite different from catecholamines, and an association with intracellular ATPase systems that compete with adenylate cyclase has been suggested.⁵

COMPARISONS OF EPINEPHRINE WITH NOREPINEPHRINE

Effects of the two catecholamines are summarized in Table 9-3.

HORMONES AND BLOOD SUGAR

TABLE 9-1
Classification of some of the Responses to Catecholamines

Structure Stimulated by Catecholamines	α -Response	β -Response
Vascular smooth muscle		
Skin	Constriction	
Mucous membranes	Constriction	
Salivary glands	Constriction	
Most abdominal viscera	Constriction	
Liver	Constriction	Dilation
Skeletal muscle	(Constriction)	Dilation
Lung	Constriction	
Other smooth muscle		
Bronchioles		Relaxation
Sphincters: gastro- intestinal, urinary	Contraction	
Pilomotor (skin)	Contraction	
Radial muscle of iris	Contraction	
Ciliary muscle (eye)		Relaxation
Stomach	Relaxation	
Intestine	(Relaxation)	Relaxation
Spleen	Contraction	
Heart		
Sinoatrial node	(Stimulation)	Rate increased
Atria		Increased conduction and contraction
Ventricles		Same as atria
Skeletal muscle		
White (Fast)		Faster, more complete contraction, slow re- laxation
Red (Slow)		Opposite to white
Neuromuscular transmission	Increased	Increased
Melanophores Glands		
Sweat	Consperion	Dispersion
Salivary	Secretion	Growth, amylase secretion
Exopancreas	Viscous secretion	Enzyme secretion
Pancreatic islets	Insulin inhibition	(Insulin secretion)
Pineal		Serotonin, melatonin synthesis
Kidney		Renin synthesis, release
Platelets	Aggregation	
Leukocytes		Antigen-induced histamine release inhibited
Calorigenesis		Increased
Glycogenolysis	(Increased)	Increased
Lipolysis	(Increased)	Increased
Toad bladder	Reduced water permeability	
Toad skin		Increased sodium, water permeability

Parentheses indicate effect of other receptor type predominates or other type in most species studied.

TABLE 9-2
Some Pharmacological Agents Used to Study Catecholamine Functions

Pharmacologically Useful Action	Agents Used
α -blockade	Phentolamine (Regitine) Phenoxybenzamine (Dibenzyline) Piperoxan (Benodaine) Dibenamine Tolazoline (Priscoline) Azapetine (Ildar) Ergot alkaloids
β -blockade	Propanolol (Inderal) Pronethalol DCI (dichloroisoproterenol) IMA (N-isopropylmethoxamine) Butoxamine Oxprenolol
Adrenergic blockade (α - and β) Catecholamine depletion	Guanethidine Reserpine (delayed effect; inhibits granule uptake) Methyldopa (inhibits synthesis) 6-Hydroxy-dopamine (destroys sympathetic neurons)
Adrenomimetic	Amphetamine Ephedrine Piperadol (action mostly central) Naphazoline (action mostly peripheral)

Both stimulate the heart, raise systolic blood pressure, decrease blood flow through skin, mucous membranes and kidney, increase blood flow through coronary vessels, and dilate the bronchioles.

Epinephrine is more useful for combating hypoglycemia; metabolic actions and dilation of skeletal muscle blood vessels make it more suitable for sustaining exercise.

Norepinephrine is more useful for protection against a fall in systemic blood pressure, since its actions on arteriolar muscle are predominantly constrictor. It stimulates the heart and quickens the rate of isolated hearts, but elevation of the diastolic pressure leads to reflex slowing of cardiac rate in the intact animal. Epinephrine, on the other hand, promotes dilation of vessels of the liver as well as of skeletal muscle; usually this results in no net change or even a lowering of diastolic pressure. Therefore stimulatory effects on

the heart of the intact animal may be more obvious, and cardiac output is enhanced.

Both elevate body temperature and metabolic rate and both promote lipolysis, but sympathetic innervation and norepinephrine secretion are more important for adaptation to low environmental temperatures.

BIOSYNTHESIS OF CATECHOLAMINES^{1, 2}

Catecholamines are synthesized in brain, sympathetic nerves and ganglia, the adrenal medulla (which is a modified ganglion) and chromaffin cells. Because of the wide distribution of sympathetic nerves, they are found in all organs. The heart is especially rich in norepinephrine, and fairly high concentrations occur in the hypothalamus.

Catecholamines are synthesized from tyrosine derived from the diet. Specific transport mechanisms facilitate its uptake from blood plasma.

TABLE 9-3
Comparison of Effects of Epinephrine and Norepinephrine

Adrenergic Response	Effect of Epinephrine	Effect of Norepinephrine
Cerebral blood flow	Increased	Little or no effect
Anxiety	Increased	No effect
Dilation of pupil	Marked	Slight
Ciliary muscle	Relaxed	No effect
Bronchioles	Markedly dilated	Slightly dilated
Cardiac force	Markedly stimulated	Markedly stimulated
Cardiac rate	Increased	Decreased (because of reflexes)
Systolic blood pressure	Increased	Markedly increased
Diastolic blood pressure	May be decreased	Markedly increased
Cardiac output	Markedly increased	May not be altered
Blood flow		
Skin	Decreased	Decreased
Mucous membranes	Decreased	Decreased
Kidney	Decreased	Decreased
Liver	Increased	No effect
Heart	Increased	Increased
Skeletal muscle	Increased	Little or no effect
Blood eosinophils	Decreased	No effect
Blood glucose	Markedly increased	Slightly increased
Blood lactic acid	Markedly increased	May be slightly increased
Oxygen consumption	Increased	Increased
Lipolysis	Increased	Increased

Tyrosine can also be obtained from dietary phenylalanine. Since the latter is a constituent of nerve tissue (and phenylalanine hydroxylase activity has been identified there as well as in the liver), some localized tyrosine formation may take place.

Tyrosine hydroxylase catalyzes conversion of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) (Fig. 9-1). This step may be *rate-limiting*; catecholamine synthesis can be blocked by administration of *α-methyltyrosine* which inhibits the enzyme.

A pteridine cofactor is required. Additional proposed sites for regulation of biosynthesis are activities of the dihydropteridine reductase and of the tyrosine transport system.

Dopamine, norepinephrine, and epinephrine inhibit the enzyme. Adrenal nerve stimulation enhances activity, possibly by promoting release of these inhibitors.

Adrenocorticotrophic hormone (ACTH) induces synthesis of the enzyme by a cAMP-

dependent mechanism. It has been proposed that either cAMP travels directly from the adrenal cortex to the medulla (via a portal system) or that ACTH promotes formation of an activator of medullary adenylate cyclase.

The enzyme is also inhibited by *iodinated tyrosines*; this probably has no physiological significance in normal individuals but can create problems for those in whom tissue dehalogenases are defective. Very high concentrations of phenylalanine are also inhibitory, and this contributes to the clinical picture of phenylketonuria. There are unresolved controversies concerning the subcellular location of the enzyme.

DOPA is decarboxylated by the cytoplasmic enzyme *dopa-decarboxylase* (dihydroxyphenylalanine decarboxylase), to yield *dopamine* (dihydroxyphenylethylamine).

Dopamine functions as a neurotransmitter in the central nervous system and participates in regulation of hypothalamic hormone secretion. It is found in high concentrations in the cerebral ganglia (es-

pecially in caudate nucleus) and in parts of the limbic system and midbrain. It also occurs in liver, intestine, and lungs where it may be synthesized locally and is a potent vasodilator. The physiological significance of release of small amounts by the medulla has not been established.

Systemic administration of DOPA leads to rapid increases in brain dopamine concentrations (since this amine crosses the "blood-brain barrier"). It is widely used by investigators to raise local concentrations of dopamine, and it is given to patients with Parkinson's disease.

Dopa-decarboxylase has been identified in brain synaptosomes. It requires pyridoxal phosphate (vitamin B₆) as a cofactor, and vitamin deficiency states impair catecholamine restoration following depletion. α -Methyldopa and α -methyl-meta-tyrosine have been used to inhibit the enzyme, but catecholamine biosynthesis is not completely suppressed since the step catalyzed is not rate-limiting.

Dopamine is taken up by sympathetic neurons and adrenal medullary granules for storage and release; most of it is hydroxylated to form norepinephrine.

The enzyme, dopamine β -oxidase, is a copper-containing protein which requires ascorbic acid as a cofactor. It is inhibited by a variety of agents, including disulfuram (Antabuse); the hypotension which often accompanies use of

this agent in treatment of alcoholism may result in part from impaired norepinephrine synthesis.

ACTH promotes rapid release of ascorbic acid from the adrenal cortex; it is possible that this plays some role in ACTH influences on the medulla.

Antibodies to the enzyme have been developed and used to produce "immunosympathectomy." A different kind of immunosympathectomy is accomplished by administration of a *nerve growth factor antibody*.

Nerve growth factor (NGF) is a peptide needed for development of the sympathetic nervous system.¹⁰ Effects on morphology and on neurotransmitter synthesis have been demonstrated *in vivo* and *in vitro*, and NGF receptors have been identified.

When very young animals are given anti-NGF sera, they suffer permanent impairment of the sympathetic nervous system. Transmission in sympathetic ganglia is affected within hours, and long-range effects include total destruction of para- and prevertebral ganglia. Sympathetic function can be seriously disrupted in adults, but recovery is possible some weeks after cessation of antibody administration.

Norepinephrine is released by both sympathetic nerves and adrenal medulla, but the adrenal medulla of most species converts considerable quantities to epinephrine. The enzyme required, phenylethanolamine-N-methyltransferase (PNMT) is

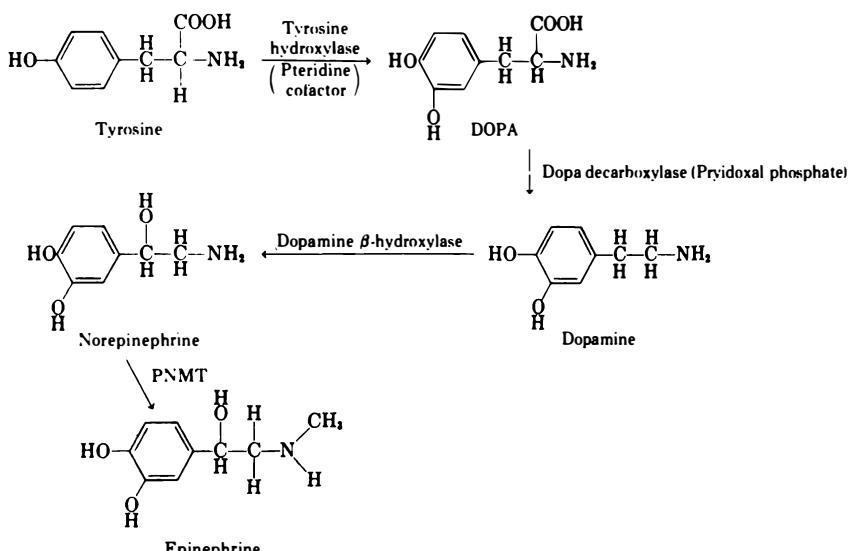


FIG. 9-1. Biosynthesis of norepinephrine and epinephrine.

also found in the brain. It requires glucocorticoids for induction.^{1, 11}

It is obvious that an early concept that the close anatomical relationship between the adrenal cortex and the adrenal medulla is fortuitous, must be abandoned. ACTH influences on the medulla mediated via the cortex were cited above, and glucocorticoids have also been directly implicated in regulation of embryonic development of the adrenal medulla.

High concentrations of epinephrine reduce PNMT activity and may thereby engage in feedback regulation of epinephrine biosynthesis.

In species producing relatively large amounts of epinephrine, it has been observed that more of this catecholamine is present in peripheral cells of the adrenal medulla, whereas norepinephrine is concentrated more internally. Most observers favor the concept that two different cell populations are present, one secreting each of the adrenomedullary hormones. However, it has been suggested that those cells with greater access to glucocorticoids produce the PNMT, while those farther from the source do not realize this potential. It has also been considered that all cells can secrete norepinephrine, but that only those that remain unstimulated for sufficient periods of time can perform the last step in epinephrine biosynthesis; the failure to achieve large changes in proportions of the two catecholamines upon prolonged stimulation seems to argue against this.

Both norepinephrine and epinephrine are stored in granules with contain relatively high concentrations of ATP and calcium, and they may exist as a complex with these substances and a granule wall protein. The granules also contain an ATPase, lysolecithin, and probably a cAMP-activated protein kinase.

Although isotope studies indicate that new catecholamine synthesis can be accomplished quite rapidly, it is likely that reserve quantities are stored for long periods of time. When the granules are severely depleted, e.g., after administration of large doses of insulin, a week or more may be needed to reestablish the normal catecholamine content. Since depletion leads to accelerated synthesis, the turnover rate of a portion of the stored hormone may be even slower.

There is some biochemical (but no morphological) evidence for existence of at least two neurotransmitter pools in adrenergic nerves, one with a turnover rate of about 2 hr that is

readily mobilized, a second with a turnover rate closer to 24 hr that requires stronger stimulation for release, and possibly even a third. Catecholamine turnover in the brain may be more rapid; it is influenced by a variety of neuronal inputs and by levels of glucocorticoid hormones.

ALTERNATE ROUTES FOR CATECHOLAMINE SYNTHESIS²

Aromatic decarboxylases catalyze conversion of tyrosine to tyramine. A *catechol-forming enzyme* promotes formation of dopamine from tyramine (Fig. 9-2). The same enzyme acts on synthesis of norepinephrine from octopamine and epinephrine from synephrine. While a physiological role for the alternate pathway has not been established, the fact that administration of agents which interfere with normal metabolism leads to accumulation of the intermediates has been cited as an indication that the pathway is normally operative. The intermediates function as "false transmitters"; i.e., they are taken up by catecholamine receptors but exert little or no transmitter-like actions. Tyramine is taken up by chromaffin granules, and the uptake leads to release of catecholamines. Tyramine is also a potent smooth muscle stimulant.

SECRETION OF CATECHOLAMINES¹

Stimuli

Acetylcholine released from *splanchnic nerve* endings provides the major physiological signal for release of adrenomedullary hormones by fully developed glands. Stimulation of the nerves directly, indirect stimulation via hypothalamic connections, and administration of acetylcholine-like agents or cholinesterase inhibitors all promote secretion. Most "stress" stimuli act through the hypothalamus.¹⁴

Angiotensin II (Section III) enhances secretion and may play a role in some stress responses; it is effective only when innervation is intact.

Histamine and *bradykinin* act directly on adrenomedullary cells, and direct activation may play a role in the physiology of developing adrenal glands. Serotonin and tyramine are also potent stimulators. (Bradykinin is a small peptide with potent vasodilator activity. It is produced when stimulation of salivary glands and some other structures leads to release of a proteolytic enzyme which catalyzes splitting of a globulin present in the blood plasma.)

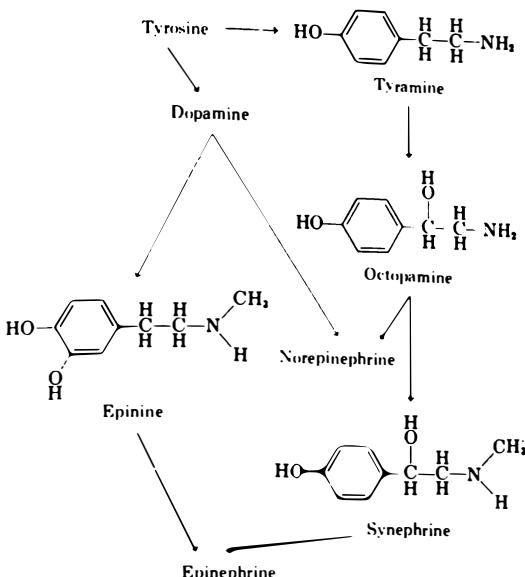


FIG. 9-2. Possible alternate pathways for biosynthesis of norepinephrine and epinephrine.

Since the relative proportions of epinephrine and norepinephrine released vary in accordance with the type of stimuli presented (see below), it is reasonable to believe that either separate nerve endings are involved or that frequency, intensity, or duration of stimuli affect the quality of response. Attempts to clarify this have been frustrating. Evidently there are species differences and probably also differences in response related to physiological state at the time of testing.

Small amounts of catecholamines are probably secreted intermittently under basal conditions. Release is rapidly augmented in response to such things as strong emotions (fear, anxiety, anger, intense pleasure), loud noises, cold exposure, hypoglycemia, anoxia, reduced barometric pressures, muscular exertion, burns and other trauma, and hypotension. Thyroid hormones enhance both sensitivity to stimuli and peripheral actions. Doses of epinephrine barely effective for stimulation of the cardiovascular system of euthyroid animals can prove lethal to hyperthyroid animals.

The stimuli affect synthesis as well as release. Activity of tyrosine hydroxylase is increased after strong or prolonged stimulation, and long-range influences include

synthesis of new enzyme. It has been shown that fighting depletes catecholamines of experimental animals, but repeated bouts lead to increased size of the gland, catecholamine content, and enzyme activity. Repeated "psychosocial stimulation" induces a rise in adrenal choline acetyltransferase activity.*

Epinephrine vs. norepinephrine secretion

The relative percentages of epinephrine and norepinephrine secreted vary widely with age and species, and differences in absolute amounts released may be even greater.¹⁴ Fetuses of many mammals (including humans) put out norepinephrine exclusively, and there is a gradual developmental change which may culminate in almost exclusive epinephrine release under most conditions. (Norepinephrine from the adrenal seems to participate in physiological regulation of blood pressure until the time when sympathetic nerves develop sufficiently to take over this function.)

Several generalizations have been made: e.g., that the tendency to secrete epinephrine increases as the evolutionary scale is ascended¹; and that carnivores are norepinephrine secretors whereas herbivores release mostly epinephrine. But many excep-

HORMONES AND BLOOD SUGAR

tions to all rules can be found. Percentages of norepinephrine released by adrenal glands of rabbits, guinea pigs, hamsters and rats have been reported as between 2 and 11 while values as high as 55 and 88, respectively, were found in the lion and whale.

It has been suggested that animals that tend to run away when threatened are epinephrine secretors, while those more likely to attack secrete predominantly norepinephrine. The concept is at least colorful. But chickens put out norepinephrine; either they are far more aggressive than some of our language usage suggests, or they are too low on the evolutionary scale to fit into the proposed scheme.

A related concept is that humans reacting to stress with anxiety put out more epinephrine than those reacting with aggression. This seems to conflict with one study suggesting that relative amounts of epinephrine secreted are greater in subjects that are angry than in those frightened.⁶ (Unfortunately, autonomic responses were measured in that study, rather than catecholamines.) Other investigations utilizing measurements of urinary catecholamines and their metabolites point to greater epinephrine output when a stress situation involves either fear or unpleasantries (than when it simply presents a challenge or requires sustained effort), but still others indicate that individuals putting out greater quantities of epinephrine are the ones best able to cope with difficult situations.¹

Mechanisms

Acetylcholine and related stimuli *depolarize* the adrenal cell membrane, and the action has been likened to acetylcholine influences on synapses and neuromuscular junctions. High extracellular potassium ion concentrations also depolarize and promote catecholamine secretion.

While most effects of splanchnic nerve stimulation are "nicotinic," or of the autonomic ganglion type (mimicked by nicotine and blocked by hexamethonium), some are of the "muscarinic" or postganglionic type (mimicked by pilocarpine). Attempts have been made to demonstrate that different types of endings affect proportions of the two catecholamines released.

Calcium ions are required in the extracellular fluid; they *move into the cell* and are believed to travel down a concentration gradient through the cytosol. Other stimuli are ineffective in the absence of extracellular calcium, and just elevation of the calcium ion content of plasma can promote secretion if other normal constituents are present. (Barium can act like calcium, but magnesium is inhibitory.) Sodium ions move into stimulated cells and have been implicated in facilitation of calcium entry.

Cytoplasmic calcium has been implicated in the polymerization of microtubules, in the binding of secretory granules to the microtubules, in contraction of an actomyosin-like protein which may be required for granule movement and extrusion, and also in the binding of the secretory granule membrane to the plasma membrane. The granule lysolecithin may be involved in the last step and in the extrusion of the catecholamine.

Hormone secretion is probably mediated via cAMP. Elevated concentrations of the nucleotide have been found following stimulation, and agents which retard cAMP degradation (theophylline, caffeine) promote hormone secretion. cAMP has been proposed to function in phosphorylation of microtubule proteins and also in phosphorylation of membrane proteins.

There are conflicting interpretations of studies investigating tubular function; e.g., there are questions concerning whether colchicine and cytochalasin B impair secretion through actions on the tubules or alternately through effects on either receptor sensitivity or on calcium redistribution.⁷

Secretion requires ATP energy which may be utilized in several ways. The granules contain high concentrations of ATP and also an ATPase. Hormone secretion is accompanied by release of adenine nucleotides and especially of AMP.

Granules also contain specific proteins (chromogranins, and especially chromogranin A) which may be present in a complex with the ATP and calcium, probably a cAMP-dependent protein kinase, and enzymes utilized in catecholamine biosynthesis. Chromogranins and dopamine hydroxylase are released with the hormones.

Pervading evidence favors a process of exocytosis similar to that described for other endocrine glands, in which secretory granule and plasma membranes fuse and granular contents are extruded. The granule membranes and adhering molecules are then evidently taken up by lysosomes for subsequent destruction. However, some investigators believe that direct release of some cytoplasmic (nongranular) catecholamines, or indirect release, through prior extrusion from the granule to the cell interior, has not been ruled out.

METABOLISM OF CATECHOLAMINES

Very small amounts of epinephrine and norepinephrine are present as such in the blood plasma, along with even smaller quantities of catecholamines which have been conjugated with sulfates or glucuronic acid. Minute amounts of all of these appear

in the urine. Epinephrine binds to plasma proteins and especially to the albumin fraction, but norepinephrine shows little tendency to bind.

The catecholamines have a very short duration of action, and this is largely attributable to the fact that they are rapidly taken up by sympathetic nerve endings and soon incorporated into granules (from which they can be later released). Fairly large amounts are taken up by the richly innervated heart and spleen. Uptake is an active, energy-consuming process. Catecholamine entry into neurons and catecholamine incorporation into granules are separate processes.

The increased sensitivity of denervated structures to exogenous catecholamines is largely explained on the basis of inability to take up excessive quantities.

Reserpine interferes with incorporation of

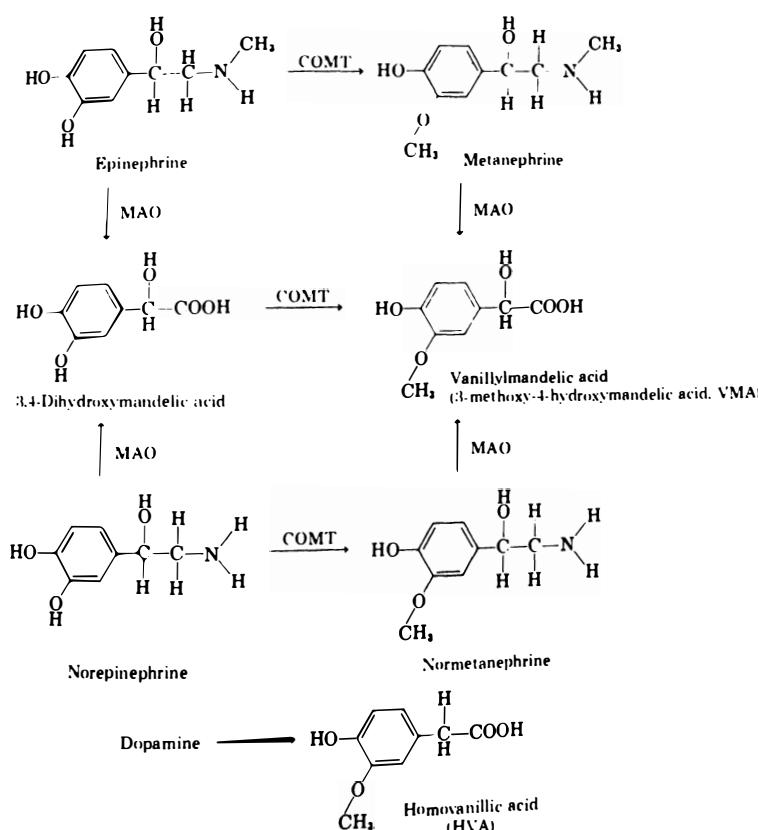


FIG. 9-3. Metabolic degradation of catecholamines.

catecholamines (and serotonin) into granules and therefore induces early signs of stimulation; long-range depressant effects result from exhaustion of the amine supply, since presence in the cytoplasm favors metabolic degradation. *Ammphetamines* also interfere with granule sequestration and may therefore create a situation of stimulation followed by some signs of exhaustion; but (unlike reserpine) they also exert sympathomimetic actions. *Tyramine* acts primarily through promotion of release of catecholamines from the granules. None of the preceding influence passage of the catecholamines from the plasma into the cytoplasm. Agents which interfere with passage across the cell membrane (and thereby potentiate early effects of adrenergic nerve stimulation) include cocaine, imipramine, and guanethidine.

Catecholamines sequestered within granules are protected against metabolic degradation. Those present in the cytoplasm are taken up by mitochondria, in which monoamine oxidase (MAO) catalyzes their conversion to *3,4-dihydroxyphenylacetic acid* (Fig. 9-3) in the case of both epinephrine and norepinephrine and *3,4-dihydroxyxypyruvic acid* when dopamine is the substrate.

Inhibitors of MAO (pargyline, imipramine) promote accumulation of catecholamines in peripheral neurons and in the brain, and increase the formation of false transmitters (p. 102). Antidepressant actions of these agents have been attributed to their ability to elevate brain stores of catecholamines and of serotonin.

MAO has little or no influence on effects of stimulation of peripheral adrenergic nerves or on the responses to systemically administered catecholamines. Amines released (or injected) into the blood plasma are acted upon by a different enzyme, *catecholamine-O-methyltransferase (COMT)* which is also present in liver and kidneys and to some extent in sympathetic neurons. Epinephrine and norepinephrine are converted to *metanephrine* and *normetanephrine*, respectively. Actions of COMT are quantitatively more important than those of MAO. The enzyme is inhibited by pyrogallol. (Almost all of the pharmacological agents cited exert additional actions, some of which affect autonomic nervous system function.)

Most catecholamine metabolites that reach the kidney have been acted upon by both enzymes, and the most abundant urinary product is *3,4-dihydroxyphenylacetic acid* (*vanillylmandelic acid, VMA*), but conjugation products of metanephrine and normetanephrine also appear in small amounts. The related aldehydes and alcohols may also be formed and excreted.

If sufficient *dopamine* is available for action of the enzymes, the major metabolite is *homovanillic acid*; under normal conditions, not enough free dopamine is present to yield measurable quantities of the metabolite in the urine.

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10. Somatotrophin

THE NEED FOR SOMATOTROPHIN (GROWTH HORMONE)

Metabolic Role during Periods of Food Deprivation

Glucocorticoids protect against hypoglycemia during the intervals between meals, but excessive or unopposed actions can lead to widespread tissue destruction (Chapter 7).

Somatotrophin (STH) secretion increases when plasma glucose levels of previously well nourished animals fall during limited periods of food deprivation^{20, 9} and during sleep. The hormone promotes amino acid uptake and incorporation into protein, lipid mobilization and catabolism, and glucose formation from hepatic glycogen and pyruvate; it also modifies gluconeogenic influences of the glucocorticoids.^{2, 4, 5, 6}

The timing of secretion and the metabolic actions suggest that STH contributes substantially to the maintenance of blood sugar levels and to protection against tissue destruction during fasting. The presence of relatively high concentrations in adults^{2, 5, 9} supports the concept.

Growth hormone influences may be "permissive" rather than direct; they seem to prolong and intensify, rather than initiate the metabolic adjustments. Fatty acids can be mobilized (although less efficiently) in deficiency states; moreover, STH secretion is not consistently elevated when conditions demand stepped-up fat catabolism and decreased carbohydrate utilization.

STH and Somatic Growth

STH supports growth of young animals.^{2b} The hormone is especially effective

when nutrition is adequate; it also stimulates the appetite. Important influences are exerted on DNA synthesis and cell proliferation. Fasted (but not starved) animals retain more nitrogen and metabolize more fat when given STH. Actual starvation blunts the responses;¹ this provides for diversion of limited metabolic reserves away from growth processes into those essential for prolonging survival.

Growth-promoting actions are most obvious between birth and puberty. Embryonic growth proceeds rapidly before STH secretion commences; fetuses secrete the hormone but can develop without it. STH affects growth and function of structures involved in reproduction and lactation, but sexual maturation is not totally arrested in its absence.

Plasma concentrations of STH are not consistently higher in actively growing animals, but the latter show greater sensitivity than adults to some of its actions.

STH and Stress

Growth hormone is released in response to many forms of stress, and it counteracts some of the otherwise deleterious effects of high glucocorticoid titers at such times. Actions of the two hormones on the thymus gland are in many respects antagonistic (Chapter 25).

EFFECTS OF STH ON THE WHOLE ANIMAL

Young animals deprived of STH soon stop growing. Effects on the bones, musculature, and viscera are most obvious, but the nervous system continues to develop. Food intake (and especially of foods that must be sought out or that require consid-

erable effort to eat) is diminished. Weight may be gained for a time (at a reduced rate), but this is attributable largely to fat accumulation. Sensitivity to hypoglycemic actions of insulin is enhanced.

Replacement doses of STH rapidly restore appetite and growth rate. Nitrogen, potassium, sodium, calcium, and phosphate are retained in greater quantities (although urinary calcium may increase), while blood glucose concentrations rise and fat stores are depleted.

Chronic administration of large doses of STH to young, well fed animals induces *gigantism* (and a similar condition develops in children secreting too much of the hormone). Effects on the long bones are the most obvious. However, it is apparent that muscles, skin, and blood vessels must accommodate to bone growth and that the increased metabolic demands can only be met by enlargement of the liver, expansion of the blood volume, and an increase in both blood cells and plasma constituents. Larger lungs and kidneys are needed, and the endocrine system must make appropriate adjustments.

Gigantism cannot be induced in adults of most species, since the growing ends of the bones (epiphyses) calcify, but the bones become thickened. (A few species, e.g., rats, continue to show bone elongation after attainment of sexual maturity.)

Patients suffering from *acromegaly* develop characteristic deformities of the fingers, elbows, and knees, and thickening of the jaws and facial cartilage. Inward growth of the skull compresses the brain, and this usually leads to development of headaches and visual disturbances. (If the condition arises because of a pituitary gland tumor, growth of the tumor contributes to the compression.) Psychic depressions commonly seen probably result from a combination of mechanical pressure, metabolic disturbances, and reaction to a progressively deforming and incapacitating condition.

The visceral structures enlarge, and the liver tends to become infiltrated with fat, while depot fat is depleted. *Hyperglycemia* develops early and is partly attributable to insulin resistance, although insulin secretion is usually augmented.

The term "idiohypophysial diabetes" has been applied to early disturbances of carbohy-

drate metabolism which develop in animals overdosed with STH; it is reversible if the hormone is withdrawn.^{20, 21} Continued administration of large amounts of growth hormone leads to development of a permanent impairment, *metahypophysial diabetes*, in which the islet cells of the pancreas become exhausted from prolonged excessive insulin demands. The condition cannot be induced in very young or in pregnant animals of most species.²²

Although onset of clinical juvenile diabetes often follows a rapid growth spurt, a previously suspected role of STH in its etiology is now questioned. No consistent elevations of plasma STH levels have been found during pre-adolescence.²³ STH hypersecretion is not a common cause for development of the maturity onset diabetes mellitus condition.¹⁰

MECHANISM OF ACTION OF STH

Special Problems of Studying STH Actions

For reasons cited below, STH is in many ways the most difficult hormone to study.

It is Difficult to Induce a Specific Deficiency. Hypophysectomy removes a whole spectrum of hormones, and physiological replacement of other factors is not readily achieved.

No pharmacological agents (comparable to alloxan for the islet cells or thiouracils for the thyroid gland) are available to "specifically" destroy STH-secreting cells or to impair their synthesis of hormone. STH antibodies are available. However, there is evidence for multiple active sites on the hormone molecule, and relationships between components involved in antibody binding and hormone action are not clear.

A hypothalamic peptide which inhibits STH secretion has been identified (see below), but unfortunately (from the viewpoint of experimental design) it exerts other influences on the endocrine system.

There Is No Specific Target Organ. STH affects virtually every cell type. It has been stated that the thymus gland responds specifically to STH, and that that structure in turn affects pituitary STH cells²⁴; however, thymectomized animals do not become acromegalic, and thymus gland implants or extracts do not provoke STH deficiency (see also Chapter 25).

There Are Marked Species Differences in Molecular Structure of STH,^{25, 4, 5, 6} and Presumably Also in the Hormone Receptors. Hormone derived from one ani-

mal type may be totally devoid of "typical" biological activity in another. Usually it is impossible to obtain sufficient native hormone to perform definitive studies.

Preparations are usually obtained from cattle, sheep, and hogs because the animals have large pituitary glands which are available at relatively low cost from slaughter houses. They are studied most often in small laboratory animals because the latter are easy to handle and presumably require small doses.

Humans are totally unresponsive to the readily available preparations (bovine, ovine, porcine) which are potent growth stimulants in rodents. They respond to STHs derived from human and other primate sources.

STH is Antigenic. This can seriously interfere with chronic studies because of loss of effectiveness and also because of complications associated with activation of immune mechanisms.

There Are Similarities between STH and Prolactin, both in Chemical Structure and Biological Properties. Some stimuli which affect release of one simultaneously affect release of the other, and both influence secretion and actions of other hormones.

Conditions Affecting STH Secretion Are Highly Variable. Concentrations in the blood plasma (and changes in response to stress) vary widely *between species*, between *individuals*, and *within the same individual* at different times. They are affected by previous *nutritional history* (both qualitative and quantitative), by the *nutritional status* at the time of testing, and by the *hour of the day*. Responses to the hormone are *sex* and *age* dependent and responsive to changes in metabolic milieu. In addition to recognized rhythmic secretion patterns linked to sleep-activity and feeding-fasting cycles, there are unexplained spurts.

Methods for Bioassay Are not Completely Satisfactory. They depend upon indirect, incompletely defined responses, and most involve subjective interpretation.

Immunoassay procedures have been developed which are sensitive, but discrepancies between immuno- and bioassay await future clarification,⁵ especially in animals exposed to stress.

Few STH Actions Can Be Studied In Vitro. Usually the hormone is administered to the whole animal and the tissues

are excised later for study. Some *in vitro* actions that have been observed are difficult to relate to whole animal physiology.

Most STH Actions Are Seen Only after a Long Latent Period. In addition to the usual problems this poses for hormone studies, there is the added difficulty that STH induces formation of humoral mediators during the latent period. There are also indications that "active fragments" may be split off in a variable manner from the STH molecule. Moreover, there are numerous *biphasic* responses in which delayed effects are opposite in direction to those seen earlier.

Influences of STH on Cell Membranes

Reasons for the belief that other peptide hormones exert their actions on plasma membranes of target cells have been presented, and similar studies have been performed with STH (an even larger molecule that is known to bind strongly to the membranes).

When STH is administered *in vitro* for just 1 min and the preparation is then thoroughly washed, metabolic influences can be demonstrated some time later.⁶ This is consistent with a *triggering action* and formation of a "second messenger."

Although some STH actions require elevation of cAMP concentrations, the hormone may not directly affect adenylyl cyclase. There are indications that STH promotes formation of a protein required for cAMP to be effective.

Early Influences on Sugar Uptake. STH promotes a *very early increase in the rate of glucose uptake* by adipose tissue, heart and skeletal muscle cells, and this is associated with a transient fall in blood glucose concentrations. The effects are reminiscent of those described for insulin. Uptakes of mannose, galactose, and arabinose are also increased, and the action is not blocked by inhibitors of RNA or of protein synthesis.

Delayed Influences on Glucose Uptake. Some hours later, glucose utilization is reduced by a mechanism which can be blocked with the inhibitors, and this is associated with *decreased rate of sugar uptake*.

It has been proposed that a delayed inhibition of *phosphofructokinase* activity leads to accumulation of fructose 6-phosphate and that the

latter is rapidly converted to glucose 6-phosphate. Inhibitory influences of glucose 6-phosphate on the hexokinase reaction may then secondarily reduce glucose entry into the cells.*

Uptake of Amino Acids. Approximately 20 min after addition of STH to *in vitro* preparations, the rate of uptake of certain amino acids by skeletal muscle, kidney, fibroblasts, and other structures can be demonstrated. In the whole animal, this influence is reflected by a fall in plasma amino acid concentrations.

Preincubation of tissues with puromycin does not affect the basal rate of uptake, but it blocks STH stimulation. This suggests that some new protein which mediates amino acid transport must be synthesized.

Although STH promotes large increases in protein synthesis, such action is separable from effects on amino acid transport (which extend to nonmetabolizable amino acids such as α -aminoisobutyric). Amino acid uptake certainly supports the later increase in more generalized protein synthesis.

Effects of STH on amino acid uptake differ from those of insulin (which can be seen within 5 min) (Chapter 5) and they also obviously differ from effects of STH on sugar uptake.

STH and Hepatic Gluconeogenesis

Glucocorticoid promotion of gluconeogenesis from amino acids via induction and sustained high activities of tyrosine-amino-transferase (TAT) and tryptophan oxidase (TO) was described in Chapter 7. Glucocorticoid influences are exaggerated in growth hormone-deficient animals.

STH promotes a very transient increase in activities of the enzymes; this is soon followed by early fall-off and *sustained inhibition*. (According to one prevailing hypothesis, STH accomplishes this via formation of a repressor of enzyme induction.*)

Sustained inhibition of transaminase activity contributes importantly to STH protection against protein-catabolizing influences of glucocorticoids, and it facilitates shunting of the amino acids into hepatic protein synthesis. The influence is reflected in reduced plasma concentrations of urea and in positive nitrogen balance. Effects on amino acid uptake (described

above) supplement the actions, since they reduce the availability of amino acid substrate for degradation within the liver.

Plasma glucose concentrations are elevated when STH inhibits gluconeogenesis from amino acids. This has been attributed to stimulation of glucose formation from liver (but not muscle) glycogen, pyruvate, and lactate (and also from glycerol), and through increased rate of release of glucose from the liver.

Glycogenolytic actions result in part from potentiation of glucagon and epinephrine actions, and glucose release from antagonism to insulin actions. Glucose synthesis from non-protein sources results from increased activities of fructose diphosphatase and glucose 6-phosphatase, while actions on lipid metabolism (see below) reduce conversion of pyruvate to acetyl-coenzyme A and increase availability of glycerol.

STH stimulation of the appetite probably also contributes substantially to influences on plasma glucose concentrations.

Influences on Adipose Tissue^{2d, 4, 9}

STH-stimulated lipolysis leads within a few hours to elevation of plasma fatty acid concentrations and within days to marked depletion of depot fat. Loss of "chubbiness" has been repeatedly described in children exhibiting growth responses.³

The action requires the presence of glucocorticoids and probably also of thyroxine. (It can be elicited in hypophysectomized animals only if they are treated with ACTH (or glucocorticoids) and thyroid-stimulating hormone or thyroxine.) When the other hormones are present, STH enhances lipolytic influences of epinephrine and of theophylline (which retards cAMP degradation). It has been proposed that STH activates a cAMP-dependent lipase.

Lipolytic actions depend at least in part on previously mentioned inhibition of glucose uptake by adipose tissue cells; they cannot be elicited when plasma glucose concentrations are very high or in the presence of inhibitors of protein synthesis.

They are characteristically preceded by transient lipogenesis in adipose tissue during the time when glucose uptake is rapid (p. 109), and in liver when fatty acids enter at a rapid rate.

Effects on Protein Synthesis

STH influences are exerted at several levels. Increased rate of amino acid uptake (p. 110) not only provides substrate, but high amino acid concentrations within the cell favor *aggregation of ribosomes* and *initiation* of peptide synthesis. However, STH stimulates protein synthesis in ways not dependent on amino acid transport. *Incorporation* of amino acids into proteins can be shown under conditions in which transport mechanisms are blocked, and also when concentrations in the extracellular fluid are very high (and therefore not rate-limiting).

Effects of STH on liver and muscle protein synthesis *in vitro* can be demonstrated within 1/2 hr after hormone administration; only certain proteins increase. The findings contrast with influences on hypophysectomized animals which require 2 days to develop and involve generalized stimulation of protein synthesis associated with augmentation of cell size.

Growth hormone-deficient animals have *reduced numbers of ribosomes per cell*, and a *smaller fraction* of those present aggregate to form *polysomes*. It has been proposed that such animals have *reduced ability to attach mRNA to ribosomes*, but there is also a *reduced rate of roll-off* associated with defects in *chain elongation*. The problems can be corrected by administration of STH.

Cell fractionation and reconstitution studies reveal that the defects resulting from STH deficiency are quite different from those found after insulin deficiency (Chapter 5). Something is reduced in the *cell sap fraction*, because relatively larger quantities of sap from hypophysectomized man from normal animals are required to support protein synthesis *in vitro*. There is also a defect in the *smaller subunit of the ribosome* which may involve a conformational change that affects function. (By contrast, preparations from insulin-deficient animals seem to have normal cell sap components but a defect in the larger subunit of the ribosome.)

While certain effects on protein synthesis can be consistently demonstrated in the essence of actinomycin D, long range actions of STH involve very marked stimulation of RNA synthesis. Usually at least 13

hrs are required for demonstration of late effects which are believed to depend heavily on prior protein synthesis. Increased *RNA polymerase* activity has been shown to be independent of synthesis of new RNA polymerase molecules or of uncovering template, but has been related to greater ability of the RNAs to bind to the DNA template. A late effect is promotion of synthesis of many forms of RNA, and even later DNA synthesis and cell proliferation, are enhanced.

A relatively early, but dramatic and sustained rise in hepatic ornithine decarboxylase activity can be demonstrated within a few hours after administration of STH. Activity falls off rapidly in the presence of cycloheximide, but "super-induction" of the enzyme with actinomycin D has been described.⁴

Influences of STH on Bone and Cartilage and on Somatomedin Formation^{4, 6, 8, 9, 14}

STH does not directly stimulate growth of cartilage when administered *in vitro* even after prolonged exposure of the tissue to the hormone alone or in combination with thyroxine and glucocorticoids.

The serum of intact animals promotes *in vitro* growth of cartilage. However, the serum of growth hormone-deficient animals and of some human dwarfs lacks something which can be restored by *in vivo* administration of STH for several days.

Normal serum stimulates incorporation of sulfate into chondroitin sulfate in cartilage preparations, and the term "sulfation factor" (SF, sulfomedin) has been applied to the active principle. The term "thymidine factor" (TF) describes stimulation of incorporation of thymidine into DNA. Serum of animals previously exposed to endogenous or administrated STH also promotes incorporation of leucine into chondromucoprotein and of uridine into RNA, and it accelerates conversion of proline into collagen hydroxyproline. For all of the preceding, tissues from young or embryonic animals are more responsive than those of adults.

It was once believed that all effects depended upon a single mediator. Now it appears that there are several growth-promoting peptides, and the term "somatomedins" has been applied to the group. All

exert some *insulin-like actions* and compete with the hormone for receptors in some cell types (e.g., those of placenta and adipose tissue) but not others, notably cartilage. Three have been identified and partially characterized.

Somatomedin A is a neutral peptide (molecular weight approximately 7000) which may be identical with sulfation factor (since it is a potent stimulator of sulfate uptake by chick cartilage). *Somatomedin B* is a slightly smaller acidic peptide (molecular weight about 5000) which is bioassayed on the basis of thymidine incorporation into human glial cells in culture. *Somatomedin C* seems to be an arginine-rich basic peptide containing something like 50 amino acid residues which binds with strong affinity to placental membrane receptors different from those binding insulin. Evidence for STH induction of somatomedin C is stronger than for specific STH induction of somatomedins A and B.¹⁴

In serum, all of the factors seem to be associated with a larger binding protein. The somatomedin-protein complex which has a molecular weight of approximately 50,000 is known as "big somatomedin."

The somatomedins may be components of a still larger family of growth-promoting peptides. There are unanswered questions about the appropriateness of inclusion of certain other serum factors in the somatomedin classification. Suggested candidates include a small peptide isolated from calf serum which stimulates multiplication of fibroblasts in tissue culture and therefore named multiplication-stimulating activity (MSA); it behaves like the somatomedins and competes with them for placental receptor sites, but induction by STH has thus far not been established. *Nerve growth factor*, *epidermal growth factor*, and *erythropoietin* possess similar properties but do not compete for the placental sites; however, erythropoietin is under STH control.

Parallel activities of sulfomedin preparations with those of nonsuppressible insulin-like activators (NSILA, Chapter 5) have been found in *in vitro* studies. Both types exert insulin-like actions, e.g., stimulation of glucose uptake by adipose tissue and skeletal muscle, promotion of protein synthesis, and competition with insulin for receptor sites on isolated fat cells and on liver membranes. However, somatomedins act on cartilage preparations poorly responsive to insulin, are a potent lymphocyte stimulant, and exert actions on skeletal muscle which do not seem to involve insulin receptors.

There have been suggestions that somatomedins are active fragments split off (or derived

from) STH, but evidence is against this; they do not share antigenic determinants with STH. Some human dwarfs with deficient STH exhibit increases in plasma somatomedin activity and growth responses, when treated, whereas others with high endogenous STH fail to show growth or somatomedin responses. This suggests that the serum factor is not derived from the hormone (although a deficiency of one or more enzymes required to act on the STH molecule is not ruled out).

A substance produced by cat tapeworm larvae which differs chemically and immunologically from STH induces somatomedin formation and promotes growth in hypophysectomized rats (and obesity in mice). It seems to compete with STH for receptor sites in the liver. (Somatomedins are believed to be produced there and possibly also in the kidney.)

Animals infested with the tapeworm larvae give responses similar to those injected with STH, except that lipolysis is not stimulated. The terms "worm factor" and "plerocercoid growth factor" (PGF) have been used to designate the growth-promoting agent found in sera of infested animals.

A form of human dwarfism has been described in which both STH and somatomedin levels in the plasma are within the normal range, but there is insulin hypersensitivity. It has been proposed that the condition involves defective receptors for both STH and somatomedins and that the condition provides evidence for existence of very different receptors for insulin.

Somatomedins are powerful inhibitors of adenylate cyclase in particulate fractions of fat cells, liver, spleen, and cartilage, and they counteract activity of hormones which activate the enzyme. This finding has broad implications for more generalized processes of growth regulation. STH plays a role in etiology and aggravation of some forms of malignant growth, and it is possible that somatomedin-mediated influences on adenylate cyclase are directly related. It should be noted, however, that some actions of STH not linked with somatomedin production require elevation of cAMP concentrations; these include stimulation of lymphocyte proliferation, lipolysis, and antagonism of insulin inhibition of hepatic glucose release.

It has been reported that STH does not stimulate formation of somatomedins in starved or severely protein-deficient infants and children and that the ability to

synthesize the substances can be restored after adequate refeeding.^{1, 5} The observations could explain indications that several days of refeeding are required before substantial effects of STH on skeletal system growth can be achieved.

The ability to suppress somatomedin synthesis may have important survival value, since such suppression leads to diversion of meager metabolic supplies into essential pathways (rather than into growth which might be considered far less important under the circumstances). In very young animals, at least some ability to respond to STH administration when food becomes available is retained.

In hypophysectomized animals, there is both reduced somatomedin synthesis (or possibly release) and reduced responsiveness to administered peptides.

The existence of regulators which inhibit both synthesis and actions of the somatomedins has been proposed. A nondialyzable, heat-labile factor which is susceptible to trypsin destruction has been identified in the serum of hypophysectomized rats. It antagonizes actions of administered somatomedins on cartilage incorporation of radioactive sulfur. High concentrations of fatty acids are also inhibitory and may account for some effects seen during starvation. It is likely that additional regulators will be identified in the future.

Glucocorticoids are growth inhibitors. Large doses reduce STH secretion, the amount of somatomedin formed in response to STH administration, and effects of somatomedins on cartilage. It is possible that amounts sufficient to promote such changes are released by the adrenal cortex in times of stress.

High doses of estrogens also retard growth. They reduce somatomedin synthesis but do not inhibit its actions. Although estrogens can stimulate STH secretion, they have been useful in treatment of acromegaly. Some effects of excessive STH have been observed in a limited number of women taking oral contraceptives (Section VI).

Surprisingly few influences of androgens on STH secretion and on somatomedin generation have been found. Such observations support the rather widely accepted hypothesis that the preadolescent growth spurt in males is largely attributable to

direct influences of the androgens on growth processes.

CHEMISTRY^{2a, 4, 5}

Human growth hormone (HGH) is a linear protein containing 191 amino acid residues; it has a molecular weight of approximately 21,000. The molecule contains two disulfide bridges (Fig. 10-1); unlike the situation for insulin, the bonds do not seem to be essential for biological activity.

Growth hormones of the rat, ox, sheep, pig, whale, dog, rabbit, monkey, and other mammals all have molecular weights of about 22,000, but they vary considerably in amino acid composition, isoelectric point, and immunological properties.

Growth hormones and prolactins have overlapping biological activities and many structural similarities. Human chorionic somatomammotrophin (HCS, described in Section VI) is a placental hormone which is even more closely related to HGH.

The pituitary gland contains at least two different biologically active STH molecules, designated as "big" and "little" STH. Both are found in the plasma, and an additional "intermediate" STH has also been described. Big STH could be a complex of STH with another protein.

Since growth hormone-deficient humans do not respond clinically to STHs of non-primates, attempts have been made to modify the readily available preparations of STH (and also of prolactins and chorionic hormones) for therapeutic use. Proteolytic procedures have yielded some interesting derivatives and have also provided information on the nature of active sites.¹⁴ Attempts are also being made to obtain fragments with specific properties, e.g., low antigenicity, special effectiveness for treatment of hypercholesterolemia, "stress ulcer" bleeding, and obesity, and with reduced "diabetogenic" actions.⁵

It has been suggested that an abnormal form of STH (or a "diabetogenic fragment") is involved in etiology of some forms of clinical diabetes mellitus. A related concept is that a genetic defect leads to formation or retention of abnormally large quantities of a fragment produced in small amounts by healthy persons (Chapter 5).

HORMONES AND BLOOD SUGAR

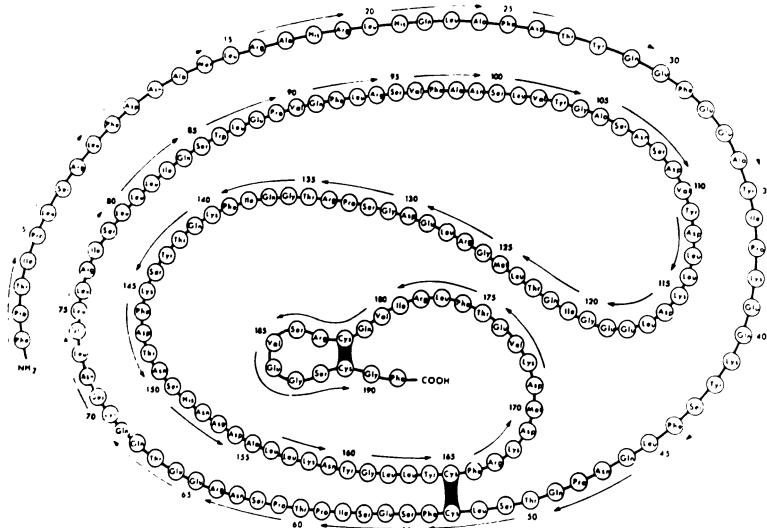


FIG. 10-1. The amino acid sequence of HGH. All amino acids are here given in the L form. (From Li, C. H. Current concepts in the chemical biology of pituitary hormones. *Perspectives Biol. Med.*, 11: 498, 1968. Copyright 1968 by the University of Chicago Press.)

REGULATION OF STH SECRETION^{2, 5, 7, 8, 11, 18}

Hypothalamic Stimulatory Hormones

Somatotrophs of the pituitary gland are described in Section VII. Synthetic and secretory functions are regulated by hypothalamic hormones which reach the somatotrophs via the hypothalamo-hypophyseal portal system. The terms "somatotropic hormone-releasing factor" (SRF, SHRF), "growth hormone release factor," (GRF, GHRF), "growth hormone-releasing hormone" (GH-RH) have been applied to an as yet unidentified factor in hypothalamic extracts which promotes STH secretion. It is not known if a single mediator affects both synthesis and release of STH (or if one of the processes is secondary to the other).

A decapeptide with the following structure has been isolated from porcine hypothalamus:



The decapeptide was reported to be highly effective for promoting depletion of STH from rat pituitary glands and to elevate plasma STH concentrations when measurements are based on a tibia bioass-

say procedure. It is also said to promote degranulation of somatotrophs (visible under electron microscopy) and to promote STH synthesis and release when studied on rat pituitary tissue incubated or maintained in tissue culture.

Conflicting interpretations have been advanced to explain failure to confirm the described rat studies with others employing immunoassay procedures. The tibia bioassay has been questioned on the basis of variability of the test data and because of uncertainty about just what is measured. There are indications that hypothalamic preparations contain contaminants which influence the tibia test, and that pituitary glands contain substances other than STH which stimulate mitosis in chondrocytes.

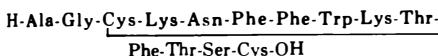
It has been suggested that hypothalamic extracts can promote conformational changes in the STH molecule within the pituitary gland which affect its biological activity without changing the binding sites involved in the immunoassay.

There have also been suggestions that immunoassay procedures do not in fact measure STH in rat plasma, but recent studies indicate that electrical stimulation of parts of the hypothalamus implicated in regulation of STH secretion leads to an increase in whatever it is that the immunoassay picks up, and that this is associated with STH actions.

The decapeptide does not seem to promote STH secretion in humans, monkeys, and some other mammals, and serious doubts have arisen concerning identification of the decapeptide with SRF.

Hypothalamic Inhibitory Hormones¹⁵

More definitive information is available on a hypothalamic principle which *inhibits* STH secretion. A peptide containing 14 amino acids and a disulfide bond with the following structure has been isolated from sheep hypothalamus:



The name "somatostatin" is most widely used, but the peptide has also been referred to as somatotrophic hormone inhibitory factor (SRIF) and growth hormone inhibitory factor (GIF and GH-IF). In small doses, it inhibits STH secretion by pituitary cells in tissue culture. It is also effective *in vivo* and has been shown to abolish STH responses to a wide variety of otherwise effective stimuli, including hypothalamic principles.

There is evidence that stimulation of STH secretion by hypothalamic hormones is mediated via cAMP. While somatostatin seems to reduce cAMP generation and to block responses to agents which raise cAMP concentrations (e.g., prostaglandins and theophylline), it also counteracts effects of dibutyryl cAMP. In addition, it elevates cGMP concentrations.

Somatostatin performs broader functions (Section VII). It has been identified in many parts of the brain (including cerebral cortex and hindbrain), and it influences behavior. It inhibits release of thyrotrophin release factor (TRF) and blocks thyrotroph (but not lactotroph) responses to TRF. It has also been reported to inhibit secretion of prolactin but not of follicle-stimulating or of luteinizing hormones. Direct influences on both α and β cells of the pancreatic islets have also been demonstrated.

Brain Regions Implicated in Regulation of STH Secretion⁶

Control of STH secretion seems to be exerted primarily by components of the *ventromedial* and *arcuate* nuclei of the hypothalamus. Electrical stimulation of

those regions consistently leads to increased STH secretion in rats, while similar stimuli applied to the anterior, supraoptic, lateral, and mammillary areas are ineffective. (There have been reports that stimulation of paraventricular nuclei may also promote STH secretion.)

In view of the biological actions of STH, it is interesting to note that the ventromedial region is also implicated in regulation of food intake (Section V) and in blood glucose regulation (Section VII). It receives neuronal inputs from several other parts of the brain and is evidently under both stimulatory and inhibitory control. Damage to the medial basal hypothalamus sharply reduces STH secretion. Deafferentation does not markedly affect *basal* secretion but abolishes responses to the usual stimuli.

Stimulation of the *basolateral amygdaloid region* (which connects, via the lateral hypothalamic area, to the ventromedial nucleus) leads to prompt release of STH, and it seems likely that such stimulation directly promotes release of SRF. Stimulation of the *corticomedial amygdala* (which makes indirect connections with the ventromedial nucleus) reduces STH secretion; this has been attributed to release of somatostatin.

Lesions of the ventromedial region abolish responses to stimulation of the amygdala. However, *direct* stimulation of the region promotes first a fall and later a rise in STH release; this is interpreted to mean that both SRF and somatostatin secretion can be promoted, but that the SRF effect predominates.

The hippocampus connects with the arcuate nucleus via the fornix. Direct stimulation of fornical pathways is ineffective (and no afterdischarge has been found), but stimulation of the hippocampus is followed by an afterdischarge which leads to increased STH secretion.

Neurotransmitters

Norepinephrine and *dopamine* clearly influence STH secretion. α -adrenergic receptors (Chapter 9) have been directly implicated in stimulation of STH release and β receptors in inhibition. Injections of either of the catecholamines into the lateral ventricle is followed by prompt

HORMONES AND BLOOD SUGAR

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responses. Serotonin is ineffective when administered in this way but appears to be involved in neuroendocrine pathways ultimately leading to release of SRF. It has been implicated in regulation of circadian rhythms of STH secretion and in release of the hormone during sleep.

There is limited information on control of the circadian patterns. Metabolic changes associated with feeding and sleep patterns may play a role. Data on blinded and anosmic animals indicate the importance of visual and olfactory cues. Pharmacological agents affecting catecholamines seem to exert little influence.

Short Feedback Control

Administration of STH leads secondarily to reduced secretion of the hormone. STH may directly influence the pituitary or hypothalamus, but somatomedins have been implicated in the mechanism.

Stress

Humans, monkeys, and some other mammals increase STH secretion in response to a wide variety of stressing stimuli, but there are marked species differences among the common laboratory mammals. The mouse has been reported to respond to a brief (16-hr) period of food deprivation but not to prolonged fasting, cold exposure, or insulin hypoglycemia. It has been suggested that the high metabolic rate maintains STH secretion at such an elevated level that a further increment may have little physiological importance. Cats do not increase STH secretion in response to cold exposure and some other stimuli; rats and rabbits sometimes exhibit inhibition of STH secretion when exposed to conditions which enhance output of the hormone in other species.

Stress influences may involve a variety of different mechanisms. Catecholamine release is probably important. Vasopressin (Section III) is released in response to certain forms of stress. It is known that administration of large doses enhances STH output; however, as noted above, stimulation of the supraoptic nuclei (which produce the hormone) does not affect growth hormone secretion.

Metabolic Control

Declining plasma glucose concentrations constitute an important stimulus even when absolute concentrations do not fall into the hypoglycemic range. STH responses to this stimulus are greater in the morning than at night in daylight-active species. The intracellular glucose concentrations of "glucoreceptor" neurons in the ventromedial hypothalamus seem to directly affect STH release, but severe hypoglycemia probably acts additionally through catecholamine release.

During fasting, hypoglycemia may contribute to stimulation of STH release, but falling plasma concentrations of free fatty acids also signal the need for STH participation in responses to food deprivation.

Growth hormone is also released in response to elevation of plasma content of certain amino acids and especially of arginine. This may be important for support for growth-promoting actions of STH and for protection against excessive glucocorticoid influences. Responses to exercise can be mediated by metabolites sited above, but a specific factor release during muscular contraction has been proposed to stimulate hormone secretion.

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III

HORMONAL REGULATION OF BODY FLUIDS

11. Aldosterone

Sodium, potassium, and water metabolism are discussed together in this section because of numerous functional interrelationships. Any influence (hormonal or otherwise) which markedly alters the quantity or distribution of one inevitably affects the balance of the other two. Approximately equal quantities of sodium and potassium are dissolved in the extracellular and intracellular fluids; distributions and functions are different but interdependent.

Since all hormones regulating water and electrolyte balance exert influences on blood vessels and circulating blood volume, some effects of hormones on systemic blood pressure are also considered.

PHYSIOLOGICAL FUNCTIONS OF SODIUM AND POTASSIUM

Sodium is by far the most abundant cation of the extracellular fluids, and it is the major determinant of the quantity of water retained within the organism. It is an essential component of blood, lymph, interstitial fluids, cerebrospinal fluid, sweat, tears, saliva, gastric secretions, intestinal and pancreatic juices, bile, urine, cartilaginous matrix, and bone. The buffer system of the blood plasma depends on the $\text{NaHCO}_3:\text{HHCO}_3$ ratio, and conditions which promote retention of sodium (in excess of chloride) induce metabolic alkalosis. Renal regulation of acid-base balance depends heavily on adjustment of the relative quantities of NaH_2PO_4 , Na_2HPO_4 , and NaHCO_3 excreted, and on the exchange of sodium and ammonium ions. Excretion of potassium ions requires establishment of sodium-dependent electrochemical gradients within the kidney.

Varying quantities of sodium are found within cells, and cytosol sodium concentration affects (among other things) the uptake and distribution of calcium ions and the maintenance of resting potentials. Much cellular energy is expended for operation of "sodium pumps" which regulate cytosol sodium content.

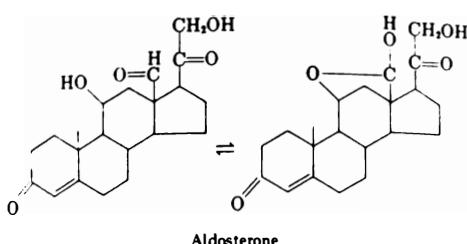
Action potentials, secretory processes in endocrine and exocrine glands, and many hormonal responses depend directly upon movements of sodium and potassium ions across plasma membranes.

Potassium is the most abundant *intracellular* cation, and it is the major determinant of cellular hydration and resting potential. Movements of potassium ions are involved in activities of muscle, nerve and gland cells, and optimal potassium ion concentrations are required for proper function of many enzymes. Excessively high concentrations in the blood plasma lead to weakening of the force of *myocardial contraction* and disruption of electrical events of the cardiac cycle; they can interfere with excitability of cells by increasing the tendency for depolarization. Potassium retention by the organism as a whole is an invariable accompaniment of growth, but excessive retention disrupts sodium-potassium balances within the tissues.

THE ROLE OF MINERALOCORTICOIDS IN THE MAINTENANCE OF SODIUM, POTASSIUM, AND WATER METABOLISM

Aldosterone is by far the most important hormone for regulation of sodium, potassium, and water metabolism. It is secreted by the adrenal cortex of mammals and by

steroidogenic tissues of most other vertebrates. It has the following structure:



Hormone biosynthesis in the adrenal cortex was described in Section II. Most of the discussion which follows refers to mineralocorticoid function in mammals. Identical steroids are produced by vertebrates of all classes, but functions differ, especially among aquatic forms.

Deoxycorticosterone (DOC) has approximately 3% of the sodium-retaining potency of an equimolar quantity of aldosterone.¹² Certain species (e.g., the dog) produce large amounts, while other mammals may secrete enough to affect salt metabolism under special conditions.

This steroid (and its acetate) was available for clinical and experimental use long before aldosterone had been identified. It has been largely replaced clinically by the more potent, orally and topically effective synthetic steroid *fludrocortisone acetate* which is chemically related to 9α-fluorocortisol (Fig. 7-6D). It is still widely used in some laboratories because of its availability and low cost, and also because (unlike aldosterone) it is totally free of glucocorticoid potency even when administered in very large doses.

The DOC concentration is higher in the plasma of female than male fishes of some species. There are indications that the hormone is produced in the ovary and serves reproductive functions.

Other adrenocortical steroids implicated in alteration of water and electrolyte metabolism and of systemic blood pressure, especially under pathological conditions, include *11-deoxycortisol* and *18-OH-DOC*. There are controversies concerning amounts produced, potencies, and importance in such conditions.

All naturally occurring glucocorticoids exert some mineralocorticoid actions, but they also affect kidney and fluid balance in opposing ways.

The most obvious actions of aldosterone are exerted on the kidney. Tubular *reabsorption* of sodium (and secondarily of water) is increased, while urinary *excretion* of potassium is enhanced. The physiological importance can be appreciated from observations of animals deprived of the hormone.

Within hours after adrenalectomy, laboratory rats begin to excrete large volumes of urine containing high concentrations of sodium and reduced amounts of potassium. Circulating blood volume is soon diminished, and blood pressure falls. Recruitment of fluids from extracellular spaces helps to sustain the animals for a short time, but this hastens cellular dehydration. Appetite fails, and lost fluids are not replaced.

If the situation is permitted to continue, falling blood volume and pressure reduce the blood supply to some regions, and weakening of the heart (because of plasma potassium accumulation) further speeds the onset of circulatory failure. Acidosis develops rapidly because of tissue anoxia and sodium loss. In time, glomerular filtration is seriously impaired, and the ensuing renal failure leads to accumulation of acid metabolites and nitrogenous wastes in the blood plasma. Untreated animals succumb within about 2 days under average conditions. Young animals are more susceptible than older ones, and death occurs earlier when environmental conditions are unfavorable. Adrenalectomized animals of other species exhibit similar difficulties, but some differences in time course may be observed. For example, dogs may become inactive and refuse food before exhibiting major changes in plasma composition.

Adrenalectomized animals of many species can be kept alive for months without hormone treatment, if their drinking water is replaced by a solution of sodium chloride (0.9–1.0%). Rats and some other animals will select saline solutions adequate for their needs if given a choice of drinking fluids. The animals drink very large quantities of saline and excrete several times as much sodium and water as do intact animals drinking tap water.

The salt does not correct the hormone defect, but it does protect against most consequences by replacing materials the

HORMONAL REGULATION OF BODY FLUIDS

animal is unable to conserve in a normal manner. The increased sodium load to the kidney aids in excretion of excess potassium. The gastrointestinal tract is maintained in good condition, and appetite is adequate.

Salt administration also mitigates some effects of glucocorticoid deficiency resulting from adrenalectomy. Food intake and absorption help combat the dangers of hypoglycemia; water retention minimizes the need for glucocorticoid influences on water balance (Chapter 13), and sodium retention helps maintain smooth muscle tone. The animals are, however, susceptible to stress and must be kept under optimal conditions.

Too much mineralocorticoid causes excessive water and salt retention, expansion of blood and extracellular fluids, elevated blood pressure and tissue edema, as well as metabolic alkalosis. Potassium depletion aggravates the condition, since it removes a physiological depressant of the myocardium.

Animals given a balanced diet and tap water may show a form of adaptation: they retain considerable quantities of sodium and water and suffer moderate elevation of the systemic blood pressure, but ability to excrete some of the excess sodium is regained in spite of continued hormone overdosage. The "escape phenomenon" has been attributed by some to effects of altered electrolyte balance on renal hemodynamics, by others to release of a *natriuretic hormone* (Chapter 13), and by still others to depletion of mineralocorticoid receptor molecules.

Escape is seen less often if animals are given DOC than if aldosterone is used. Exaggerated thirst may exacerbate the condition, and further difficulties are imposed if animals are fed a diet high in sodium and low in potassium.

While mineralocorticoid actions on the kidney are the most obvious, these hormones affect all kinds of cells which engage in net transport of monovalent ions. Aldosterone decreases the sodium content of sweat, saliva, milk, and other secretions and increases the potassium concentration. It affects ion transport across the intestine and colon and promotes movement of sodium into and potassium out of skeletal muscle and other cells.

All of the actions in mammals tend to elevate the sodium content (and reduce the potassium) of extracellular fluids and to promote net retention of water. Extra-renal actions are easily demonstrated in adrenalectomized animals exhibiting early (and therefore reversible) deficiency symptoms. When aldosterone but no saline is administered, water is transferred from cells to extracellular fluids, sodium and potassium are redistributed, and improvements of the circulatory system soon become apparent.

In nonmammalian vertebrates, aldosterone similarly promotes sodium and water retention but it does not affect the kidneys of all species. Predominant actions are exerted on sodium-transport across the skin and urinary bladder of amphibians, while actions on gills of some of the fishes are most obvious. In some nonmammalian vertebrates, aldosterone is secreted but corticosterone may be quantitatively more important. (See also Chapter 13.) In a few terrestrial nonmammalian vertebrates, aldosterone promotes salt excretion through specialized glands.

Additional influences of mineralocorticoids which have received less attention may contribute substantially to changes seen in the whole animal. It has been reported that aldosterone promotes the renal excretion of magnesium,¹⁴ and some magnesium retention in the plasma of adrenalectomized animals probably adds to other causes of skeletal muscle weakness.

To fully appreciate the importance of mineralocorticoid hormones, it would be useful to have animals deficient in such hormones but possessing normal glucocorticoid function. Animals of this kind cannot be prepared by performing adrenalectomies and then administering glucocorticoids, because all of the latter have mineralocorticoid potency. Some success has been achieved with the use of specific inhibitors of aldosterone. The *spironolactones* compete with aldosterone and other mineralocorticoids for nuclear binding sites but do not seem to interfere with known glucocorticoid functions. They have been used with some success in treatment of clinical hyperaldosteronism. No effects of these agents on water and electrolyte metabolism of adrenalectomized animals deprived of mineralocorticoid have been described. *Triamterene* exerts influences antagonistic to those of mineralocorticoids and was once believed to interfere directly with actions

of the hormones, but it is now known that triamterene also affects otherwise untreated adrenalectomized animals. Structures of these agents are shown in Figure 11-1.

MECHANISM OF ACTION OF ALDOSTERONE

The urinary bladder of the toad functions as a reservoir for water and electrolytes. It has been extensively used for aldosterone studies because electrolyte transport responses are similar to those of mammalian kidney, and there are fewer technical difficulties involved.

Binding of the Hormone to the Receptor Site¹⁸

In common with other steroid hormones, aldosterone actions are initiated by binding of the hormone to specific cytoplasmic receptor molecules. Properties of the cytoplasmic macromolecule are then altered, and an "activated" steroid-receptor complex enters the nucleus.

When concentrations of aldosterone within the physiological range are presented, specific high affinity binding can be demonstrated in responsive cells (e.g., of the kidney, toad bladder, and intestine) but not in "nontarget" organs such as the liver. Radioactively labeled aldosterone is displaced by unlabeled aldosterone, by steroids exhibiting mineralocorticoid activity (DOC, fluorocortisol), and by specific antagonists such as the spironolactones. Chemi-

cally related steroids (cholesterol, testosterone) which do not affect sodium transport across kidney and toad bladder fail to compete. Progesterone can act as a weak mineralocorticoid; in very large amounts it competes with low concentrations of aldosterone.

The binding process is saturable. After administration of labeled aldosterone, maximal binding in the cytoplasm has been observed within 15 min. The quantity of label detectable in the cytoplasm then declines, but the amount within the nucleus rises and reaches a peak 30 min after exposure to the hormone.

Since binding can be impaired with certain proteolytic enzymes but not with lipases, RNases or DNases, the aldosterone receptor seems to be (or contain) a protein. The concept is supported by studies of the complex isolated from responding cells. The cytoplasmic receptor is also known as aldosterone-binding protein (ABP).

The Latent Period and Aldosterone-induced Protein Formation

Aldosterone actions are not seen until a characteristic latent period has elapsed. The time lag (about 1 hr for tissues in which maximal binding is completed in about $\frac{1}{2}$ hr) cannot be shortened by use of higher hormone concentrations and is not influenced by washing the tissues once binding has occurred.

The time is required for synthesis of a specific *aldosterone-induced protein* (AIP) which promotes sodium transport. Inhibitors of protein synthesis which do not affect the basal level of sodium transport block actions of aldosterone.

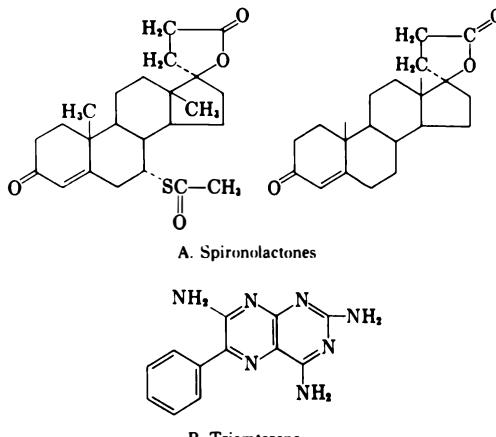


FIG. 11-1. Some aldosterone antagonists.

HORMONAL REGULATION OF BODY FLUIDS

An AIP has been identified in toad bladder.⁷ However, recent findings suggest that at least three additional proteins affecting sodium transport are synthesized in response to aldosterone.

Induction of the protein seems to require synthesis of a specific messenger RNA.

Actinomycin D blocks the ability of mineralocorticoids to promote sodium transport. Moreover, it has been demonstrated that aldosterone stimulates the rate of incorporation of labeled uridine into RNA. (Some investigators have questioned the significance of the last mentioned finding, since other hormones can increase RNA synthesis in the same tissues without stimulating sodium transport.)

Movement of Sodium Ions

Sodium enters the *mucosal* or *apical* surface of transporting cells (the side of the toad bladder in direct contact with bladder urine or the surface of the kidney cell closest to the tubular urine). Entry is *passive* (in the direction of the concentration gradient via processes which do not consume energy). It is believed that the sodium is *actively pumped out* at the

opposite (serosal or basal) surface in contact with extracellular fluids (Fig. 11-2). A magnesium-activated Na-K-ATPase has been implicated in operation of the "sodium pump."

When concentration gradients favor passive entry of sodium, a basal rate of transport across the serosal membrane is maintained in absence of the hormone. Aldosterone increases the rate of transfer of sodium from urinary to extracellular fluids. (Aldosterone will not promote sodium transport in the opposite direction if the concentration gradients are reversed.)

If the system is depleted of energy-yielding substrate, no influence of aldosterone on sodium transport is observed. Energy depletion evidently interferes with actions of AIP, but it does not seem to affect generation of AIP. Addition of glucose or other substrate at the time when the latent period is usually completed is followed by prompt stimulation of sodium transport.

The Role of AIP

While there is general agreement on what has just been presented, there is

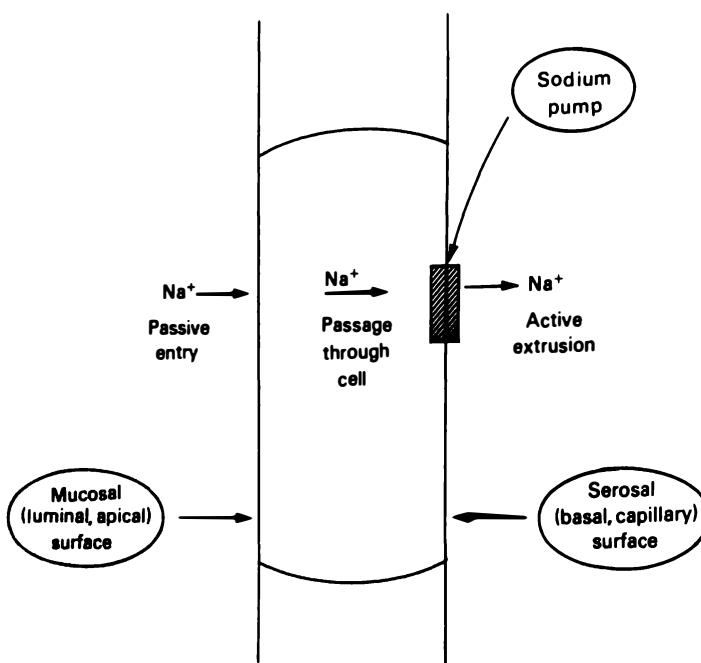


FIG 11-2. Direction of sodium ion transport influenced by aldosterone.

considerable controversy concerning the mechanism of action of AIP. Several hypotheses have been proposed along with arguments for and against each of them.

ATP-generation Hypothesis. Aldosterone increases the amount of ATP energy available to the sodium pump through stimulation of substrate utilization.

In particular, it has been suggested that AIP acts on enzymes catalyzing reactions which promote formation of NADH.⁹ Glucose, pyruvate, oxaloacetate, and certain other substrates have been observed to support aldosterone actions, whereas succinate (which utilizes a flavoprotein dehydrogenase) does not. Moreover, inhibitors for utilization of the first group (e.g., oxythiamine) block aldosterone actions, whereas malonate (which inhibits activity of succinic dehydrogenase) does not.

Decreased activities of mitochondrial citrate synthetase, glutamate dehydrogenase, and NADP-isocitrate dehydrogenase in kidneys of adrenalectomized rats have been described along with enzyme activity restoration following aldosterone treatment. There is also evidence that aldosterone induces synthesis of citrate synthetase and two other mitochondrial proteins.

One argument against the ATP-generation hypothesis states that such influences cannot be primary, since ATP accumulation cannot be demonstrated when responding tissues are treated with aldosterone in the absence of sodium. A counterargument is that AIP does stimulate ATP synthesis, but in the absence of sodium the ATP is not utilized. Therefore ADP depletion soon shuts off further ATP synthesis.

The "Permease" Hypothesis. AIP is or induces formation of a "permease" which increases the rate of passive entry of sodium either through direct influences on the mucosal membrane or because the permease acts as a sodium carrier. One possibility is that AIP regulates activity of a membrane protein phosphatase. Studies utilizing pharmacological agents have provided support for the hypothesis.

Amphotericin B increases sodium transport in aldosterone-sensitive tissues. Influences on ion transport are associated with substrate utilization similar to that seen after aldosterone.¹⁰

Amiloride¹¹ blocks actions of amphotericin B on sodium transport. Pretreatment with aldosterone prevents the block. It has been assumed that amiloride

impairs binding of sodium to its carriers in the mucosal membrane.

However, unlike the hormone, amphotericin B is effective when applied to the mucosal surface, and a stimulatory influence of amphotericin B on sodium transport in substrate-depleted bladders (which are unresponsive to aldosterone) has been reported.⁹ Moreover, assumptions concerning the mechanism of action of amiloride have been questioned, and it has been pointed out that the increased radioactivity of bladders described above has not been shown conclusively to be intracellular.

The Sodium Pump Hypothesis. AIP could stimulate sodium transport by either increasing the efficiency of existing "sodium pumps" or by promoting formation of additional ones.

Ouabain is said to be a fairly specific inhibitor of Na-K-ATPase activity. There are concentrations of ouabain that effectively block the stimulatory influence of aldosterone without appreciably influencing basal rates of sodium transport.

Spironolactones (which antagonize aldosterone) inhibit ATPase activity, and the effect can be overcome with very large doses of mineralocorticoids.

That adrenalectomy lowers ATPase in aldosterone-responsive tissues or that all transepithelial sodium transport depends upon the enzyme has not been conclusively established. Very large doses of aldosterone can increase ATPase activity, but the effect is preceded by influences on sodium transport.

Localization of Aldosterone Influences on Mammalian Kidney

While actions on the most distal portion of the distal tubule are best known, there are indications that the hormone also affects collecting tubules. An effect on sodium transport in the proximal tubule has been accepted by some observers and rejected by others.¹²

Influences on Potassium Transport

Aldosterone influences on sodium-dependent electrochemical gradients within the kidney can indirectly affect potassium excretion. However, under some conditions, mineralocorticoid influences on potassium precede the onset of measurable sodium changes, and they may persist after

"escape" blunts the sodium response. Moreover, potassium responses to aldosterone are not consistently blocked by actinomycin D. Aldosterone also seems to directly affect potassium uptake by skeletal muscle and other cell types.

REGULATION OF ALDOSTERONE SECRETION^{2, 3, 4b}

Role of ACTH

Adrenocorticotrophic hormone (ACTH) regulation of adrenal growth and glucocorticoid secretion was described in Chapter 8. ACTH can affect sodium and potassium metabolism in this way since all glucocorticoids have some mineralocorticoid potency.

The direct influences of ACTH on aldosterone secretion are difficult to assess because of marked species variations^{2, 3} and because responsiveness to the pituitary hormone changes with physiological conditions. Very large doses of ACTH acutely stimulate mineralocorticoid secretion; the amounts required may not be greater than those released during times of stress. Endogenous ACTH secreted at other times may synergize with more effective stimuli for aldosterone regulation.

Hypophysectomized dogs exhibit defective sodium conservation and blunted adrenocortical responses to sodium deprivation which can be corrected by administration of ACTH. Humans suffering from pituitary insufficiency have similar problems. It has therefore been proposed that ACTH plays a supportive role in the maintenance of aldosterone function in these species.

It has been stated that the laboratory rat maintains normal electrolyte metabolism and aldosterone secretion following hypophysectomy and that aldosterone-secreting cells are not stimulated by physiological concentrations of ACTH. However, it has been pointed out that the rat is characteristically offered and chooses to consume a sodium-rich diet. High sodium intake is known to reduce sensitivity of zona glomerulosa cells of several species (including man and dog) to a variety of stimuli. Both ACTH and stress promote aldosterone secretion in sodium-depleted intact and hypophysectomized rats. Similar doses of ACTH are totally ineffective in sodium-replete animals.

Beef adrenal glands studied *in vitro* do not put out greater quantities of aldoster-

one when ACTH alone is added, but they do respond to the same concentrations of ACTH given in combination with amounts of either potassium or of angiotensin II (see below) which are not by themselves effective.

Chronic high concentrations of ACTH actually may be inhibitory. Human subjects with ACTH-secreting tumors tend to have high glucocorticoid but either normal or low mineralocorticoid levels. When ACTH is added to zona glomerulosa (aldosterone-secreting) cells in culture, the cells become transformed into zona fasciculata (glucocorticoid-secreting) type cells (Chapter 8).

ACTH can also promote secretion of DOC by fasciculata-reticularis regions; large amounts of this mineralocorticoid may be released under conditions when glucocorticoid output is below normal.¹² Sodium- and water-retaining influences of DOC could indirectly lead to suppression of aldosterone secretion and thereby mask stimulatory influences of ACTH on the zona glomerulosa.

Direct Influences of Sodium Concentrations on Aldosterone Secretion^{2, 3}

High concentrations of sodium in plasma or incubation fluids have been shown to depress aldosterone output by adrenal glands of the dog, ox, and rat. Under similar conditions, nonspecific alterations of osmotic pressure (resulting from addition of nonmetabolizable sugars) are apparently without effect.

The physiological significance of the findings has been questioned for two reasons. First, the plasma sodium ion concentration is a rather poor indicator of sodium balance and the need for sodium conservation or removal in the intact animal. There is a strong tendency to adjust the water content of the body to the available sodium; thus, conditions of sodium retention are more likely to be reflected in expanded plasma and extracellular fluid volume than in elevated sodium concentration. Second, the changes in sodium concentration needed to influence aldosterone secretion are quite large, and impressive effects are obtained only when physiological ranges are greatly exceeded. Moreover, effects of sodium ions seem to be exerted late in the biosynthetic pathway at the level of conversion of corticosterone to aldosterone.¹²

Small changes in plasma sodium could, however, synergize with other stimuli. They may also affect sensitivity of the zona glomerulosa cells to other stimuli; but just how the message affects the cells is not clear. One suggestion is that sodium depletion leads indirectly to cell proliferation and formation of increased numbers of receptors.

Direct Influences of Potassium Concentrations^{2, 3, 4b, 12}

Over a fairly wide range, aldosterone secretion may vary directly with the potassium ion concentration of the plasma or incubation fluid. Effects of small changes have been demonstrated in several species (cattle, dogs, sheep, rats) under a variety of experimental conditions (intact and hypophysectomized animals with adrenal transplants, and also *in vitro*). They can be demonstrated in sodium-replete animals but are augmented by sodium depletion.^{3, 4a} Some influences in intact animals may be indirect, since potassium loading can lead to sodium loss. Further complications in intact animals arise from influences on the renin-angiotensin system (see below).

There is a strong tendency to maintain plasma potassium ion concentrations within narrow limits when relatively large shifts in total body potassium are induced. However, variations within the physiological range are sufficient to affect aldosterone secretion.

Potassium seems to act exclusively on cells of the zona glomerulosa (with no known influences on glucocorticoid-secreting cells). The conversion of cholesterol to pregnenolone is accelerated, but there are indications that potassium may also promote conversion of corticosterone to aldosterone and that it affects 11β -hydroxylase activity in the zona glomerulosa.¹²

An effect on the cell membrane is suggested by the observation that ouabain blocks potassium influences. Potassium may also stimulate protein synthesis in the adrenal cortex since its influences on aldosterone secretion can be blocked by administration of inhibitors of protein synthesis but not by actinomycin D.

The Renin-angiotensin System^{4, 12}

Renin and the Juxtaglomerular Apparatus. In most mammalian species the major control over aldosterone secretion is

exerted by *angiotensin II* (A-II) which is derived from a plasma protein when the kidney releases the enzyme *renin*. As its name suggests, A-II acts on blood vessels to elevate systemic pressure in addition to providing a stimulus for aldosterone secretion.

Near the point of entry into the glomerulus, the afferent arteriole of the nephron contains a group of granular and agranular cells which form what has been called an *arteriolar cuff (polar cushion, Polkissen)*. These modified smooth muscle (myoepithelial) cells make up the *juxtaglomerular (JG)* component of the *juxtaglomerular apparatus (JGA)*. Renin is synthesized here and stored in granules for later release into the circulation.

Fluorescent renin antibodies localize in the vicinity of the granules, which increase in number following adrenalectomy or salt depletion and decrease after salt loading or administration of mineralocorticoids.

Large, clear cells within that portion of the distal tubule of the same nephron which is adjacent to the polar cushion are known collectively as the *macula densa*. These are believed to contain *chemical sensors* involved in regulation of renin secretion. (They have been implicated by some in renin synthesis).

The macula densa, JG cells, and associated portion of the afferent and efferent arterioles make up the JGA (Fig. 11-3).

Modified smooth muscle cells located in some of the large arteries, the arterioles of the splanchnic bed, and in salivary, adrenal and thymus glands, and the uterus also produce renin or a related enzyme, but such renin is believed to act *locally*. Only the JGA renin seems to contribute to plasma content; changes in the plasma component in response to the usual stimuli are lost after nephrectomy. The term "pseudorenin" designates enzymes in plasma and nonrenal tissues which act on renin substrate but which differ in properties such as optimal pH for catalysis. Pseudorenins do not seem to participate in regulation of aldosterone secretion.

Renin Substrate. Renin acts on a glycoprotein synthesized by the liver and secreted into the blood plasma. It is associated with the α -2-globulin fraction and is most commonly known as *renin substrate*. Since it was originally studied in several independent laboratories, it has been

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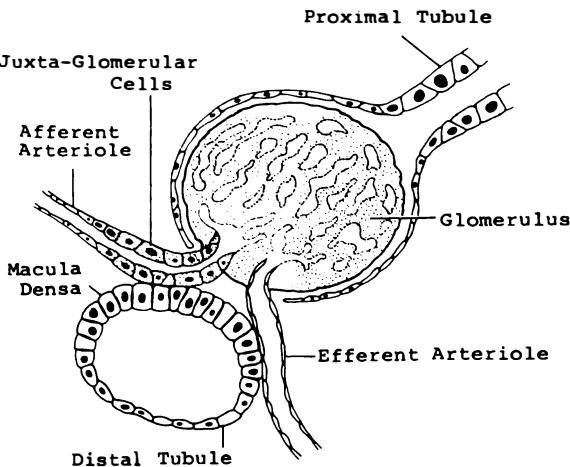


FIG. 11-3. Diagrammatic representation of juxtaglomerular apparatus.

known under other names, including *preangiotensin*, *angiotensinogen*, *angiotensin precursor*, and *hypertensinogen*.

The structure of the tetradecapeptide component of human substrate to which renin attaches is shown in Figure 11-4A. An identical peptide sequence is found in horses and dogs, but the bovine peptide differs in one amino acid moiety.

Renin substrate concentrations are lowered following hypophysectomy or adrenalectomy and elevated after administration of glucocorticoids or estrogens. The steroids probably stimulate hepatic production of the glycoprotein directly; excess quantities have been found in some women taking oral contraceptives.

Renin substrate is destroyed by the kidney (but not excreted directly). High concentrations in nephrectomized animals have been attributed variously to reduced degradation, to accumulation because renin is not secreted, and to metabolic disorders.

Renin catalyzes splitting of the substrate to yield the decapeptide *angiotensin I* (Fig. 11-4B); the latter does not seem to have *direct* activity but serves as the precursor for A-II.

The rate of renin release usually determines the rate of A-II synthesis, but large changes in plasma substrate concentration can be important. A-II may participate in regulation of substrate synthesis.

Converting Enzymes. Peptidases in blood plasma, lungs, kidneys, and proba-

bly other tissues catalyze conversion of angiotensin I to A-II. Angiotensin I is completely transformed during a single passage through the lungs.

Structures of human, horse, and hog A-II are shown in Figure 11-4C. The octapeptide is also known as *angiotonin* and *hypertensin*.

Angiotensin III.* A heptapeptide, *angiotensin III*, and an enzyme catalyzing its formation from A-II have been identified in the rat. It is said to be at least as potent as A-II for stimulation of aldosterone secretion, but with only 22% as much influence on blood pressure, and even lower activity on rat uterus.

A-II Antagonists. Synthetic derivatives (e.g., 1-sarcosyl-8-alanyl-angiotensin II) which block actions of A-II have been synthesized and proven useful for investigative and clinical purposes.

A-II is rapidly degraded by enzymes (angiotensinases) present in blood plasma and many tissues. It has a half-life of not more than 1-3 min and is rapidly destroyed in the liver.

Role of the Renin-angiotensin System in Regulation of Aldosterone Secretion

There is little doubt that the renin-angiotensin system plays a major role in regulation of aldosterone secretion in humans, dogs, sheep, opossums, frogs, and other species. Influences of mineralocorticoids

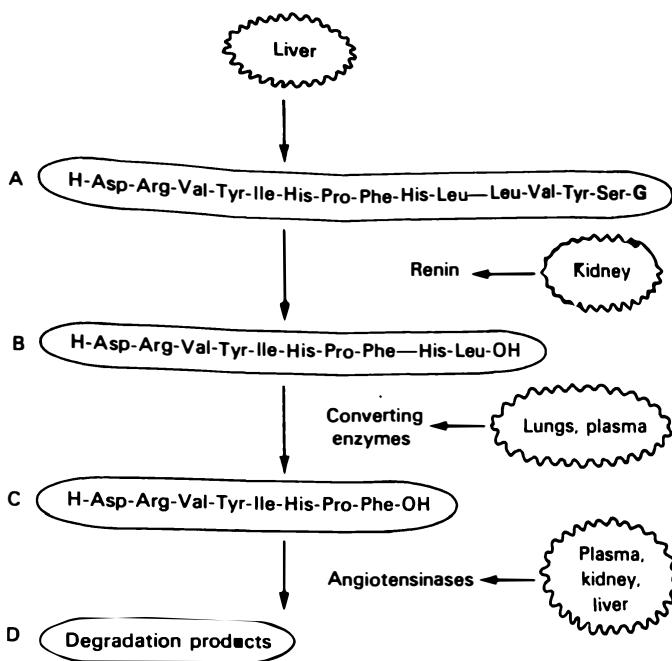


FIG. 11-4. Components of the renin-angiotensin system. A. Renin substrate; B. angiotensin I; C. angiotensin II; G. glycoprotein component.

and electrolytes or JG cell granulation were cited above, and it is also known that renin administration leads to increased secretion of aldosterone and to widening of the zona glomerulosa of the adrenal cortex.

Actions of A-II on the zona glomerulosa have been compared with actions of ACTH on the fasciculata-reticularis regions. Both peptides attach to specific receptors, promote growth, and stimulate secretion. But there are important differences. The glomerulosa is capable of maintaining structure and function without tonic hormonal stimulation. A-II seems to function only for enhancing growth and secretory activities in times of special need, e.g., in response to salt deprivation or when blood volume is diminished.

The role is readily demonstrated in most species, but there are controversies concerning what happens in rats. These animals secrete A-II (and A-III), and carefully executed studies in which each animal serves its own control have demonstrated that large doses of renin stimulate aldosterone secretion. Negative find-

ings have been reported in studies utilizing unphysiological conditions (deep anesthesia, nephrectomy, hypophysectomy, and combined operations) and pooled samples, but similar studies have yielded positive data in dogs. The high sodium intake of rats may blunt responses to small doses of renin.

Perhaps too little attention has been directed at the fact that a certain amount of angiotensin II is present most of the time (in the rat and also in other species), and that concentrations of the octapeptide too small to directly elevate aldosterone secretion may quite effectively synergize with other stimuli to the zona glomerulosa. Moreover, direct effects of small amounts of angiotensin II on the kidney (see below) may contribute to the effectiveness of low plasma levels of aldosterone.

In common with ACTH and potassium, angiotensin seems to increase the rate of conversion of cholesterol to pregnenolone. It may also stimulate conversion of corticosterone to aldosterone.

cAMP has been implicated in actions of

angiotensin on the zona glomerulosa, but its role has not been defined. (On the other hand, cAMP is clearly involved in stimulation of renin release.) The observation that angiotensin seems to be ineffective when added to homogenates of adrenocortical tissue supports the concept that the octapeptide acts directly on cell membrane functions. There are controversies concerning effects of angiotensin on rate of uptake of potassium by zona glomerulosa cells.

Other Actions of Angiotensin II^{1b}

On a molar basis, A-II is the most potent agent known for elevation of the systemic blood pressure (and this action extends to aquatic vertebrates in which no influence on sodium excretion can be demonstrated). The peptide acts directly on smooth muscle cells of arterioles and possibly also on the precapillaries. Vasoconstriction is beneficial at times when blood volume is reduced (e.g., after water deprivation or hemorrhage). Actions of A-II on the adrenal cortex synergize with vasoconstrictor effects because aldosterone promotes retention of water and salts (which aids in restoration of the blood volume) while elevated plasma sodium concentrations increase the sensitivity of the arterioles to A-II stimulation. The peptide is also a powerful dipsogen (Chapter 13).

Angiotensin has been implicated in the etiology of some forms of hypertension; it may play some role in physiological regulation of blood pressure. Direct actions on the heart have also been described.

Hemodynamic influences on the kidney can lead to reduction of glomerular filtration rate and of renal plasma flow with shunting of a greater part of the blood through the medullary portions of the kidney. Small doses reduce urine volume, sodium, potassium, and water. These actions contribute to protection against falling blood volume.

However, very large doses of angiotensin actually increase sodium excretion possibly via depression of the rate of tubular reabsorption.

Part of the vasoconstrictor action of angiotensin in intact animals results from release of norepinephrine. Effects of both angiotensin and norepinephrine can be antagonized with adrenergic-blocking agents. But angiotensin can also act independently of the catecholamines, and the mechanism of action seems to involve some different molecular component.

An influence on Na-K-ATPase is suggested by the fact that the action can be antagonized by ouabain (Ouabain does not abolish stimula-

tory effects of other hormones, e.g., vasopressin, on smooth muscle.)

A-II also stimulates smooth muscle in many other parts of the body and may perform localized functions related to this action.

The importance of catecholamines during strenuous exercise was discussed in Chapter 9. A supporting role for A-II is suggested since it stimulates sympathetic nerves, promotes release of epinephrine, and inhibits insulin release. Such actions lead to elevation of plasma glucose concentrations (and hypoglycemia is an effective stimulus for renin secretion). A-II also seems to inhibit catecholamine uptake by sympathetic nerves and may thereby prolong effects of adrenergic stimulation.

A stimulatory influence on autonomic ganglia with increased acetylcholine release has been described, but peripheral effects of vagal stimulation are antagonized.

A-II promotes secretion of ACTH directly and possibly also through release of vasopressin. (The latter, in turn, inhibits renin release.)

Pharmacological doses of A-II provoke glycosuria through direct inhibition of reabsorption. This probably has no physiological significance.

Regulation of Renin Release

Role of the Juxtaglomerular Cells. The myoepithelial cells (which are part of the renal arteriole) function directly as stretch or baroreceptors and exert a tonic inhibitory control. When the blood pressure falls in this vicinity, the inhibition is lifted and renin is released. If the fall in blood pressure results from reduction of blood volume, this mechanism provides effective protection; it is also self-limiting since the angiotensin II that is soon produced reestablishes blood pressure through actions on the adrenal cortex, drinking mechanisms, and blood vessels.

Inhibition is also lifted when a localized fall in blood pressure results from constriction of renal blood vessels. A form of hypertension is produced in experimental animals by clamping the renal artery.

Role of the Macula Densa Cells. The macula densa cells respond to reduction in the rate of presentation of sodium ions to

the distal tubule. The cells are therefore affected by changes in blood volume and glomerular filtration rate. Since (as was noted above) sodium depletion is more likely to lead to reduction of plasma volume than to reduction of plasma sodium concentration, macula densa cells probably mediate most effects of sodium depletion on aldosterone secretion.

Effects of Potassium Depletion. Low plasma concentrations of potassium may lead (via influences on the renal proximal tubule) to reduced presentation of sodium to the macula densa cells. However, a sodium-independent influence of potassium ion concentration has not been ruled out. It should be noted that whereas low potassium increases renin release, high potassium stimulates aldosterone secretion.

Role of the Nervous System. The JGA is richly innervated, and there is good evidence for enhanced rate of renin release following stimulation of renal nerves and when levels of circulating catecholamines are increased. Moreover, renin release occurs under a variety of conditions associated with activation of the sympathetic system, including postural changes, anoxia, hemorrhage, and hypoglycemia.

β -adrenergic receptors have been implicated. Administration of β -blocking agents is known to interfere with renin secretion; effects of adrenergic blockers are more controversial. Administration of N°,O° -dibutyryl cyclic AMP (D-cAMP) and of theophylline has been reported to enhance renin secretion.

A direct *secromotor* function of adrenergic nerves has been proposed, but catecholamines may also reduce renal blood flow and thereby affect the JG cell receptors. Sodium depletion seems to influence function of the nerves, but some renin responses to sodium depletion can be elicited in denervated kidneys.

Other Influences on Renin Secretion. A-II may exert limited negative feedback control over renin secretion. However, the very high plasma A-II concentrations in some pathological conditions suggest that either stimuli promoting renin release can effectively overcome the inhibition or that the feedback regulation is impaired in such conditions.

Vasopressin elevates blood pressure

(Chapter 12), but inhibitory influences of this hormone on renin secretion may participate in protection against hypertension.

Factors implicated in regulation of renin secretion are summarized in Table 11-1, and interrelationships between electrolytes, aldosterone, and the renin-angiotensin system are shown in Figure 11-5.

Pathological Secretion of Renin. JG cells have no mechanism for distinguishing between reduction of blood pressure in their immediate vicinity resulting from diminished circulating blood volume, and reduction of blood pressure secondary to myocardial insufficiency. When cardiac force is inadequate for delivery of normal amounts of blood to the kidney, renin secretion can be detrimental. The resulting vasoconstriction and fluid retention create an additional burden for the heart; this leads to augmentation of the stimulus for release of more renin. Further problems arise when anoxia develops since this too enhances renin secretion.

Thus, a condition initiated by a weakened myocardium sets off a train of events in which progressively increasing demands on the heart can lead ultimately to circulatory failure. Under such circumstances, it

TABLE 11-1
Factors Implicated in Regulation of Renin Secretion

Renin secretion increased
Low circulating blood volume
Low pressure in renal afferent arterioles
Low sodium concentrations in plasma
Low potassium concentrations in plasma
Stimulation of renal nerves
High concentrations of epinephrine in plasma
Anoxia
Hypoglycemia
Some forms of stress
Muscular exercise
Upright posture
Renin secretion decreased
Expanded blood volume
High pressure in renal afferent arterioles
High sodium concentrations in plasma
High potassium concentrations in plasma
Adrenergic blocking agents
Horizontal position
High concentrations of angiotensin II
Vasopressin (antidiuretic hormone)

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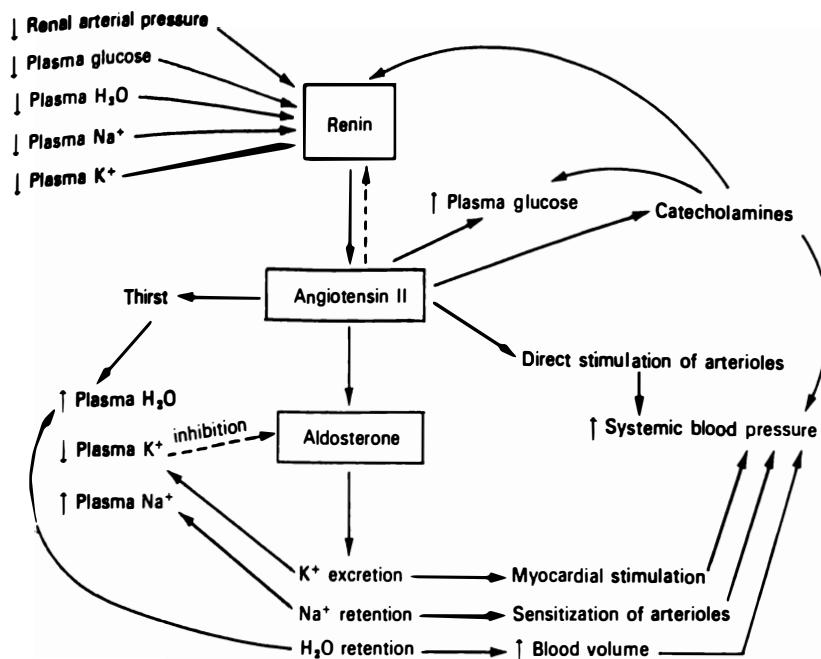


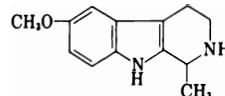
FIG. 11-5. Some interrelationships involved in regulation of plasma water and electrolyte content and arterial blood pressure.

becomes necessary to administer pharmaceutical agents. Cardiac stimulants (e.g., digitalis) can improve the circulation, while judicious use of diuretics may reduce excessive blood volume. (Precautions are needed to avoid further stimulation of renin secretion.) Angiotensin inhibitors may prove to be the most useful agents in such conditions.

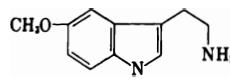
When renin hypersecretion results from problems originating in the kidney (e.g., stenosis of the renal artery), blood pressure can rise to dangerous levels. Paradoxically, although sodium contributes in several ways to the hypertension, salt restriction may be hazardous since it leads to augmentation of renin output.

Influences of the Pineal Gland on Aldosterone Secretion^{2, 3, 4}

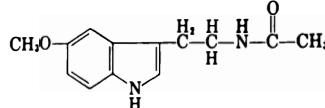
A substance isolated from pineal gland extracts and named *adrenoglomerulotropin* (Fig. 11-6A) was reported to stimulate aldosterone secretion in *decerebrate* dogs. Studies in other laboratories failed to confirm the finding in *hypophysectomized*



A. Adrenoglomerulotropin (Glomerulotropin)
(1-Methyl-6-methoxy-1,2,3,4-tetrahydro-2-carboline)



B. 5-Methoxytryptamine



C. Melatonin

FIG. 11-6. Adrenoglomerulotropin and possible precursors.

dogs, and the significance of the initial observation was questioned.

It was later demonstrated that the substance in question can be formed *in vitro* in pineal extracts from either 5-methoxytryptamine (Fig. 11-6B) or from melatonin (Fig. 11-6C) precursors.

ers present in the glands. Doubt has been cast on the existence of adrenoglomerulotropin in normal pineal glands, and the proposed structure has been disputed.

The pineal gland contains high concentrations of serotonin (Fig. 11-7A). Since this amine can stimulate aldosterone synthesis by acting at some point between cholesterol and pregnenolone, it has been suggested that serotonin accounts for aldosterone-stimulating actions of pineal extracts. However, dosages of serotonin needed are far in excess of quantities expected to travel from the pineal gland to the adrenal cortex. If serotonin does play a role in regulation of aldosterone secretion, it is not necessary to implicate the pineal gland; serotonin occurs in high concentration in mast cells, enterochromaffin cells, blood platelets, and in the mesencephalon.

Melatonin has been implicated in both stimulation and inhibition of ACTH release (Sections II and VIII); presumably, this can secondarily affect aldosterone secretion.

Several authors have suggested that the pineal secretes inhibitors of aldosterone secretion. In one study aldosterone secretion was significantly elevated 17 and 30 days after pinealec-tomy (although the rats continued to respond to sodium depletion). In another study on dogs, no influence of pinealec-tomy could be found on either basal aldosterone secretion or on responses of the adrenal to constriction of the thoracic vena cava.

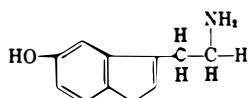
Lipid extracts contain ubiquinone which de-

creases aldosterone secretion. But this substance cannot be considered a pineal hormone, since it is found in other parts of the body, including kidney and liver. The structures of serotonin and of ubiquinone are compared in Figure 11-7.

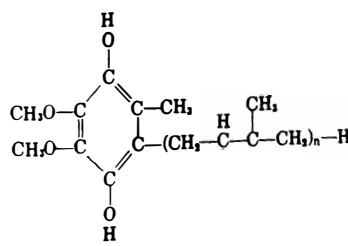
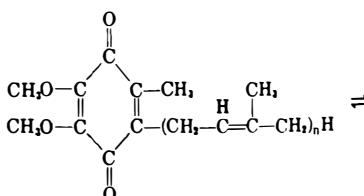
Several kinds of explanations have been offered for seemingly contradictory findings. The pineal gland may exert both stimulatory and inhibitory influences on the zona glomerulosa cells with prevailing conditions determining the predominating effect. The pineal probably modifies sensitivity to other stimuli; it may also exert influences on electrolyte metabolism via mechanisms independent of the adrenal cortex. Species differences in pineal function further complicate the picture (see also Section VIII).

Possible influences of Somatotrophin and of Other Adenohypophysial Hormones

Hypophysectomized animals seem to have defective aldosterone secretion responses to several stimuli, which are only partially corrected by administration of ACTH. Growth hormone (STH) alone does not restore the responses, but STH in combination with ACTH can be effective. STH in combination with pituitary ex-



A. Serotonin (5-Hydroxytryptamine)



B. Ubiquinone (Coenzyme Q)

FIG. 11-7. Structures of serotonin and of ubiquinone.

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tracts devoid of ACTH activity has also been reported to be effective. It has been proposed that the adenohypophysis secretes an as yet unidentified *aldosterone-stimulating hormone* (ASH).

Heparin

Heparin is a sulfated acid mucopolysaccharide synthesized in many parts of the body including lung, liver, heart, spleen, and connective tissues. Its functions are only partially

understood; they seem to include participation in regulation of bone calcification, lipid transport and metabolism, and mast cell functions. While probably best known for its ability to prevent blood clotting and widely used for this purpose in the laboratory, it is not believed to be a *physiological anticoagulant* since concentrations needed far exceed those present in the circulation. (The one known exception is during peptone shock in dogs.) Moreover, it affects the clotting mechanism late in the train of events.

An interest in a possible role for heparin in

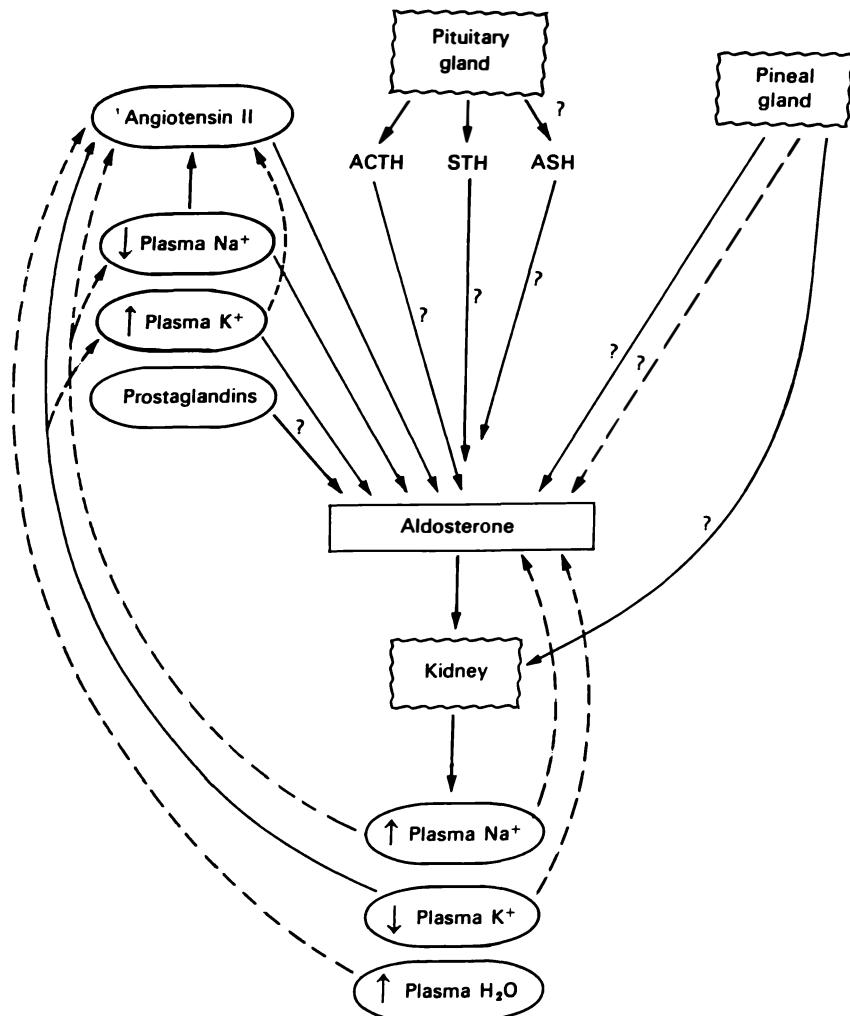


FIG 11-8. Known and suspected influences on aldosterone secretion. →, increase; —, decrease; ?, controversy regarding influence.

regulation of aldosterone secretion arose from observations that patients given pharmacological doses for prevention of intravascular clotting excreted large quantities of salt and water. In some patients with myocardial problems associated with edema, it was possible to discontinue diuretic therapy.

Reproducible influences of heparin and related agents on sodium and water excretion have been demonstrated in patients and in experimental animals, and chronic administration of large doses can induce structural changes in the zona glomerulosa. However, repeated attempts to demonstrate an *in vitro* influence of heparin on adrenal glands have been uniformly unsuccessful. Moreover, this agent can promote salt and water excretion in adrenalectomized animals. It is unlikely that heparin directly influences mineralocorticoid secretion.^{3, 10} (See also Section VIII.)

Prostaglandins

Prostaglandins of the A series may exert important influences on renal function (Chapter 13). Since other prostaglandins promote release of epinephrine, they may act on the renin-angiotensin system. There are conflicting reports concerning both stimulatory and depressant direct actions on the adrenal cortex.

Other Influences on Aldosterone Secretion

Aldosterone may exert feedback inhibition on its own synthesis; if the mechanism exists, it is consistent with known influences of other steroids on steroid biosynthesis. This cannot be an important site for regulation because numerous stimuli can tremendously increase aldosterone secretion.

Direct nervous control does not seem to be important since transplanted adrenal glands exhibit typical aldosterone responses to the usual stimuli. However, *renal nerves* function indirectly, via the renin-angiotensin system.

Vasopressin (antiuretic hormone) actions are described in Chapter 12. In addition to direct actions on water and electrolyte metabolism which secondarily affect the adrenal gland, high concentrations affect release of pituitary hormones.

Regulation of aldosterone secretion is summarized in Figure 11-8.

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12. Antidiuretic Hormone

ANTIDIURETIC HORMONE (ADH) AND VASOPRESSIN

ADH promotes water conservation by the mammalian kidney and thereby reduces urine volume. Since diuretics are agents which enhance urine flow, the name refers to the most obvious physiological function of the hormone.

ADH is also a smooth muscle stimulant; in high dosage it elevates the systemic blood pressure. The term "vasopressin" describes such action; it is now used to designate chemical as well as biological properties.

It is unlikely that ADH functions as a physiological vasoconstrictor in mammals; very high concentrations are required, the action is indiscriminate (extending to the coronary vessels), and the amounts required would also promote excessive contraction of smooth muscle in the gastrointestinal tract and elsewhere. Related peptides do participate importantly in regulation of blood pressure of some nonmammalian vertebrates.

Pharmacological actions of ADH on uterus and arterioles have been extensively employed in bioassay procedures. Actions on smooth muscle have limited therapeutic application, for example, in restoration of the tone of gastrointestinal muscle (which has been impaired by prolonged surgical manipulation) and for promoting localized vasoconstriction during surgical procedures on the esophagus.

SITES OF SYNTHESIS

Most ADH is synthesized by *magnocellular neuroendocrine cells* of the hypothalamus and especially those located within the *supraoptic nuclei*. It is packaged into granules along with specific *neurophysin* proteins. The granules travel down long axons and are stored in the neural lobe ("posterior pituitary gland") for later release into the circulation. The magnocellular components of the hypothalamus, the axon groups (nerve tracts), the neurophysins, and minor sites of ADH synthesis are described in Section VII.

CHEMISTRY

In most of the mammals ADH has the structure shown in Figure 12-1 and is known as *arginine vasopressin*. The numbering system for amino acid components is useful for making comparisons between this molecule, related hormones, and synthetic analogs. Nine positions are shown, but ADH is classified as an *octapeptide* since cysteine residues at positions 1 and 6 combine to form cystine.

While arginine vasopressin is the antidiuretic hormone of most mammals (including the marsupials and monotremes), the domestic pig produces *lysine vasopressin* (in which lysine substitutes for arginine at position 8), and other members of the Suiforme family (wart hogs, peccaries, and hippopotamuses) synthesize both octapeptides.

The difference in ADH structure is of interest to those studying evolution of the neurohypophyseal peptides. There is speculation concerning the physiological significance of the presence of the structural modification of a water-conserving hormone in species preferring aqueous habitats. Lysine vasopressin is only about two-thirds as potent as the arginine hormone for promotion of antidiuresis in the mammalian kidney, but secretion of greater amounts of hormone should present no special problem. Lysine vasopressin has also been identified in one strain of (nonaquatic) mouse.¹

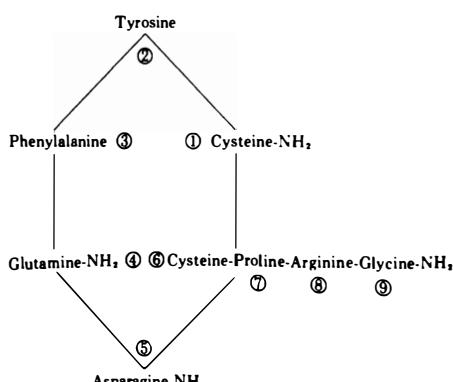


FIG. 12-1. Structure of arginine vasopressin: antidiuretic hormone for most mammals.

EFFECTS OF ADH DEFICIENCY

ADH deficiency can be studied in animals with *genetic aberrations* which preclude synthesis of the hormone, and after performance of *surgical or electrolytic procedures* which interfere with normal physiological mechanisms.

A clinical syndrome, diabetes insipidus (DI) resulting from ADH deficiency, has been recognized in patients and has been successfully treated with hormone replacement therapy. Insipidus means tasteless. The term "diabetes insipidus" distinguishes excessive urine flow (diabetes) resulting from inability to conserve water, from diabetes mellitus in which urine flow is enhanced because water is drawn osmotically when hyperglycemia leads to glycosuria.

The Brattleboro strain of rats has been especially useful for studies of ADH deficiency. The animals also have defective synthesis of specific neurophysins.⁷

Destruction of the supraoptic nuclei leads to permanent loss of ADH function. If the nerve cell bodies are left intact but the tracts are cut, the axons degenerate and a temporary deficiency ensues. However, proximal portions of the neurons regain ability to synthesize the hormone and to liberate it into the bloodstream. Limited ability to store and to selectively release the hormone develop, but fine control and ability to adjust to acute disturbances of water and electrolyte balance may be defective.

If the neural lobe is excised, a similar deficiency syndrome develops. In time, a "miniature neural lobe" forms, and much of the function is regained.

For reasons which are only partially understood, severe diabetes insipidus does not develop if the entire pituitary gland (including the adenohypophysis) is removed and supraoptic nuclei are damaged. Deficiencies of adenohypophysial hormones partially "compensate" for ADH loss.

In ADH deficiency, *very large volumes of dilute, hypotonic urine* are excreted (polyuria), and *compelling thirst* leads to the ingestion of very large volumes of water (polydipsia). If the fluid supply is ade-

quate, the condition is compatible with survival but certainly not with convenience. The human totally deficient in ADH may excrete more than 20 liters of urine per day and will have to drink enough to replace the fluid loss. The serious disturbances of sleep and of most activities can be appreciated when one recognizes that the presence of 150 ml of urine in the bladder is sufficient to evoke the desire to void, and that the usual beverage cup has a capacity of about 180 ml.

Water deprivation can reduce the urine volume, but the ensuing thirst is severe. If water deprivation is prolonged, inability to concentrate urine soon leads to severe dehydration and hemoconcentration. The symptoms include elevation of body temperature and disturbances in mental function. Ultimately, cardiovascular collapse can terminate in death.

Effects of too much ADH can be predicted from the deficiency syndrome. Urine volume is sharply reduced, and water retention leads to hemodilution, increased blood volume, elevated blood pressure, and tissue edema.

The most important actions of ADH are exerted on the kidney. However, it is possible to demonstrate that ADH affects fluid and electrolyte exchanges in other tissues including skeletal muscle.

RENAL MECHANISMS FOR CONCENTRATION OF URINE

Cortical nephrons of the mammalian kidney have glomeruli and convoluted portions of the proximal and distal tubules located within the *outer two-thirds* of the renal cortex. Straight parts of the tubules extend for varying distances toward the renal medulla; the *Henle loops* are relatively *short* and may not include a thin segment.

By contrast, the *juxtamedullary* nephrons are located within the *inner part* of the renal cortex adjacent to the medulla. The straight parts of the tubules are *long* and the thin segments well developed. Collecting ducts for both types extend to the inner medulla.

The ability to concentrate urine is related to the *number and length* of the *long Henle loops*.⁸ Some desert mammals have

only juxtamedullary type nephrons.³ Humans with moderate concentrating ability have relatively few (approximately 14%); much of the renal tubular blood flow is sent through them during times of water deprivation, while shunting of blood through the cortical nephrons when fluid intake is high favors water excretion.

Human kidneys produce approximately 120 ml of glomerular filtrate each minute. Solute and water reabsorption in the proximal tubules removes about 65–80% of the volume. (If no additional water were removed, the urine volume could exceed 30 liters per day.)

The uppermost parts of the Henle loops are surrounded by interstitial fluid that is isotonic with the entering filtrate (and with the bulk of the blood plasma). As the loops descend deep into the medulla, they come in contact with progressively more concentrated fluids.

Water is osmotically drawn out of filtrate passing down the *descending limb*; the filtrate therefore becomes concentrated and reduced in volume (Fig. 12-2B). Passive entry of sodium and chloride (Fig. 12-2C) adds to the concentration.

The ascending limb is relatively *impermeable* to water. Sodium is actively pumped out of filtrate passing up the limb (and chloride is passively extruded). The volume of the filtrate changes little, but the fluid gradually becomes more dilute and is actually *hypotonic* when it exits toward the distal tubule of the nephron.

The net effect of passage through the Henle loop is therefore loss of both water and sodium chloride (with proportionately more of the latter). Something like 15% reduction of volume is usual. If no additional water were reabsorbed, human urine volume could exceed 15 liters per day.

Small net exchanges of water can occur in the distal tubule (the major site of aldosterone regulation); ADH exerts minor influences^{ab} here but major influences at the collecting tubules and ducts.

The collecting ducts descend deep into the renal medulla and are surrounded by the same fluids as those which bathe the Henle loops. ADH increases the water permeability of the collecting ducts. Under its influence, large amounts of water are osmotically drawn from the future urine and returned ultimately to the blood. Usu-

ally the volume leaving the ducts to enter the bladder is equivalent to about 1% of the original filtrate. In diabetes insipidus, very little water is reabsorbed from the (impermeable) collecting ducts, and other disturbances of fluid and electrolyte balance can further disrupt renal functions.

MECHANISM OF ACTION OF ADH ON WATER TRANSPORT

Frog and toad skin and urinary bladder respond to ADH in much the same manner as mammalian kidney. Since they are less difficult to study than mammalian kidney, they have been widely used for ADH research.

While much valuable information has been gained, it should be noted that amphibians do not secrete ADH. A related neurohypophysial peptide, *arginine vasotocin*, influences water and electrolyte metabolism in these and most other non-mammalian vertebrates; it is described later in the chapter.

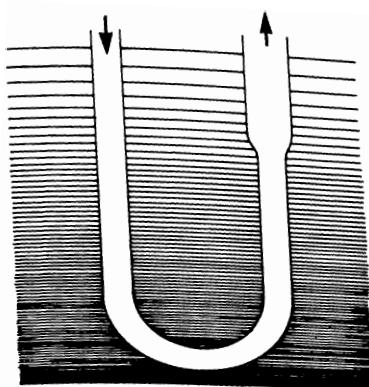
ADH increases water permeability in distal tubules and collecting ducts of the mammalian kidney and exerts similar actions on amphibian skin and bladder epithelium. Permeability to urea is increased in some tissues but not in others.

All available evidence is consistent with an action on *apical* membranes (those in contact with tubular or bladder urine); but the hormone is effective only when applied to the *basal* surface (on the side of the interstitial fluids and blood capillaries). Basal (serosal) surfaces have high water permeability in the absence of ADH.

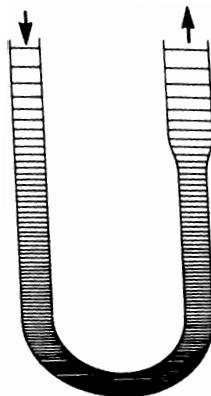
ADH combines specifically with a receptor on the basal surface. This is soon followed by activation of adenylate cyclase. Evidence for cAMP mediation of ADH actions is strong, and cAMP-dependent protein kinases have been identified in frog urinary bladder and in rabbit and dog kidney.^{ab} However, information on the mechanism of action of cAMP is fragmentary. Generation of cAMP near the basal surface may lead to later phosphorylation of an apical membrane component involved in regulation of water permeability (possibly through intermediate formation of an additional "messenger"). cAMP influences on microtubules leading to translocation of granules which affect properties of

ANTIDIURETIC HORMONE

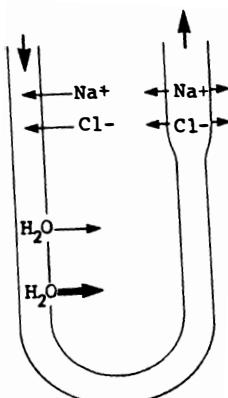
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A. Sodium Chloride Concentrations of
Interstitial Fluid Surrounding Henle Loop.



B. Osmotic Changes in Tubular Fluids Passing
through the Henle Loop.



C. Movements of Water and Ions Contributing to B.

FIG. 12-2. Water and electrolyte exchanges in Henle loops.

the apical membrane may enhance water transport.¹²

Comparisons of influences of ADH on movements of water with effects on solutes support the concept that these substances must pass two barriers before entering the cells. The outer is highly permeable to water but less so to solutes, and is unaffected by ADH. The inner membrane is described as porous. In some way, ADH increases the effective size of the "pores." Some investigators believe that existing pores are enlarged or modified, but most favor the concept that new channels are opened under the influence of the hormone.

Water that has entered the cells may exit through basal membranes or through intercellular passageways. (There is electron microscope evidence for intercellular channels.¹³)

Some of the now discarded concepts of the mechanism of action of ADH are of theoretical and historical interest. It was once suggested that sulfur atoms of the hormone attach to sulphydryl acceptors on the apical membrane and thereby induce conformation changes affecting permeability. Inhibitors of sulphydryl binding were shown to abolish effects of ADH. The concept fails to explain why ADH must be applied to the basal surface. There is no evidence that ADH actually enters the cells; moreover, interpretations of mechanism of action of the sulphydryl inhibitors have been revised.

Another idea was that ADH activates hyaluronidase and thereby promotes destruction of mucopolysaccharide cement substances surrounding collecting duct cells.

In addition to direct influences on collecting duct permeability, ADH may act on blood vessels of the renal medulla to slow blood flow and thereby enhance ability to maintain sodium chloride gradients.

Neurophysins are secreted along with ADH. Proposed functions include participation in synthesis, packaging, storage and release of the hormone, and protection against metabolic degradation. Suggestions that these proteins are essential carriers of ADH in the blood plasma, that they facilitate binding of the hormone to target tissues, or that they synergize with hormone actions, have not been supported by experimental data.⁷

ADH AND SODIUM TRANSPORT

Neurohypophyseal hormones promote sodium transport in nonmammalian vertebrate tissues. While ADH can stimulate sodium transport in mammalian kidney,

the physiological significance of the finding remains equivocal. The hormone seems to favor passive sodium entry by affecting apical membrane permeability, but the possibility that it affects affinity of a sodium carrier for the ion or that it activates "sodium pumps" has not been ruled out. The actions are separable from influences on water transport.

While cAMP has been implicated in both processes, cGMP affects sodium transport only. High concentrations of either calcium or manganese at the serosal surface inhibit ADH actions on water but not on sodium transport. The ions do not impair responses to exogenous cAMP. The data are consistent with the existence of two kinds of receptors. Calcium and magnesium could be interfering with either binding of the hormone to one type of receptor or with the coupling of hormone-receptor interaction to mechanisms leading to generation of cAMP.

COMPARISONS OF ACTIONS OF ADH WITH THOSE OF ALDOSTERONE ON SODIUM TRANSPORT

Actions of ADH on sodium transport seem to be very different from those of aldosterone. When the two hormones are presented simultaneously, effects are additive; moreover, responses to ADH can be enhanced by pretreatment with aldosterone.

Aldosterone effects are not manifested before completion of a prolonged latent period, and actions can be blocked by prior administration of puromycin or actinomycin D. By contrast, ADH effects are measurable within minutes and are not blocked by inhibitors of protein or nucleic acid synthesis. If a specific protein is involved in mediation of ADH actions, it may arise from activation of an existing precursor.

Substrate requirements for the two hormones differ. ADH continues to be effective in the presence of inhibitors of oxidative phosphorylation which completely abolish aldosterone stimulation of sodium transport. (But substrate utilization is required for all active transport of sodium.)

REGULATION OF ADH SECRETION

Water deprivation provides a potent stimulus for ADH synthesis and release. It reduces the extracellular fluid volume and

can, if severe, elevate the sodium ion concentration and osmotic pressure of the blood plasma.

The existence of hypothalamic osmoreceptors is established, but there are questions about the number of different kinds and about the nature of the stimuli to which they respond.^{2b}*

Elevation of the osmotic pressure of the blood supplying the supraoptic region of the hypothalamus (by addition of sodium chloride or glucose to carotid artery blood) leads promptly to ADH release even when total blood volume is maintained within normal limits. Some (or all) of the ADH-secreting cells may be directly sensitive to changes in the sodium ion content of the blood, and additional cells within the supraoptic nuclei and perinuclear zones which synapse with them may function as osmoreceptors. There is also evidence for the existence of "sodium sensors" within the third ventricle that respond to changes in composition of the cerebrospinal fluid.

At least some of the osmoreceptors may be activated by localized dehydration resulting from loss of intracellular fluid to hypertonic environments. The concept arises from observations that hypertonic solutions of urea do not constitute an effective stimulus in certain kinds of experiments. (Urea is known to penetrate into many cell types.)

Interpretation of studies involving elevation of the urea content of cerebrospinal fluid is controversial. While low concentrations of urea do not appear to cross the "blood-brain barrier," high concentrations may injure the barrier and thereby affect membrane properties.

Some "nonspecific" cells of the hypothalamus involved in ADH release seem to respond to a variety of noxious stimuli (including those of an osmotic nature)* while others appear to be more directly concerned with responses to changes in plasma sodium content.

Osmoreceptors within the olfactory bulbs may supplement functions of the supraoptic sensors. The olfactory bulbs do not play a dominant role in regulation of water balance, but they may function importantly in mediation of nonosmotic stimuli affecting ADH release.

ADH decreases the secretion of renin. The resulting reduction in stimulation of cells of the zona glomerulosa could contrib-

ute to reestablishment of normal sodium ion concentrations.

There are conditions (e.g., hemorrhage, fasting, and heavy sweating) which deplete both salt and water. Under such circumstances, blood volume may be reduced without elevation of osmotic pressure. The need for water conservation would not then be sensed by osmoreceptors.

It is widely believed that *volume or pressure receptors* located within the vascular system, and especially within the left atrium, relay messages via the vagal nerves to the supraoptic nuclei. While an ADH response to blood volume depletion can be readily demonstrated, the location of the receptors has been questioned.*

Reduction of blood volume and of blood pressure trigger the release of renin (Chapter 11). Actions of renin (via angiotensin) on blood vessels, kidney, adrenal cortex, and drinking behavior contribute to reestablishment of fluid balance and maintenance of blood pressure. Effects of baroreceptors on renin release are evidently far more effective than are inhibitory influences exerted by ADH.

The secretion of ADH is increased following exposure to hot environmental temperatures, and decreased following cold exposure. The responses contribute to body temperature-regulating mechanisms, since increased blood volume resulting from water conservation by the kidney supports vasodilation and sweating or panting and therefore heat loss, while decreased blood volume following cold exposure helps to mitigate blood pressure-elevating effects of peripheral vasoconstriction. (Attempts to demonstrate a direct influence of ADH on sweat glands have yielded negative data.) Body temperature influences on ADH secretion may be mediated via direct neuronal pathways arising in thermoregulatory parts of the hypothalamus, but the full significance of species differences in temperature-regulating mechanisms will have to be clarified before a full understanding is reached concerning specific neuronal pathways.

It has been pointed out that those forms with relatively large areas of exposed skin (humans, laboratory primates, rabbits) depend on cutaneous receptors for awareness of cold environments, while responses to heat result from

stimulation of temperature-sensitive neurons in the preoptic region of the hypothalamus. In heavily furred animals, on the other hand, cutaneous receptors cannot play an important role in temperature perception. When such animals are exposed to hot environments, the preoptic region receives blood which has been cooled by passage through structures involved in panting. This could explain why cooling the preoptic region increases ADH release in the monkey but reduces it in the goat.

Muscular exercise usually increases ADH secretion. This can be useful for maintaining blood pressure when skeletal muscle vessels are dilated and there is simultaneous need for heat dissipation. *Postural changes* are also important stimulants in humans and other species which assume upright positions. Effects are apparently mediated via volume receptors; increased blood volume contributes to vascular adjustments to the pull of gravity.

ADH release is triggered by a wide variety of *noxious* stimuli, loud noises, pain, strong emotions, and pharmacological agents acting on the central nervous system (nicotine, caffeine, barbiturates, cholinergics, and also morphine in some species). Failure to appreciate influences of anesthetics led to misinterpretations of ADH physiology in some of the early work.

Ethyl alcohol usually suppresses ADH release; perhaps the resulting polyuria and subsequent thirst facilitate excretion of alcohol and its metabolites. (A few individuals increase ADH secretion and experience discomfort from water retention.)

While no specific hormone controls ADH synthesis and release, many hormones affect responses of ADH cells to other stimuli.

Angiotensin II enhancement has been described, but there are seemingly contradictory findings. *Glucocorticoids* protect against excess ADH probably through influences on glomerular filtration rate and exchange of water between cells and extracellular fluids. Hydrocortisone seems to elevate the threshold for osmotic stimulation of ADH release without affecting responses to other stimuli, but early suggestions that it is a major regulator have not been substantiated. Glucocorticoids do not directly antagonize ADH influences on the kidney. (An old concept that ADH increases, while glucocorticoids decrease hyaluronidase activity in the kidney and thereby exert antagonistic influences

on permeability of the collecting duct cells, has been discarded.)

On the other hand, adrenalectomized animals have abnormal rates of ADH secretion during water loading, and the mechanism has not been elucidated.

Mineralocorticoids may exert a "permissive role" in support of ADH influences on water and sodium transport. (An inhibitory influence on phosphodiesterase has been proposed.)

Influences of *epinephrine* have been described, and epinephrine has been implicated in the effects of exercise, anxiety, and anoxia. However, interpretation of epinephrine studies requires careful evaluation of effects of the catecholamines on the vascular system (and especially in the kidney) and on the rate of their penetration into structures involved in mediation of the responses.

Adrenergic agents have been reported to antagonize responses of kidney and toad bladder to ADH (but not to cAMP) without influencing baseline permeability. The inhibitory effects are abolished by administration of α (but not of β) adrenergic blocking agents. Interestingly, seasonal variations in responses to isoproterenol were found.

Prostaglandins have been suspected of influencing sodium and water excretion by mechanisms involving, and others independent of interrelationships with ADH.

Cells of the supraoptic nucleus accumulate estrogen, and histological changes in the cells following castration have been described which are consistent with increased activity levels. Thresholds for the usual stimuli seem to be altered by hormonal changes associated with estrous and menstrual cycles and pregnancy. ADH release may also be triggered by stimuli affecting secretion of *oxytocin*, and a transient rise in blood ADH levels has been found after coitus.

Inappropriately high rates of ADH secretion after water loading in hypothyroid animals have been attributed to loss of *thyroid hormone* influences on osmoreceptor function.

Neurons promoting ADH release seem to be predominantly cholinergic, while adrenergic stimuli are mostly inhibitory. (But some cholinergic inhibitory fibers have been described.)

Some factors affecting ADH secretion are shown in Figure 12-3.

OTHER ACTIONS OF ADH

High concentrations of ADH affect a wide variety of tissues, and most influences seem to be mediated via activation of adenylate cyclases. While some actions are purely *pharmacological*, others may con-

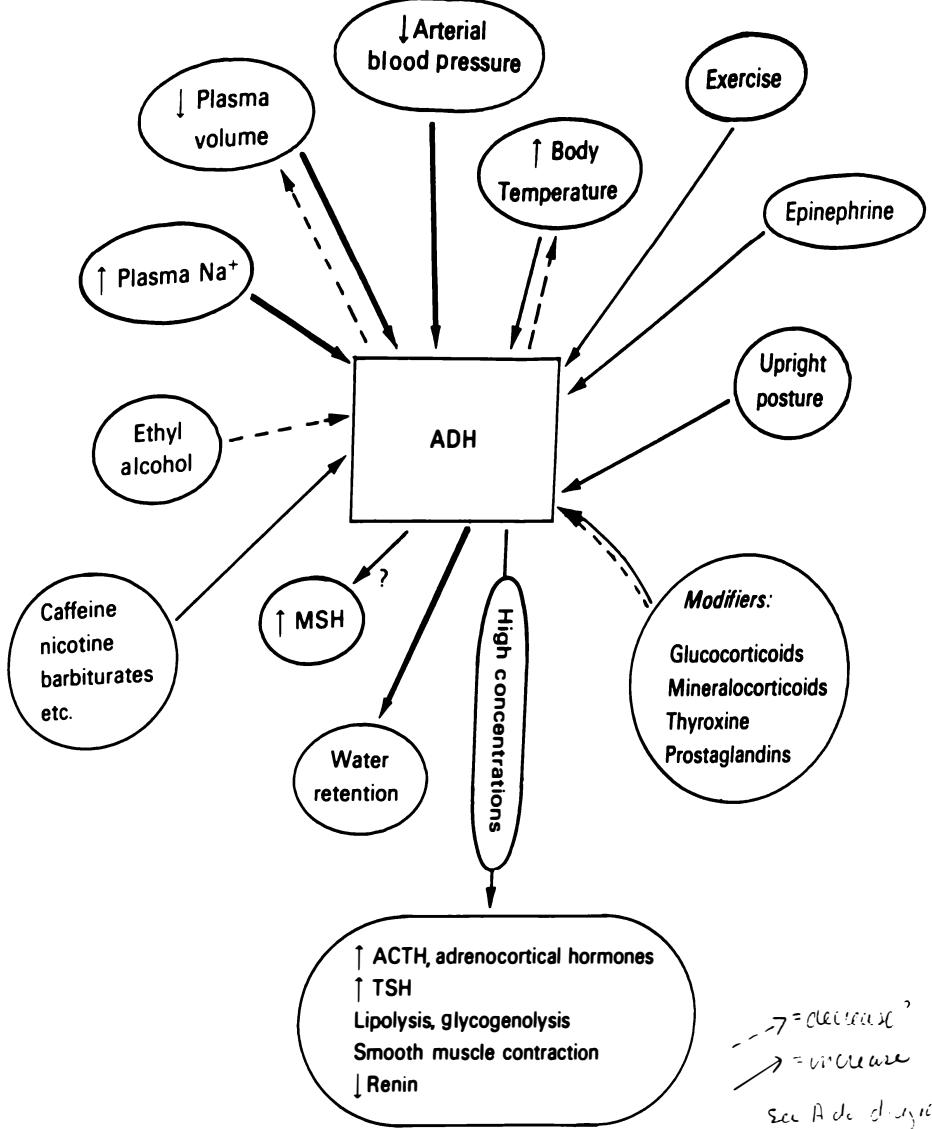


FIG. 12-3. ADH secretion.

tribute to responses to stress. (Interactions with the corticotrophin-releasing factor-adrenocorticotrophic hormone-glucocorticoid system were described in Section II.)

Effects of large doses include release of TSH and other pituitary hormones, elevation of plasma glucose concentrations (through activation of hepatic phosphorylase), and stimulation of lipolysis in adipose tissue. In skeletal muscle, water content is increased while electrolyte content is decreased. Pharmacological doses stimulate smooth muscle of the gastrointestinal tract, uterus, and blood vessels. Blood pressure is elevated in this way through synergism

with other vasoconstrictors and because of the augmented blood volume.

Some ADH-secreting neurons terminate in the vicinity of the pars intermedia; the latter has been implicated in regulation of water and electrolyte balance (Chapters 13 and 23).

Actions on the reproductive system have not been studied in depth. Changes in plasma concentrations of the hormone associated with the menstrual cycle have been described. ADH secretion is increased during the last few days of pregnancy. This could be related to nonspecific stimulation associated with release of oxytocin;

↑ p89

↓ = decrease
↑ = increase
see A de chrysanthemum
Ch:

however, ADH may synergize with oxytocin during labor. It has been implicated by some in precipitation of unwanted abortion, and administration of ethyl alcohol (which suppresses ADH release) has proven helpful in many cases.

SOME COMPARATIVE ENDOCRINOLOGY OF WATER AND ELECTROLYTE METABOLISM REGULATION^{1, 4, 6}

Mechanisms utilized by different kinds of animals for regulation of water and electrolyte metabolism (and especially by animals with special problems of adaptation) provide valuable information on the roles and limitations of hormones.

Terrestrial Mammals

Water conservation can be a major problem when the supply is intermittently available. Storage capacity is limited while tolerance to dehydration is low. There is continuous loss of water through the respiratory system and structures involved in maintaining body temperature. The problems are exaggerated for animals exposed to the high temperatures and low humidity of desert habitats. Considerable quantities of water are also needed for functions of the gastrointestinal tract and kidney.

The role of ADH in water conservation has been described. Over a wide range, a direct relationship can be demonstrated between the percentage of tubular water reabsorbed by the mammalian kidney and the quantity of ADH secreted.

The laboratory rat exhibits good resistance to short term water deprivation. It can increase the ADH content of the neurohypophysis 10-fold over usual values after a 24-hr period without water, and can excrete urine 9 times as concentrated as its blood plasma.

The kangaroo rat (a desert rodent which can survive without *any* drinking water) normally has ADH levels higher than those of the dehydrated laboratory rat, can store 6 times as much ADH, and is capable of producing urine 14 times as concentrated as the blood plasma.

It should be noted, however, that the ability to concentrate urine is limited, *not* by the amount of ADH, but rather by the structure of the kidney. Desert animals have well developed Henle loops and col-

lecting ducts which permit them to respond maximally to hormones. The laboratory rat cannot achieve the urine-concentrating ability of the desert rodent no matter how much ADH is injected.

It is often stated that animals such as the kangaroo rat and the gerbil can survive without drinking because they obtain water from the metabolism of fats and carbohydrates. However, for most animals living in arid climates, the uptake of oxygen and loss of carbon dioxide needed to sustain the metabolism requires evaporative loss through the respiratory tract of a quantity of water equal in magnitude to that gained from the food utilized.⁶ (And increases in water production are directly associated with accelerated heat production.)

Desert animals have specially constructed respiratory passages which provide for cooling of expired air so that water loss is minimized. Much of the moisture condenses on walls of the nasal passages. Very long airways could cool expired air to ambient temperatures (through countercurrent exchange), but further cooling of nasal mucosa is achieved when inhalation of very dry air promotes evaporative heat loss. (Humans with short nasal passages expire air which is almost at body temperature and laden with water vapor.)

Some desert animals have hemoglobins with especially high affinity for oxygen, and this reduces the quantity of inhaled air needed to sustain metabolism. (This is not the case for the kangaroo rat.) A variety of other water-conserving mechanisms are seen among the mammals including ability to excrete very dry feces. Many do not have sweat glands but depend upon behavioral adaptations for maintenance of body temperature. Nocturnal animals can live in moist, shaded burrows during the daylight hours, and some reduce their metabolic rate during the hottest part of the day when they are inactive.

Animals with well developed urine concentrating ability secrete large quantities of *aldosterone*. The steroid enhances ability to conserve water by promoting retention of the sodium chloride that is needed for maintaining steep osmotic gradients in the renal medulla, and by augmenting effects of ADH on sodium transport. Water loss is thereby minimized.

Some animals cool their bodies by spreading saliva (which evaporates) over the fur, and others depend upon sweating. Aldosterone minimizes sodium loss through sweat and saliva and can promote sufficient potassium loss to permit withdrawal of small amounts of intracellular water when extracellular volume is reduced. It also acts on the colon to reduce sodium and water loss in the feces.

Most animals can adjust aldosterone secretion rates over a wide range. A 13-fold rise has been described in sheep responding to salt depletion. Kangaroo rats and other desert animals are known to have high secretion rates.

The dromedary camel is truly a remarkable beast, capable of survival for up to 20 days without drinking while in the desert. Special adaptations include ability to tolerate fluid losses equivalent to 30% of its body weight (compared with a maximum of 12% for humans). Special properties of red blood cell membranes confer high resistance to elevation of the osmotic pressure of the blood. The camel can function well with a body temperature as low as 34°C at night and as high as 41°C in the heat of the afternoon. This permits substantial reduction of the amount of water used by sweat glands. Location of the sweat glands on the undersurface of the body confers a further advantage, while high temperatures can be tolerated over surfaces exposed to the sun.

Most animals succumb to heat because the brain cannot withstand elevation of blood temperatures much above 40°C. But the camel sends its blood through respiration-cooled areas before returning it to the brain, while less sensitive regions are exposed to warmer blood. The camel can lessen heat production by reducing its metabolic rate. When adequate water is available, camels utilize sweating at a maximal rate of 260 g of water per square meter per hour (compared with the relatively heat-resistant merino sheep's maximum of 32 g per m² per hr). Perhaps at least as important as any of the preceding, the camel has a truly amazing ability to ingest and store water. Severely dehydrated animals have been observed to ingest 100 liters within minutes!

The camel utilizes ADH and aldosterone, and these aid in production of highly

concentrated urine and feces, and of sweat low in sodium. The hormones are, of course, essential for survival. But without the described anatomic adaptations and regulatory mechanisms, hormonal influences would be inadequate to sustain life.

Kangaroos and other marsupials utilize some different mechanisms for resistance to desert conditions. Body temperature is regulated at a lower level, and there is some tolerance to elevations above the normal 35°C during hot afternoons. Some are nocturnal. Steroid and neurohypophyseal hormones are utilized as in placental mammals.

It was noted above that the antidiuretic hormone of most mammals is *arginine vasopressin* (Fig. 12-4A), while a few species, including semiaquatic members of the pig family, produce *lysine vasopressin* (Fig. 12-4B). Since the quantities of neurohypophyseal peptides synthesized and released can be varied over a wide range, the fact that the lysine form of the hormone is in some ways only two-thirds as potent as arginine vasopressin in humans and rats (and much less in the dog) does not seem to be important. No physiological significance (other than differences in receptor structure) has as yet been attached to the species differences, but the possibility that future research will reveal *new functions* of ADH for which the lysine moiety confers special advantage cannot be ruled out.

Birds

Birds that fly have special water and salt balance problems. Limitations on body weight restrict amounts of reserve water that can be carried, while high metabolic rates associated with activity of flight muscles increase the need for heat dissipation. Those engaging in long flights must forego drinking for many hours. On the ground, other kinds of problems arise from the need to produce eggs containing sufficient fluids and electrolytes to nourish the embryos.

Most birds maintain body temperatures 3–4° above those of placental mammals. Ability to withstand higher temperatures reduces use of water for cooling and increases the rate of heat loss to the environment at most ambient temperatures; however, the associated high respiratory rate tends to increase loss of water, especially

HORMONAL REGULATION OF BODY FLUIDS

	1	2	3	4	5	6	7	8	
A. Arginine vasopressin	Cys	Tyr	Phe	Gln	Asn	Cys	Pro	Arg	Gly-NH ₂
B. Lysine vasopressin	Cys	Tyr	Phe	Gln	Asn	Cys	Pro	Lys	Gly-NH ₂
C. Arginine vasotocin	Cys	Tyr	Ile	Gln	Asn	Cys	Pro	Arg	Gly-NH ₂
D. Oxytocin	Cys	Tyr	Ile	Gln	Asn	Cys	Pro	Leu	Gly-NH ₂
E. Mesotocin	Cys	Tyr	Ile	Gln	Asn	Cys	Pro	Ile	Gly-NH ₂
F. Ichthyotocin (Isotocin)	Cys	Tyr	Ile	Ser	Asn	Cys	Pro	Ile	Gly-NH ₂
G. Glumitocin	Cys	Tyr	Ile	Ser	Asn	Cys	Pro	Gln	Gly-NH ₂

FIG. 12-4. Neurohypophysial peptides. Circled amino acids indicate those different from amino acids in arginine vasopressin.

since birds do not have the anatomical specializations of the nasal tract described above for desert rodents. (Birds do, however, have anatomical arrangements which provide for a high rate of panting without production of respiratory alkalosis, for a high rate of oxygen extraction from inspired air, and also for countercurrent heat exchange with environments that are cooler than body temperature.)

The mobility of birds enables them to obtain drinking water from distant sources. Some can rapidly ingest very large quantities of water, and it has been suggested that this confers protection in regions where predators endanger the process of leisurely drinking. Many species can utilize seawater.

In common with mammals, birds have kidneys with Henle loops, and most are able to concentrate urine. They secrete *arginine vasotocin* (Fig. 12-4C), a peptide produced by most vertebrates and recently identified in mammalian embryos and in pineal glands and other structures of mammals.

Arginine vasotocin is less potent than vasopressin for promoting water uptake by distal tubules and collecting ducts, but high concentrations of vasotocin reduce urinary water loss via influences on glomerular filtration rate.

Vasotocin can reduce filtration rate through alterations in renal blood flow (which affect

the number of glomeruli active at any given moment) and through reductions in systemic blood pressure. There are questions concerning whether birds actually secrete sufficient vasotocin to do this, or whether the presence of vasotocin is more realistically related to differences in the nature of hormone receptors in birds as compared with mammals.

Nitrogenous wastes are largely converted to *uric acid* which is poorly soluble in water (and therefore exerts little influence on osmotic pressure). Uric acid excretion in the form of a concentrated moist paste requires only a fraction of the water loss required for removal of urea. Both urine and feces are delivered into a *cloaca*; there is no urinary bladder. Salt and water reabsorption take place in the cloaca and probably also in the lower part of the intestine.

Birds secrete *aldosterone* which acts on the kidney but also exerts other actions in those species that drink seawater or ingest diets containing more sodium that can be eliminated via the urine and feces. *Nasal salt glands* put out a sodium-rich product so concentrated that it can blow off the feathers as a powder. Salt glands are cholinergically innervated in most species but require aldosterone to function. Unlike sodium sensors for ADH secretion, the salt glands respond to a wide variety of osmotic stimuli and undergo hypertrophy when birds ingest hypertonic solutions for long

periods of time. In contrast with sodium-retaining influences of aldosterone on mammalian kidney, sweat and salivary glands, the steroid promotes *sodium removal* by the salt glands.

Oxytocin (Fig. 12-4D) secreted by the avian neurohypophysis may participate in regulation of salt and water metabolism especially during egg-laying periods. (Effects of oxytocin on salt and water metabolism of mammals have been demonstrated, but the high doses required raise the possibility that the actions are purely pharmacological. Reproductive functions of oxytocin are described in Section VI.)

Reptiles

Varying degrees of poikilothermy are encountered among the reptilian forms. Lower metabolic rates and the associated lower body temperatures reduced the need for water. Evaporative loss is further reduced by the presence of impermeable skin coated with insulating materials, but water loss through the respiratory tract (and through the skin of some species) may be substantial when environmental temperatures are high.

Behavioral adaptations (warming in the sun, digging into cool burrows, changes in posture affecting the amount of body surface exposed to the environment) play a major role in regulation of body temperature and water balance. (It should be noted that this imposes limitations on activity.)

Most reptiles can rapidly ingest large quantities of water when it is available and store it for later use. In some, the urinary bladder serves as a reservoir from which reabsorption can be controlled. Succulent foods (both plant and animal) are ingested, and some species effectively utilize brackish or seawater.

Reptiles do not secrete vasopressin, nor do they have kidneys capable of producing hypertonic urine. They can, however, reduce the water content of the urine to isotonicity with the plasma by increasing reabsorption through the tubules. Probably more important, they can sharply curtail urine production by shutting down the number of active glomeruli. (Certain reptiles go for many days without excreting measurable quantities of urine.) This is made possible by high tolerance to wide fluctuations in osmotic pressure of the

blood and by the production of uric acid rather than urea. A few can even vary the relative quantities of urea and uric acid in accordance with changes in hydration states.

Many possess salt-secreting glands in the nose (nasal glands) and eye (Harderian, supraorbital, "tear" glands). Ability to effect color changes in the skin may afford additional protection against heat uptake from the environment. (Perhaps it is worth speculating on interactions between color changes and electrolyte metabolism since certain hormones seem to control both functions.)

Reptiles secrete arginine vasotocin which influences renal function. Large doses of the hormone depress systemic blood pressure; this could contribute to antidiuresis if the quantities required are, in fact, secreted. Unlike the situation in amphibians (see below), vasotocin does not seem to affect water uptake from the urinary bladder. *Mesotocin* (Fig. 12-4E) is also secreted by many reptilian forms. Little is known of its functions, but it has been implicated in direct stimulation of sodium transport.

Aldosterone and deoxycorticosterone (DOC) have been identified and have been implicated in regulation of water and salt reabsorption by kidney tubules, cloaca, and urinary bladder. Effects on salt glands are inconsistent in this class of vertebrates; in some it promotes sodium excretion while in others it favors sodium retention and potassium excretion.

Prolactin is essential for osmoregulation in certain fishes, and indications of influences of this hormone on mammalian regulation of water and electrolyte metabolism are accumulating (Chapter 13). A role in reptiles is suggested by the observation that defects in sodium metabolism of hypophysectomized lizards can be corrected by administration of this, but not of other hormones.⁴

Amphibians

Most amphibians live in or near fresh water and have low electrolyte concentrations of the blood plasma. (A 0.7% sodium chloride solution is isotonic with plasma of the grass frog, whereas 0.85–0.90% solutions are used with mammalian cells.) The highly permeable skin (which supplements respiratory functions of poorly developed lungs)

permits uptake of materials from the aqueous environment.

Excess water can be eliminated by excretion of copious quantities of hypotonic urine. (The 10-g alpine newt has been reported to put out 16 ml per day.¹) Ability to excrete water is enhanced by production of nitrogenous wastes in the form of urea and ammonium salts. Active inward transport of ions through the skin, urinary bladder, and gastrointestinal tract aids in maintenance of electrolyte balance.

When amphibians leave the aqueous environment, the urinary bladder serves as a reservoir for fluids and salts. A few terrestrial forms can resist loss of up to 50% of their body water content. (Some have become adapted to brackish water and even to marine environments, but these are the exceptions.)

Arginine vasotocin seems to be most important for survival of urodeles when they leave the water. It promotes sodium and water uptake through the skin and urinary bladder and probably also through the gastrointestinal tract, and reduces urine volume through influences on both tubular reabsorption and renal blood flow. It may also participate in regulation of blood pressure. (Effects have not been found in the purely aquatic mud puppy, and no need for such actions would seem to be indicated.)

Mesotocin and oxytocin have been found in a number of amphibians. The functions have not been defined and may vary with the species. In some they promote diuresis by increasing glomerular filtration.

Aldosterone, corticosterone, and related steroids exert important influences on sodium and water uptake by skin, gut, and urinary bladder and are probably needed to support the actions of the neurohypophyseal peptides. Other hormones implicated in water and electrolyte metabolism include *adrenalin*, reported to increase osmotic permeability of toad skin (with possible facilitation of vasotocin activity) and to stimulate chloride excretion by action on skin glands of frogs. (Adrenalin also influences movement of pigment granules in some amphibians.)

Prolactin has been implicated in many different ways.¹¹ It affects sodium and water transport across toad bladder and restores plasma sodium levels in hypophysectomized newts. It makes newts seek out

water ("water drive") during the breeding season, and this is associated with influences on the skin. It promotes proliferation of melanophores in some amphibians and seems to play a role in release of thyroid-stimulating hormone. *Thyroid hormones* have also been implicated in regulation of amphibian salt and water balance. They antagonize actions of prolactin on "water drive" and also on metamorphosis.

Fishes

The fishes are a very diverse group of animals, and discussion of water and electrolyte balance in these animals would occupy more space than seems appropriate in this text; but a few observations are certainly worth mentioning.

A most obvious difference between fishes and other vertebrates is the presence of gills with surfaces many times greater than those of the skin, across which most of the electrolyte and water exchange takes place. (Amphibian larvae and some neotenous amphibians also have gills; such forms are fishlike in many ways.)

Steroid hormones are known to affect chloride-secreting cells of the gills and to stimulate electrolyte transport across gastrointestinal epithelia; they do not seem to act on electrolyte transport in the fish kidney.

The hormone most directly implicated in ability of some euryhaline fishes to survive transfer to fresh water is *paralactin*, an adenohypophyseal protein closely related to the prolactin of "higher" vertebrates. Animals deprived of the hormone (through hypophysectomy) succumb to sodium loss but can be protected by administration of mammalian prolactin as well as by preparations derived from fish pituitary glands. An influence of the hormone on structure and function of the mucous cells has been demonstrated, and separate influences on permeability and on ion pumps have been suggested. Changes in pituitary morphology have been directly related to changes in salt content of the environment.

Thyroxine protects some species against otherwise deleterious effects of transfer from fresh to salt water. Antagonism of prolactin effects in amphibians was mentioned above, and this probably represents another example of such interaction.

Fishes secrete arginine vasotocin and

some other related peptides (including isotocin and glumitocin, Figure 12-4, and also valitocin and aspartogtocin).¹³ It is difficult to see why fresh water fishes would need to limit urine output; no antidiuretic actions of the peptides have been demonstrated. Rather, these hormones have been reported to increase renal blood flow and glomerular filtration rate and to promote diuresis. Branchial blood flow and electrolyte exchange across the gills is stimulated in some species. It is suspected that the neurohypophyseal peptides also play a role in regulation of blood pressure.

A unique structure found in fish but not other vertebrates, the *urophysis*, seems to be importantly involved in osmoregulation.¹⁰ Little is known of secretory products or mechanism of action, in part because there is no way to completely remove the structure without severing the entire tail region.¹⁴ Changes in neurosecretory cells of this region have been described after alteration of the osmotic environment.

Urophysial extracts seem to contain a number of small peptides exhibiting stimulatory actions on smooth muscle of the gastrointestinal tract and urinary bladder of fish (urotensin), on contraction rates of caudal lymph heart (lymph heart-stimulating substance, LHSS), on glomerular filtration, and on urine production. Hypertensive action in fish and hypotensive action in mammals have also been observed. The *urophysis* is also known to be rich in acetylcholine.^{15, 16}

The *corpuscles of Stannius* are also found only in the fishes. A function in secretion, modification, or storage of steroid hormones is suspected, and these structures seem to participate in osmoregulation. Hypercalcemic and hypotensive factors have been identified.

Additional mechanisms employed by some of the fishes for adaptation to marine environments include accumulation of osmotically active substances such as urea or

trimethylamine oxide in the blood plasma. The hagfish (a cyclostome) is unique in that it does not seem to possess typical vertebrate osmoregulatory mechanisms.

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13. Additional Hormonal Influences on Water and Electrolyte Metabolism; Drinking Behavior and Salt Appetite

GLUCOCORTICOIDS AND WATER TRANSPORT

Glucocorticoids play a role in water transport which is distinct from the relatively minor mineralocorticoid actions exerted by physiological concentrations.

Administration of a large oral dose of water creates several problems: (1) prolonged distention of the gut creates mechanical pressure on neighboring structures, while sustained hypotonicity of the gut contents adversely affects the mucosa; (2) too rapid absorption dangerously augments circulating blood volume and thereby elevates blood pressure and taxes cardiac muscle, as marked reduction of osmotic pressure leads to destruction of red blood cells and clogging of renal tubules with the liberated hemoglobin (avoidance of such problems requires orderly transfer of some of the water to nonvascular extracellular spaces and into tissue fluids without production of edema); (3) the excess water must be excreted by the kidney; this requires suppression of antidiuretic hormone (ADH) secretion and regulated transfer of temporarily stored water back into the circulating blood.

Intact animals can readily solve the problems, but adrenalectomized animals pretreated with salt and glucose supplements cannot cope with the situations. It is obvious that administration of mineralocorticoids could aggravate rather than improve conditions created by water loading. But administration of glucocorticoids restores the ability of adrenalectomized animals to handle the excessive water.

A "water tolerance test" is sometimes (carefully) administered to patients suspected of suffering from glucocorticoid deficiency.

Mechanisms whereby glucocorticoids

promote orderly transfer of water across membranes are only partially understood. Direct effects of the steroids on cell membranes, "permissive" actions on blood vessels and on the heart, and an increase in glomerular filtration rate are involved. Glucocorticoids may also suppress excessive secretion of mineralocorticoids and modify responses to ADH.

Natriuretic Hormone^{1, 2, 6, 17}

Factors influencing the rate of renal sodium excretion include the volume and composition of the blood and extracellular fluids, systemic arterial and venous blood pressures, tone of afferent and efferent renal arterioles, hydrostatic pressure in the ureters and peritubular regions of the kidney, renal metabolic activities, functions of the renal nerves, and actions of a large number of hormones.

Some investigators believe that a thorough understanding of clearly established influences can explain variations in sodium excretion under all physiological and experimental conditions. However, others believe there is strong evidence for secretion of at least one as yet unidentified humoral agent which influences the disposition of sodium and especially the rate of sodium reabsorption from the proximal tubules and possibly also from the Henle loops and distal tubules.

The terms "natriuretic hormone" and "hormone X" have been used to designate the unknown factor, and the first term has also been applied to more specific substances identified in tissues and body fluids. "Third factor" is a name used by some to describe a hormone other than aldosterone or ADH, and by others to indicate a locus of action within the kidney.

different from those affected by recognized hormones.

The intact dog excretes copious quantities of sodium and water when it is infused with saline. This occurs even when the animal is given large doses of mineralocorticoids and ADH, and when measures are taken to prevent a rise in glomerular filtration rate. Blood plasma taken from such dogs has been reported to induce sodium diuresis in other animals not receiving the saline infusion, and to affect kidney preparations *in vitro*.

Constriction of the thoracic inferior vena cava blunts the saline diuresis in infused dogs; when the clamp is removed there is a prompt rise in sodium and water excretion. This has been interpreted to mean that natriuretic hormone is released in undisturbed but not in clamped animals.

Constriction of the vena cava also reversibly blocks the "escape" (Chapter 11) from prolonged action of mineralocorticoids. The diuresis which follows removal of the clamp has been attributed to release of natriuretic hormone.

Several sites of origin have been proposed for the hormone. These include the kidney, the liver, and the brain.

The isolated kidney exhibits autoregulation in response to changes in quantity, pressure, and composition of perfusion fluids, but plasma from saline-infused nephrectomized animals has been reported to contain the natriuretic principle.

The hepatic site was proposed because saline infusion directly into the hepatic portal vein seems to be more effective for induction of salt diuresis than does infusion into peripheral vessels. Moreover, natriuretic activity has been found in liver perfusates.

The possibility that natriuretic hormone is formed in the brain has received the most attention. In separate studies, decapitation and destruction of the paraventricular nuclei and of posterior regions of the hypothalamus have been reported to prevent appearance of the hormone in response to saline infusion.

On the basis of studies of partially purified preparations of blood taken from venous drainage of the brains of chloralose-treated, carotid artery-occluded cats, it has been proposed that natriuretic hormone is a polypeptide produced in the posterior hypothalamus which may have some structural similarity to (but is not identical with) known hypothalamic peptides. Other workers observed that infusion of sodium into the third ventricle of goats elicited increases in

sodium and potassium excretion, but that infusions of ammonium chloride depressed sodium excretion. They speculated on the existence of hypothalamic mechanisms for regulation of sodium excretion which might involve release of a slow-acting natriuretic hormone or inhibition of secretion of an antinatriuretic substance.⁶

Some investigators believe that the relatively mild sodium diuresis seen in animals given preparations purported to contain the natriuretic principle could be adequately explained on the basis of hemodynamic alterations too small to be detected by conventional methods, and that the studies provide no real evidence for secretion of a specific humoral factor. On the other hand, the small (and sometimes inconsistent) effects have been attributed by others to such problems as short half-life or chemical instability of the hormone, its dilution by the plasma of the recipient or partial loss via the urine, improper treatment of test animals, or absence in recipients of factors normally present in saline-infused animals which contribute to actions of natriuretic hormone.⁷

More recent studies of plasma concentrates and extracts obtained from saline-loaded dogs, rats, cows, humans, goats, and sheep, and newer assay methods seem to be providing stronger evidence for the existence of at least one additional humoral factor. Preparations have been injected into renal arteries of the cat, cross circulation studies have been performed on dogs and rats, dialysates have been directly applied to frog skin and urinary bladder, and *in vitro* studies have been performed on rabbit kidney slices and on fragments of rabbit and dog renal tubules.

PROSTAGLANDINS AND ELECTROLYTE EXCRETION^{2,4}

The renal medulla contains high concentrations of prostaglandins E₁ and A₂, (PGE₂ and PGA₂), smaller amounts of PGE₁, and some PGF₂. They are believed to be produced locally, and synthesis seems to be increased after salt loading.

A renal lipid with potent vasodilator activity (but without direct action on non-vascular smooth muscle), medulin has been identified with PGA₂. It has been implicated in physiological regulation of systemic blood pressure and redistribution of the blood. Intra-arterially administered prostaglandins of the A and E series increase regional blood flow in coronary, carotid, brachial, femoral, mesenteric, pulmonary, cutaneous, and renal beds. Effects of prostaglandins of the F series are

transient because these agents are destroyed during a single passage through the lungs, but compounds of the A series are resistant to lung enzymes.

Pharmacological studies have ruled out mediation via cholinergic, adrenergic, or histaminergic nerve endings. Large doses lead to reflex increases in cardiac output.

Exogenously administered prostaglandins exert profound influences on the kidney. Very small doses promote an increase in renal blood flow and shunt blood from the medulla to the cortex. Sodium, chloride, and water excretion are thereby increased with no influence on glomerular filtration rate. Such observations have led some investigators to suggest that medullin is identical with natriuretic hormone.

Opinion is divided on whether prostaglandin-induced redistribution of renal blood flow results primarily from dilation of cortical vessels or from constriction of those in the medulla. (Prostaglandins have not been found in the renal cortex, but enzymes for inactivation of these agents have been identified there.)

In acute experiments, intrarenal doses sufficient to markedly increase sodium excretion of intact animals may exert no discernible influence on systemic arterial blood pressure. However, larger amounts in the peripheral circulation can reduce blood pressure through direct action on arteriolar muscle, and prolonged action of smaller doses on the kidney indirectly affects blood pressure by reducing blood volume.

Pituitary gland prostaglandins may play an indirect role in regulation of water and electrolyte metabolism. PGE₁ has been implicated in hypothalamic mechanisms for release of prolactin in mammals.¹²

PROLACTIN AND ELECTROLYTE EXCRETION^{3, 8}

A role of prolactin (or a closely related hormone) in osmoregulation by some of the fishes and in promotion of amphibian "water drive" was described in the preceding chapter.

In addition to established roles in reproduction and lactation (which involve influences on water and electrolyte metabolism), prolactin may directly affect kidney function. Specific prolactin receptors have been identified in the kidney and adrenal

cortex, and injections of ovine prolactin have been reported to promote antidiuresis in rats and humans. Lactogenic preparations increase glomerular filtration rates and renal blood flow in mammalian heart-lung-kidney preparations; sodium excretion is at first transiently increased and later decreased.³

Administration of hypertonic solutions to human subjects leads to elevation of plasma prolactin levels, while hypotonic solutions inhibit prolactin release.⁹

Prolactin secretion is influenced by several hormones implicated in regulation of electrolyte and water metabolism, including those of estrous and menstrual cycles and thyroid hormones. Roles in osmoregulation and development of mammalian fetuses have been proposed. Clearly, further investigation is indicated.

THE THYMUS GLAND¹³⁻¹⁸

A seasonal variation in water, sodium, potassium, chloride, and phosphate excretion has been found in salt-loaded rats. Thymectomy was reported to decrease excretions when values for control animals were low, and to increase them at times of high output. Influences of thymus gland deprivation were exaggerated by adrenalectomy. The data are consistent with some kind of fine-control regulation of water and electrolyte excretion which does not depend upon the adrenal cortex. In unrelated studies, it was shown that the thymus gland synthesizes and may secrete a heparin-like mucopolysaccharide.

Influences of heparin injection on water and electrolyte excretion were described in Chapter 11. Administration of heparin to adrenalectomized-thymectomized rats can reverse the effects of thymectomy. The data are consistent with release of a heparin-like substance from the thymus gland of salt-loaded animals which participates in regulation of electrolyte metabolism.¹⁴ (But it has not been established that sufficient heparin is in fact released to elicit such influences.)

Thymus extracts from several species have been shown to contain one or more substances which reduce blood calcium levels. Indirect evidence for secretion of a calcium-lowering principle has been obtained from studies in which thymectomy markedly exacerbated lesions of the vascular system resulted from excessive parathyroid hormone secretion in renal-damaged rats.¹⁵

Since hormones known to influence blood calcium levels have also been shown to affect renal excretion of water and monovalent electrolytes, it is possible that the thymus gland affects electrolyte excretion indirectly. High blood calcium levels interfere with the action of ADH.

THE THYROID GLAND

Thyroid hormone deficiency is associated with water retention problems and altered electrolyte metabolism, while thyroxine administration promotes diuresis and may induce dehydration. The underlying mechanisms are multiple and complex.

The "puffy" appearance of the skin (myxedema) in severe hypothyroidism has been attributed to defects in hyaluronic acid and protein metabolism which lead to subcutaneous accumulation of mucopolysaccharides, proteins, and water. Altered capillary permeability permits leakage of proteins into nonvascular extracellular fluids and into the urine. Circulating blood volume is diminished partly because of such leakage and also because of low fluid and food intake and poor gastrointestinal absorption.

Reduced force and rate of myocardial contraction combine with renal hemodynamic problems to decrease glomerular filtration rate and renal blood flow. Under chronic conditions, there is gradual reduction of kidney mass. Sluggish proximal tubular transport of water and electrolytes has been related to deficient synthesis of specific proteins involved in the processes.

Defective "sodium pumps" associated with low ATPase activity disrupt sodium-potassium balance. While sodium and chloride are retained within the extracellular fluids, the plasma sodium concentration is diminished. Negative potassium balance could result from cellular dehydration, but potassium content may retain its normal relationship to the (reduced) lean body mass.

Both the secretion rate and the sensitivity to several hormones (including aldosterone) are lowered, but renal responses to ADH and ability to concentrate urine are maintained. Difficulties excreting a water load (which cannot be corrected by administration of glucocorticoids) have been related to impaired ability of hypothalamic mechanisms to respond to osmotic changes, since ADH secretion is not appropriately reduced.

Calcium retention (which secondarily affects sodium metabolism) may result from faulty bone metabolism, while phosphate retention

seems to be related to problems in both bone and muscle. Magnesium content of the body is diminished in chronic states. Deficiency effects can be reversed by administration of thyroxine, and large doses of the hormone cause difficulties related to changes in opposing directions.

In hyperthyroidism, elevated metabolic rate may lead to dehydration via excessive water loss through the sweat glands and respiratory tract. Gluconeogenesis enhances urea formation, and this contributes to the diuresis. Glycogenolysis can induce mild glycosuria, but it is seldom of sufficient magnitude to markedly affect urine volume. Although thyroxine tends to promote magnesium retention on a long range basis, transient elevation of blood and urinary magnesium concentrations can result from mobilization of the ion from previously inactive pools.

Thyroid hormones exert numerous indirect influences on water and electrolyte metabolism since they affect secretion rates of many hormones (including the sex steroids, somatotrophin, and prolactin) and affect responses to some.

OTHER HORMONAL INFLUENCES ON WATER AND ELECTROLYTE METABOLISM

Estrogens

Estrogens tend to promote sodium and water retention, but responses within a given species are variable. The action has been attributed to stimulation of renin secretion and of renin substrate synthesis (both of which lead to increased aldosterone secretion). In susceptible individuals, effects may be troublesome during pregnancy or use of oral contraceptives. The fact that estrogens can promote salt and water retention in animals exhibiting "escape" from chronic actions of mineralocorticoids suggests that sex steroids may act at sites different from those affected by aldosterone. Estrogens are also known to promote release of STH and of prolactin.

Estrogens promote rapid water uptake in the uterus. The effect has been attributed by some to release of histamine and said by others to be mediated via activation of uterine adenylate cyclase.

Progesterone

Progesterone is chemically closely related to deoxycorticosterone, and it com-

petes weakly for aldosterone binding sites. It can, therefore, function as a low potency mineralocorticoid, but it may also bring about anti-aldosterone effects through interference with binding of the more potent hormone to target organ receptors.

Unsuccessful attempts have been made to relate water and salt retention during the luteal phase of the menstrual cycle to progesterone accumulation. However, water retention may become most troublesome at the time when progesterone levels have already declined.

Androgens

Water and salt retention often present an unwanted "side effect" of androgen therapy. Retention of sodium, chloride, potassium, phosphate, calcium, sulfate, and nitrogen have been associated with anabolic actions of the hormone. Androgens may also strongly stimulate the appetite and therefore the intake of water and ions. Actions of STH on electrolyte metabolism resemble those of androgens, but STH is less likely to promote severe water retention.

Glucagon, Parathyroid Hormone, and Calcitonin^{3, 7}

Glucagon increases urinary sodium, calcium, magnesium, and water excretion. The physiological significance of the finding has not been elucidated in mammals, but some speculation concerning a role in calcium metabolism is presented in Section IV. An electrolyte-regulating function of glucagon has been described in some of the fishes. Changes opposite in direction have been observed following administration of insulin.

It is possible that influences of glucagon on electrolyte excretion in mammals represent an "evolutionary carry-over" which has little physiological significance. Another possibility is that some interrelationships between influences on calcium and sodium excretion remain to be elucidated. (Angiotensin is known to affect calcium excretion in dosages effective on the adrenal cortex.)

Parathyroid hormone influences on renal phosphate and calcium excretion have been widely studied. But the hormone also promotes excretion of sodium, potassium, bicarbonate, and water, and high doses can induce dehydration. Parathyroid hormone also decreases excretion of magnesium and ammonium ions. High plasma calcium levels which follow administration of the hormone have been implicated in inhibition of ADH actions on the renal distal tubule and collecting ducts.

Although calcitonin induces effects on plasma calcium levels opposite in direction to those of parathyroid hormone, it promotes water and salt excretion. Its high potency and evidence for actions on the proximal tubule have led to suggestions that thyrocalcitonin is the natriuretic hormone.

Melanocyte-Stimulating Hormone

Relatively little is known of melanocyte-stimulating hormone (MSH) actions in mammals other than influences on pigment synthesis (Section VIII). Migration of pigment granules of poikilotherms depend upon sodium transport mechanisms, and some observed MSH influences on the central nervous system also seem to involve transmembrane sodium movement.

Histological changes in the pars intermedia (in which MSH is synthesized) have been described after water deprivation and after administration of hypertonic solutions; administration of hypertonic solutions of sodium chloride (but not of potassium chloride or glucose) was reported to deplete pituitary α -MSH content. This hormone promotes natriuresis in rats without affecting renal excretion of potassium. Therefore some regulatory influence on sodium metabolism seems likely.

Some axons originating within the supraoptic nuclei terminate in the vicinity of the pars intermedia; and ADH has also been identified in that part of the pituitary. ADH may affect MSH secretion.

In very large doses, oxytocin promotes renal excretion of sodium and potassium; a role in electrolyte metabolism of other vertebrates is known but may represent a purely pharmacological effect in mammals.

THIRST AND DRINKING BEHAVIOR^{8, 10, 11}

Since drinking is a behavioral phenomenon, it is controlled predominantly by neural mechanisms and can be conditioned; it is also strongly affected by availability of foods and fluids, their taste, the efforts required to attain them, and the condition of the drinker. (Debilitated animals may not drink even when severely dehydrated.)

Stretch receptors within the vascular system can sense reduction of extracellular fluid volume, while osmoreceptors in com-

munication with the central nervous system provide information on intracellular hydration. Different receptors may be involved in perception of *localized* (e.g., oropharyngeal) dehydration and in mediation of drinking associated with eating. Thermoreceptors in preoptic regions of the hypothalamus play a role in some species, since warming them can promote drinking in well hydrated animals. Additional receptors seem to be located in the respiratory system of animals that pant. Both cholinergic and adrenergic pathways affect fluid intake.

The concept of the existence of a "drinking center" in the hypothalamus is based on observations that damage to lateral regions or severing of the associated medial forebrain fibers impairs responses to the usual stimuli. Recently evidence has been presented for an important role of the subfornical organ (SFO), a structure projecting into the third ventricle of the brain at the level of the intraventricular foramina.¹⁴ Histological changes have been described after water deprivation.

The existence of inhibitory neurons in the septal region, in the posteroverentral part of the amygdala, and in the hypothalamus is suggested by findings that destruction of these areas leads to polydipsia.

Hormones can influence receptor function, responses to receptor stimulation, metabolic factors affecting the receptors, and the more general condition of the animal.

Angiotensin II is the most potent dipsogen known, and it may exert a unique action on thirst receptors. Injection of picogram quantities into the third ventricle rapidly elicits copious drinking in hydrated animals. Recent evidence indicates an effect directly on the SFO, since very small lesions destroying this structure but leaving intact all parts of the hypothalamus implicated in drinking behavior, abolish responses to angiotensin. The SFO can also be stimulated by carbachol. Angiotensin analogs interfering with responses to the octapeptide do not affect responses to cholinergic stimulation.

ADH decreases drinking. The effects are believed to be secondary to induced water retention. Most of the studies involving direct application to various parts of the

brain have yielded negative data. It has been pointed out that conditions favoring ADH release are ones in which *increased* drinking would be useful for restoration of fluid balance. ADH may lower the threshold for osmotic perception of thirst, at least in dogs.

Thirst in diabetes mellitus is secondary to dehydration resulting from glycosuria and ketonuria. However, insulin can also invoke thirst, and two explanations have been offered. Insulin tends to reduce the volume of extracellular fluid by promoting uptake of glucose (along with potassium and water) by the cells. Insulin also induces hypoglycemia, and this provides a stimulus for release of renin. In addition, insulin stimulates the appetite and may thereby increase intake of fluids as well as of food. Large doses act on the parasympathetic nervous system and may activate cholinergic fibers mediating drinking behavior.

In hyperparathyroidism, thirst has been attributed to direct effects of high plasma calcium concentrations on hypothalamic neurons, and to the dehydration which follows the diuresis. Vomiting often also contributes to dehydration in this condition.

Thirst is increased in both hypothyroidism and hyperthyroidism. In myxedema, interstitial fluid volume is increased, but the blood volume is reduced, and this may affect vascular receptors. In hyperthyroidism, water depletion results from increased loss through the respiratory tract, sweat glands, and kidney. Hypothalamic neurons may also be directly stimulated by high levels of thyroid hormones, and thermoreceptors can be activated by elevated body temperature.

Thirst is also increased in conditions of both excess and deficiency of adrenocortical hormones. In the former case, intracellular receptors respond to potassium depletion, while in the latter, sodium depletion leads to reduction of extracellular fluids.

There is growing awareness of the role of prolactin in maintenance of fluid and electrolyte balance. However, no influence of prolactin on thirst in mammals, other than a secondary one related to fluid loss in the milk, has been demonstrated.

SALT APPETITE

Reduction of extracellular fluid volume is an indication of the need for replenishment of sodium as well as water. There is

HORMONAL REGULATION OF BODY FLUIDS

some evidence that specific areas within the amygdaloid complex influence the quantity and concentration of salt solutions ingested by rats when they are given choices.¹¹

Adrenalectomized rats receiving no hormone supplements will voluntarily ingest sufficient saline to compensate for urinary losses. When presented with a choice of several salt concentrations and water, they can adjust intake to remain in balance. (However, intact rats fed a diet containing sufficient sodium tend to select a dilute sodium chloride solution in preference to tap water.) Animals on sodium-deficient diets will ingest sufficient additional saline to make up the deficit. Patients with adrenocortical insufficiency often experience salt craving and preference for sodium-rich foods. Distinct but synergistic influences of ACTH and glucocorticoids on salt appetite have been found in rabbits.¹⁰

In hypothyroidism, the increased salt appetite had been attributed to the low rate of aldosterone secretion and to insensitivity to actions of mineralocorticoids.

It is known that sodium-deficient diets lead to increased release of renin, and it is possible that angiotensin is involved in appetite for salt as well as for water. In adrenocortical insufficiency and in hypothyroidism, the decreased circulating blood volume provides a stimulus for renin secretion.

Salt appetite may also be increased by overdosage with mineralocorticoids. This appears paradoxical, but conditions favoring endogenous release of mineralocorticoids include sodium deficiency states.

One may speculate on the possibility that the same kind of "sodium receptor" is affected by both adrenocortical deficiency and adrenocortical excess. In the former, sodium content of the cell could be reduced because of generalized body depletion of sodium; in the latter, the effects of mineralocorticoids on sodium transfer out of the cells could also deplete sodium locally. It is also possible that two different kinds of receptors are involved. The fact that aldosterone decreases the sodium content of the saliva may be relevant.

Salt appetite is usually increased during pregnancy and lactation. The need for increased sodium chloride is obvious, but the underlying mechanisms are unknown.

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IV

HORMONAL REGULATION OF CALCIUM AND PHOSPHOROUS METABOLISM

14. Parathyroid Hormone

PHYSIOLOGICAL FUNCTIONS OF CALCIUM

Calcium participates in every known biological function. It has clearly been implicated in *transmission of nerve impulses* and *release of neurohumors*, in *contractile mechanisms* of skeletal, cardiac and smooth muscle, and in the *coupling* of excitation and contraction. It affects secretory processes of *exocrine* and *endocrine glands* in many ways, participates in *hormone-receptor interactions*, and mediates *hormone effects* through direct influences on key enzymes (including adenylate cyclase) and indirectly by affecting intracellular pH, phosphate and sodium content, and magnesium availability. It influences *cell proliferation* in several ways, is needed for *coagulation* of the blood, participates directly in *bone mineralization* and remodeling, and affects *oxidative phosphorylation* and ATP utilization.

Calcium is an essential component of *plasma membranes* influencing structure, deformability, permeability, and enzyme functions. Binding to mucoproteins of the glycocalyxes affects *surface properties*, while influences on *microtubules* extend to a broad range of functions. Calcium is taken up by *sarcoplasmic reticulum* and *mitochondria*.

Calcium is found in high concentrations in milk, in considerable quantities in intestinal juices, and in lower concentrations in most other secretions. It is present in blood plasma, cerebrospinal fluid, lymph, interstitial fluids, and cell cytosol.

QUANTITIES PRESENT^{9, 11}

The human adult body contains 1200–1400 g of calcium—almost 10 times the weight of sodium and potassium combined. About 99% is within extracellular matrices of hard tissues, and most of the remaining 1% is located intracellularly, with less than 1.5 g present in extracellular fluids.

The cytosol calcium ion concentration of "resting" cells has been estimated at 1×10^{-7} moles per liter, and this rises to about 1×10^{-5} after stimulation. Rapid changes depend largely upon movements across mitochondrial membranes, since mitochondria may contain up to 500 times as much calcium as the cytosol (in the form of metabolically inert but readily recruitable calcium phosphate). Smaller fluctuations result from reversible binding with plasma and other membranes, exchange with extracellular fluids, and attachment to intracellular molecules.

Blood plasma calcium is maintained within the narrow limits of 9–11 mg per 100 ml. Forty-five per cent of this is bound in nondiffusible (non-ionized) form to proteins and especially to the albumin fraction; almost half is present as free calcium ion (at a concentration of just over 1×10^{-3} M), and the remainder is bound in poorly ionizable complexes to small diffusible components, especially citrate, phosphate, and bicarbonate. Large changes in plasma concentrations of proteins or of the diffusible components can alter the fraction that is present as free calcium ion.

EFFECTS OF CHANGES IN PLASMA CALCIUM ION CONTENT

Small changes can lead to disaster. *Sudden elevation* of calcium increases the force of *myocardial contraction* and interferes with relaxation; the heart can be totally arrested in systole ("calcium rigor"). Sustained elevations of smaller magnitude lead to *deposition* of calcium in soft tissues of the *blood vessels*. The elastic recoil of the arterial system is diminished (so that pulse pressure rises excessively), and ability to adjust the caliber of arterioles to changing needs is impaired. Long term hypercalcemia leads to necrosis and aneurysm formation, and death can result from rupture of the aorta.

Calcification of *renal tubules* impairs kidney function, and high calcium ion concentrations antagonize antidiuretic hormone (ADH) actions. Renal retention of phosphates enhances the tendency for calcification, and renal failure may ultimately ensue. Calcium also deposits in smooth muscle of the *gastrointestinal tract* and in blood vessels of the *liver and lungs*.

Reduction of calcium ion concentration leads first to increased *neuromuscular excitability*; this rapidly progresses to involuntary tremors and *spasm* of skeletal and smooth muscle. The term "tetany" describes the condition. Death from asphyxia is precipitated by spasm of muscles of the *larynx* and *diaphragm*.

Chronic smaller reductions of calcium ion concentration affect the gut; loss of appetite may progress to vomiting and diarrhea with ensuing dehydration and elevation of body temperature. Hypocalcemia can lead to mental retardation in children and to irritability, paresthesias, and psychic depression in adults. Teeth do not develop normally, hair and nail growth are impaired, and the skin becomes dry and susceptible to infection.

The *free calcium ion* is the *physiologically active form* which influences cardiac contraction and neuromuscular excitability, but *total plasma calcium* (and associated content of phosphate) determines the tendency for *precipitation* in soft tissues.

CALCIUM IN THE SKELETON⁶

Sixty-five per cent of the weight of adult mammalian bone is made up of inorganic mineral deposited within the extracellular

matrix; 30-40% of this is present as *amorphous calcium phosphate*. Most of the remainder is *crystalline*, predominantly *hydroxyapatite* $[Ca_{10}(PO_4)_6 \cdot (OH)_2]$ with smaller amounts of other crystal types, e.g., *octacalcium phosphate* $[Ca_8(H_2O)_6(PO_4)_6 \cdot 5H_2O]$. The mineral phase of bone also contains sodium, magnesium, potassium, citrate, carbonate, chloride, and fluoride.

Technical difficulties involved in study of bone tissue, and divergent interpretations of X-ray diffraction and electron microscopy data have led to disagreements among investigators concerning the precise nature of bone mineral.

Some of the bone calcium is bound to "ground substance," which makes up about 1% of bone organic matter, and to extracellular granules which also contain proteins and phospholipids.

The calcium content of bone cells (and especially of osteocytes described below) is much greater than that of "typical" cells. The surrounding "bone fluid" is sequestered and differs in composition from the "bulk" extracellular fluid with which it exchanges slowly.

Estimates of bone fluid composition have been made by equilibrating powdered bone with buffer solutions. The calcium content appears to be only one-third that of the plasma, while the phosphate content does not usually differ. Elevation of phosphate content markedly increases calcium concentration.

Bone fluid potassium concentration is related to cell function and can vary independently of plasma potassium. Concentrations of 25 mM are typical (compared with 4 mM for plasma) and can go as high as 100 mM in young, growing tissue. It is maintained during times of potassium depletion severe enough to depress plasma concentrations and can be very low after hypophysectomy when plasma concentrations are in the normal range.

Bone fluid is low in sodium and magnesium but high in chloride content, and the pH has been estimated at about 6.8, compared with 7.35 for plasma.

CALCIUM INTAKE, ABSORPTION AND EXCRETION⁶

The daily dietary calcium requirement for the human adult has been placed at between 600 and 1,000 mg. Requirements are increased during pregnancy and lactation, and positive calcium balance is

needed to sustain growth and skeletal repair.

Calcium is absorbed from the small intestine by active processes described in Chapter 15. The duodenum is the major site for most species, but in a few (hamster, chick) it is transported across in the lower ileum. The rate of uptake is controlled primarily by vitamin D metabolites but is also influenced by several hormones (Chapters 15 and 16). Uptake is enhanced by the presence of hydrochloric acid, amino acids, and citrate, and decreased by large amounts of fat and by substances (e.g., phytic and oxalic acids of vegetable grains) which form insoluble complexes with calcium. For unknown reasons, absorption is increased when lactose is present in other parts of the intestine.

Calcium balance in the adult is maintained largely by fecal excretion of the equivalent of 90% of the intake. Most of the fecal calcium is derived from intestinal secretions, but some of the ingested calcium usually escapes absorption.

The kidney filters 10–11 g of calcium daily (the equivalent of over 10 times the dietary intake). Usually only about 1% (100 µg) escapes reabsorption. The amount excreted is increased by low carbohydrate diets and other factors promoting metabolic acidosis, and is affected by parathyroid hormone (see below).

Very small quantities of calcium are also lost through sweat.

CALCIUM TURNOVER

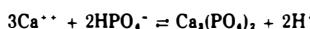
⁴⁵Ca has been widely used for turnover studies. It has been estimated that all of the extracellular fluid calcium is renewed 0–50 times a day, the calcium content of the liver and heart is exchanged every 4 hr, and skeletal muscle calcium is renewed very 8–12 hr. Although 50–60 g of bone calcium seem to be available for rapid exchange with the blood plasma, skeletal calcium turnover in the human adult has been estimated at only 3% per year.

REGULATION OF CYTOSOL CALCIUM CONCENTRATIONS¹⁰

Role of The Mitochondria

The following equation describes the reversible process by which calcium is

rapidly taken up by mitochondria, converted to a metabolically inert form, and released again upon appropriate stimulation:



The uptake requires ATP energy and can be limited by phosphate deficiency; release is facilitated by high cell pH (which is lowered as the calcium and phosphate are sent into the cytosol). In some cell types, the entire cytosol content could (theoretically) be replaced 500 times by depletion of mitochondrial stores.

Exchanges with Extracellular Fluids

This is a slow process compared with mitochondrial exchange. Most investigators believe that calcium enters passively ("calcium leak") by a process of facilitated diffusion that requires a specific carrier. Calcium efflux has been attributed to activity of "calcium pumps" in the plasma membrane. Strong evidence has been presented for the existence of a calcium-activated ATPase in some kinds of cells, while sodium-calcium exchange mechanisms have been described for others.

An important hypothesis (backed up by data) is that calcium extrusion is directly linked to the sodium pump. Energy is used to eject the sodium; sodium then diffuses back down a steep gradient and the diffusional energy supports calcium export.

On the basis of observations that cooling bone cells to 2°C stops calcium uptake but does not affect efflux, it has been suggested that in such cells the uptake is an active process, whereas egress is passive. Alternate interpretations of the data have been offered.

It is likely that several mechanisms are utilized by the various cell types and possibly by the same cell under different conditions. Calcium efflux on the "bone" side of osteocytes may be very different from calcium transfer to the extracellular fluids.

INTAKE, DISTRIBUTION AND EXCRETION OF PHOSPHORUS¹¹

The adult human body contains about 600 g of phosphorus in inorganic form and as components of essential molecules. About 85% is contained within the skeleton, and phosphate is a major determinant

of the rate of bone calcification. Mitochondrial phosphate was described above.

The list of vital substances containing phosphorus gives an indication of its metabolic role: *nucleic acids*, *nucleotides* (ATP, GTP, cAMP, NAD, etc.), *phosphoproteins*, *phospholipids*, *creatine-phosphate*, and intermediates of the glycolysis sequence. Several key metabolic enzymes are transformed from inactive to active states by acquisition or removal of a phosphate group. Pyrophosphate (P-P) is formed when adenylate cyclase acts on ATP, and as a product of numerous other ATP reactions; it is an effective inhibitor of adenylate cyclase, and it influences many other enzymes. The *buffer system* of the kidney depends heavily on the ability to excrete varying combinations of phosphate with sodium, hydrogen, and ammonium.

Approximately one-half of the phosphorus of the blood plasma occurs in the form of ionized inorganic phosphates; another one-third is present as poorly ionized complexes with inorganic cations (mostly sodium, but also calcium and magnesium); the remainder is bound to organic components.

Plasma concentrations of inorganic phosphate are influenced by diet, hormones, metabolic conditions, age, sex, and species peculiarities. Much greater variations from optimal levels can be tolerated than is the case for calcium. Excessively high concentrations indirectly exacerbate the tendency for calcification of nonosseous tissues. When high phosphate levels lead to combination of calcium with phosphate, the fraction of plasma calcium remaining in ionized form is reduced; increased secretion of parathyroid hormone follows, and this leads to secondary elevation of blood calcium concentrations, and ultimately to deposition of both calcium and phosphate.

The plasma of the healthy, well fed human adult contains approximately 4 mg of inorganic phosphate per 100 ml. Children have higher levels, and values of 8-10 mg per 100 ml are within the normal range for adult rats of some strains.

Dietary phosphate is absorbed in the form of inorganic ions by the small intestine. Since the rate is linked to calcium absorption, it is influenced by the same factors which affect calcium uptake. How-

ever, most phosphate absorption seems to take place in the jejunum.

Inorganic phosphate is freely filtered by the renal glomeruli, and a portion is reabsorbed by the proximal tubules. Additional phosphate is transported by the distal tubules. Renal phosphate excretion is variable, and rapidly influenced by hormones. The equivalent of about two-thirds of the ingested phosphate is usually removed by the kidney; phosphate balance is maintained by fecal excretion of the additional one-third.

EFFECTS OF PARATHYROIDECTOMY

The plasma calcium ion concentration falls rapidly after parathyroidectomy, and may be less than one-half normal within hours. The time required for onset of tetany varies with the species, and is shortened if food is withheld. Although plasma phosphate concentration rises, the $\text{Ca} \times \text{P}$ product (the clinical "solubility product") falls.

The effects can be mitigated by feeding a diet rich in calcium and vitamin D and low in phosphorus. Rats and some other animals can be maintained for long periods in this way, but they succumb rapidly if fasted.

Administration of parathyroid hormone (PTH) reverses the condition; urinary phosphate excretion is rapidly increased and plasma calcium levels soon return to normal ranges.

Excessive PTH further elevates plasma calcium concentrations. Prolonged administration leads eventually to thinning of the bones and deposition of calcium in soft tissues. Patients suffering from chronic high calcium suffer psychic symptoms and have been mistakenly diagnosed as schizophrenic.

SOURCES OF PARATHYROID HORMONE¹¹

The number of parathyroid glands varies; most mammals have two, but some have four. They are usually closely associated with or embedded within the thyroid glands, but in certain species some or all are anatomically separated from the thyroid. Additional PTH cells are commonly seen outside the main glands, e.g., in the

thymus or mediastinum (see also Section VIII).

The *chief* cells of the gland have been identified as the site for synthesis of PTH. "Dark" chief cells contain a prominent Golgi apparatus, well developed endoplasmic reticulum and many granules, and are believed to engage in active hormone synthesis. "Light" cells which are glycogen-rich but contain few granules are believed to be recruitable "resting" cells. No function has been assigned to acid-staining "oxyphils" seen in many species.

CHEMICAL NATURE OF PARATHYROID HORMONE^{8, 9, 11}

The cells of the parathyroid gland synthesize a *pre-pro-parathyroid* hormone which contains 109 amino acids and seems to exist for only a short period. It is soon cleaved to the 90-amino acid *proparathyroid hormone* which is packaged into granules and is secreted with the more active 84-amino acid *parathyroid hormone*. Further cleavage takes place after the hormone is released into the circulation. Pro-PTH, PTH, and a smaller peptide have been identified in blood plasma.

It was noted in earlier sections that the process of synthesizing prohormones may be related to the nature of ribosomal functions which require that molecules of a minimal size be formed, and that post-translational modification can provide regulatory sites for hormone formation and release. But other interpretations can be attached to the processes in parathyroid glands. Proparathyroid hormone has only a fraction of the biological potency of PTH; it could be released when rapidly changing metabolic needs reduce requirements for the more active molecule. It could also serve as a rapidly recruitable reserve for the more potent PTH.

PTH cleavages in peripheral tissues may be important for both further activation (or selective enhancement of certain actions) and for metabolic degradation.

It has been determined that amino acids 2 through 27 are essential for demonstration of parathyroid hormone functions. A 34-amino acid peptide with parathyroid hormone activity has recently been synthesized, and a peptide

containing amino acids 3-34 has been shown to inhibit activity of the native hormone.

Inactivation of the circulating hormone takes place in the liver and to a lesser extent in the kidney.

REGULATION OF PTH SECRETION⁸

It was noted (Section I) that a *fall in plasma calcium ion concentration* provides the major stimulus for PTH secretion. The cells seem to be responding to loss of an inhibitor molecule.

Calcium ions have been implicated in inhibition of (1) the enzyme which converts Pro-PTH to PTH; (2) release of preformed hormone; (3) uptake and incorporation of amino acids into new hormone protein; and (4) growth of the parathyroid gland. Calcium may also facilitate degradation of Pre-Pro-PTH and also of Pro-PTH.

Two secretory phases have been described: an early rapid, puromycin-insensitive one which seems to involve release of preformed hormone, and a delayed, puromycin-sensitive phase which depends upon new hormone synthesis.

Vitamin D metabolites have also been suspected of influencing degradation of PTH and its precursors. They may act through elevation of cytosol calcium concentrations within the gland.

Parathyroid hormone secretion seems to be mediated via cAMP. The adenylate cyclase is apparently extremely susceptible to inhibition by concentrations of calcium ion that do not inhibit adenylate cyclase in most other cell types.

The only other endocrine cells known to increase their secretion in response to *reduction* of plasma calcium levels are those secreting glucagon. The α cells of the pancreas also respond to changes in magnesium ion concentrations. Effects of changes in magnesium concentration beyond the physiological range have been shown to influence parathyroid gland cells, but magnesium is not believed to be involved in physiological regulation of parathyroid hormone secretion. Glucagon promotes release of PTH, possibly through stimulation of adenylate cyclase.

Calcitonin (Chapter 16) also promotes release of PTH. It may act indirectly via

lowering of plasma calcium ion concentrations and also directly (since it lowers intracellular calcium and may therefore activate parathyroid adenylate cyclase).

BONE PHYSIOLOGY AND CELL TYPES

Bone provides the major long range source of calcium when dietary intake is inadequate for maintaining plasma calcium concentrations; it also takes up calcium when too much is provided by a combination of high calcium diet and moderate vitamin D intake, or if calcium is injected intravenously.

It was once thought that plasma-bone exchange provides the single important mechanism for rapid regulation of calcium concentrations; this concept has been challenged.

The mineral phase confers rigidity to bone; if it is dissolved away, a flexible structure with considerable tensile strength that retains the original contours remains. (This is readily demonstrated by soaking a chicken femur in hydrochloric acid or in a solution containing chelating agents; the bone can soon be tied into a knot.)

About 95% of the organic matter of bone (30-33% of its weight) is *collagen*, which is considered below. Bone matrix also contains small quantities of a "ground substance" consisting largely of sulfated and nonsulfated mucopolysaccharides and non-collagen proteins, and small quantities of lipids. There is growing evidence that the ground substance is highly structured and intimately related spatially to other matrix constituents.

Mesenchymal and Other Surface Cells

Bone surfaces are covered by what appears to be a continuous layer of cells. *Mesenchymal* components are characteristically spindle-shaped, with poorly developed endoplasmic reticulum, glycogen-rich cytoplasm, and small, dark, elongated nuclei. These cells undergo mitosis and are known as *osteoprogenitor* cells since they give rise to other bone cell types.

Fibroblasts and "transitional cells" are also frequently distinguishable on bone surfaces. The latter are sometimes called *preosteoblasts*, but use of this term is discouraged by some

investigators since it implies that they will soon become osteoblasts.

A functional membrane separates the "bulk" extracellular fluid (in communication with the blood capillaries) from the small volume of "bone fluid" which bathes the osteocytes. The membrane may consist of mesenchymal, transitional, and osteoblastic cells.

There are unanswered questions about whether communication between the bulk extracellular fluid and bone extracellular fluid is accomplished via small channels between the lining cells, by passage through the cytoplasm, or by a combination of both (involving passage through the cells in one direction and between them in the other).

Osteoblasts

Osteoblasts Synthesize and Secrete Collagen. They lie between the mesenchymal cells and the fully formed bone, and may participate in bone alignment. They send vertical processes to the underlying osteocytes and may therefore be part of a giant syncytium.

Osteoblasts are plump cells with basophilic cytoplasm that contains a moderate number of mitochondria and numerous mucopolysaccharide-laden vesicles. The nuclei are rich in RNA and typically contain three or more nucleoli. The outstanding characteristics are an especially well developed rough endoplasmic reticulum with numerous ribosomes, and prominent Golgi vesicles. Alkaline phosphatase content is high. Young osteoblasts are rich in glycogen, but the carbohydrate is gradually depleted during the process of collagen formation.

Transitional forms which have assumed some of the morphological characteristics of the osteoblasts but have not yet begun to secrete collagen are classified as *preosteoblasts*.

The term "resting osteoblast" is sometimes used to designate cells which resemble osteoblasts morphologically, but which are not participating in collagen synthesis. It may lead to confusion since osteoblast is usually defined functionally as a cell which produces collagen.

Osteocytes

Osteoblasts become surrounded by the extruded extracellular matrix and are transformed into osteocytes. During the transformation (which is called *modula-*

tion, rather than differentiation because osteoblasts are already highly differentiated cells), the cytoplasm becomes more dense, much of the endoplasmic reticular structure disappears, and lateral processes are sent out to neighboring cells. Numerous mitochondria with well developed cristae and electron-dense granules appear. The granules are rich in calcium phosphate. Lysosome-like vesicles form. These contain acid hydrolases implicated in bone dissolution, but collagenase enzymes appear to be present within the cytosol rather than enclosed in the lysosomes.

Mature osteocytes are completely surrounded by matrix and lie within small cavities, or *lacunae*, in contact with lacunar fluid shared with other cells of mature bone. The cells are capable of undergoing rapid changes in size in response to local concentrations of ions or hormone. Sometimes they appear shrunken and pycnotic, but at other times they almost completely fill the lacunae.

Osteoblasts and osteocytes make up approximately 95% of the bone cells.

Osteoclasts

These are the largest and least numerous of the bone cells. They are characterized by the presence of an extensive "ruffled," "brush," or "striated" border made up of cytoplasmic extensions at sites of active bone resorption. Osteoclasts commonly contain 4-5 nuclei, but cells containing up to 300 nuclei (large enough to be seen with the naked eye) and others with but a single one have been described. The cells often have many lobes connected by narrow cytoplasmic strands which may pinch off so that smaller cells are formed. The numerous vesicles are believed to be lysosomes rich in acid hydrolases. Mitochondria laden with electron-dense amorphous calcium phosphate accumulate at the surfaces. Little or no endoplasmic reticulum is visible, but the Golgi apparatus is well developed.

COLLAGEN SYNTHESIS^{8, 10, 11}

Collagen is widely distributed in skin, tendon, dentin, cartilage, blood vessels, and basement membranes. There is considerable interest in the question of why

bone collagen mineralizes while that of other tissues normally does not. The known small differences in collagen chemistry have not yet provided the answers.

The amino acid composition of all collagens differs markedly from that of "typical" body proteins. The glycine content is especially high (up to one-third of the amino acid makeup) while almost equal amounts of proline, arginine, and alanine make up another third. Collagen contains hydroxylated amino acids (hydroxyproline and hydroxylysine) not known to occur elsewhere, and there seems to be a complete absence of tryptophan.

Several stages in the process of collagen synthesis have been recognized.

Protocollagen Synthesis

Pro- α chains containing approximately 1200 amino acids are synthesized on ribosomes. They combine in groups of three to form left-handed spiraling helices.

Two distinct chain types, α -1 (I) and α -2 have been identified in collagen of bone, dermis, and tendon. They differ in amino acid composition and surface charge. It is believed that both types are synthesized simultaneously, and that two of the first combine with one of the second to form the triple helix. (By contrast, cartilage collagen contains predominantly α -1 (II) chains.)

Procollagen Formation

Transformation of procollagen into collagen involves hydroxylation of some of the amino acid components of the chains, followed by addition of carbohydrate groups.

There is evidence that pro- α chains are hydroxylated while still attached to the ribosomes. A peptidyl proline hydroxylase adds OH groups to those prolyl residues which immediately precede glycyl residues, while a peptidyl lysine hydroxylase catalyzes hydroxylation of the lysine residues. Both enzymes require ferrous ions, ascorbic acid, molecular oxygen, and the conversion of α -ketoglutarate to succinate. The very specialized energy source requirements are probably related to intracellular mechanisms for regulation of collagen synthesis, but the rate-limiting step has not been established. It has been suggested that hydroxylation serves to stabilize the amino acid chains and to direct formation and stabilization of the triple helices.

Enzymes catalyzing addition of galactose and glucosyl-galactose to certain of the hydroxylysine residues have been identified. Glycosylation seems to occur after helix formation but prior to release of procollagen from the ribosomes.

Some authors believe that glycosylation is essential for subsequent extrusion of the molecules into the extracellular spaces, but others have cited the importance of the location of sugar moieties for determination of polymerization patterns. Another concept is that sugar molecules play a role in binding of phosphates and in initiation of mineralization.

Because of uneven distribution of components, the procollagen molecules exhibit orientation of charges which give them "heads" and "tails." Trimers are held together by hydrogen and hydrophobic bonds.

While evidence favors direct extrusion of procollagen helices, the possibility that some of the procollagen is converted to collagen while still within the cell has not been ruled out.

Mechanisms for extrusion are incompletely understood. The helices may be packed into vesicles and the entire vesicles discharged. A role of microtubules in the process has been proposed.

Tropocollagen Formation

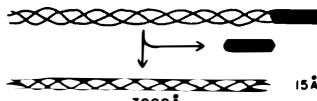
Procollagen undergoes limited proteolysis which slightly shortens the helices and yields tropocollagen as the product (Fig. 14-1, A and B). Tropocollagen is a rodlike cylinder approximately 2800 Å long and 13.6–16 Å in diameter, with a molecular weight of about 290,000.

Most (if not all) of the proteolysis is catalyzed by extracellular enzymes. The process is believed to be necessary for exposure of groups involved in subsequent polymerization.

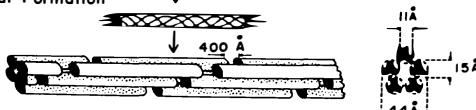
Microfibrils

Tropocollagen cylinders polymerize to form *microfibrils* which are larger cylinders having a diameter of approximately 44 Å and an estimated core size of 30 Å (Fig. 14-1B). The tropocollagen units are arranged in a longitudinal direction to form very long fibrils with gaps 400 Å long between the units. The tropocollagen trimers are arranged so that the gaps are

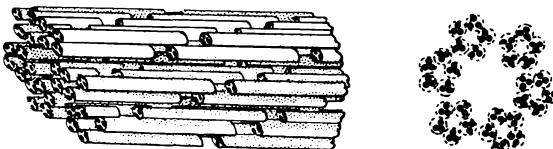
A. Procollagen to tropocollagen



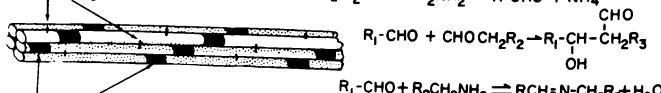
B. Microfibrillar Formation



C. Fibril Formation



D. Cross Linking



E. Calcification

FIG. 14-1. Formation of collagen fibrils. (Reprinted with permission from Ref. 6.)

staggered. The exact three-dimensional structure of the microfibrils has not been clearly established, but there are indications that the five units wind about each other in a right-handed spiral.

Collagen Fibers

Microfibrils polymerize into highly ordered fibrils (fibers) 150–1,500 Å in diameter (Fig. 14-1C), having a repeating pattern 640 Å long which can be demonstrated with electron microscopy. Polymerization seems to be a spontaneous process (requiring no enzymes) dependent upon location of surface charges and sugar moieties.

The collagen fibers are then further organized within a supramolecular extracellular matrix which has been the subject of considerable analysis but whose structure is not yet fully defined.

Maturation of the Matrix

Collagen undergoes modifications which render it less soluble than the newly formed material. It has been proposed that dehydration leads to reduction in distance between adjacent molecules and thereby strengthens intramolecular forces. The number of covalent bonds increases with age.

Mineralization of Bone

A characteristic time interval elapses between initiation of collagen synthesis and the beginning of mineralization. (A period of 8–10 days has been observed in human tissues.)

There is some evidence that deposition of calcium salts occurs first within the 400 Å gaps between tropocollagen units of the microfibrils (Fig. 14-1C), and that the minerals soon form a continuous phase which is chemically associated with the collagen. It is estimated that not more than 5–18% of the mineral is exposed directly to the bone extracellular fluids.

According to one hypothesis, mineralization starts with ionic or covalent bonding of phosphate groups to serine or sugar moieties of the collagen; other researchers believe that the initial binding involves calcium.

MECHANISM OF ACTION OF PARATHYROID HORMONE: HISTORICAL PERSPECTIVES

Parathyroid hormone physiology has a fascinating history, partly because information gaps permitted broad speculation on the meaning of existing data, and partly because it is possible to view the problems from many sides. Early investigators with logical and creative minds utilized the limited factual material to formulate concepts which were discarded when new information became available, but many of the older ideas have since been resurrected, modified, and incorporated into current hypotheses. Since the full story of just what parathyroid hormone does not yet unfold, it is instructive to examine the older concepts in the light of present thinking.

Some General Aspects of the Problem

Blood drawn from a healthy subject contains calcium and phosphates in physically stable forms which exhibit no tendency to precipitate on standing. However, if a bone spicule is added, calcium and phosphate deposit on it. On the other hand, plasma treated with agents which markedly lower the $\text{Ca} \times \text{P}$ product will draw minerals out of the bone. The ion concentrations at which an equilibrium between precipitation and dissolution is established depend on such factors as the quantities of proteins, hydrogen ions, citrate, etc. that are in the plasma.

In *fasting parathyroidectomized* animals, bone takes up calcium from the plasma to the point where plasma concentrations of the ion fall below levels compatible with survival. (Bone also takes up phosphate, but the plasma phosphate does not fall because renal excretion of phosphate from nonosseous sources is impaired in parathyroid hormone deficiency.) If the animal can be kept alive, the calcium content of the blood stabilizes at a point which may be one-half that of normal animals.

Administration of PTH (without calcium) restores the plasma ion concentrations, and it can be shown that some calcium is transferred from bone to plasma. It seems logical to conclude that

PTH somehow "persuades" bone to less avidly take up minerals.

The "Renal Theory" of PTH Action

One of the earliest and most famous hypotheses (presented in the 1920's) was based on the repeatedly confirmed observation that potent parathyroid gland preparations induce a prompt increase in renal phosphate excretion, leading to reduction of plasma phosphate concentrations.

According to the "renal theory," bone gives up calcium to the blood plasma because primary actions of PTH on the kidney lead to a lowering of the "solubility product" ($\text{Ca} \times \text{P}$) of the plasma with which it comes in contact.

A modification of the hypothesis states that the phosphate content of the extracellular fluids is the determining factor for bone mineralization. When PTH promotes phosphate removal, calcium that would otherwise be taken up by bone is sent back to the plasma.

The renal theory in its *original form* is no longer regarded as adequate for explaining ability of PTH to elevate plasma calcium concentrations. For one thing, it offers no reason for the continued effectiveness of high doses of the hormone on bone when the "solubility product" becomes elevated; for another, it cannot explain why vitamin D-deficient animals respond to the hormone by excreting phosphate, but do not exhibit substantial elevation of plasma calcium.

Recent research indicates that exchanges between bone fluid and blood plasma are slower than would be required to support the concept. Moreover, newer interpretations have been attached to the importance of alterations of plasma phosphate.

When plasma phosphate falls, some of the plasma calcium previously held in poorly ionized complexes becomes available as *free calcium ion*. Therefore, some relief from tetany may be obtained with no change in *total* plasma calcium.

Since PTH usually elevates plasma levels, the amount of calcium filtered by the renal glomeruli and passed ultimately into the urine is usually *increased*. PTH also acts directly on the kidney to *enhance calcium reabsorption*. It has just recently been recognized that calcium-conserving

actions of PTH play a role in moment-to-moment calcium homeostasis and that they can be of great importance in parathyroidectomized animals and at other times when plasma calcium is low.⁷

Older Concepts of a Direct Influence of PTH on Bone

Early Evidence for a Direct Influence. Suspicions that parathyroid hormone exerts a direct action on bone which may be independent of effects of the hormone on the kidney were confirmed by a simple but imaginative experiment. Parathyroid tissue was placed in direct contact with bone in the cerebral cavities of mice. After a time, localized resorption occurred on the side of the bone in contact with the parathyroid tissue. More elegant tissue culture studies by other investigators confirmed the finding.

For a time there were suggestions that the parathyroid gland secretes two separate hormones, one affecting the kidney and the other acting on bone. But all early procedures for purifying parathyroid extracts yielded preparations in which potency for renal effects paralleled potency for actions on bone.

Attempts were then made to define the nature of the action on bone.

The "Citrate Theory." PTH administration leads to accumulation of citrate in bone, elevation of plasma citrate concentrations, and increased urinary excretion of this substance. Citrate solutions directly draw minerals from bone tissue, and citrate injections elevate plasma calcium concentrations of intact and parathyroidectomized animals.

It was proposed that the primary action of PTH is to promote accumulation of citrate in bone cells and its extrusion into the surrounding calcified matrix. This presumably leads to solubilization of bone mineral and transport of the readily diffusible (but poorly ionized) calcium citrate to the plasma. Citrate can then be metabolized or excreted while the calcium remains in the plasma.

The concept was questioned when it was observed that elevations of plasma calcium ion concentrations preceded elevations of plasma citrate concentrations in parathyroidectomized rats treated with parathyroid extracts.

Other doubts arose on the basis of comparisons between the quantities of citrate calculated to be produced after administration of parathyroid extracts and amounts needed to solubilize bone, because of discrepancies between effects of vitamin D and glucocorticoid hormones on citrate vs calcium changes, the failure of the citrate theory to explain some aspects of bone phosphate mobilization, failure to substantiate early reports of low bone content of isocitric dehydrogenase, and because of evidence (see below) that calcium mobilization after administration of parathyroid preparations is associated with destruction of the bone matrix.

However, it is now known that parathyroid hormone *does* promote inhibition of isocitric dehydrogenase in bone (although the effect may be indirect). More recent thinking about the importance of citrate is presented below.

Lactate Formation and Related Concepts. Other investigators called attention to the increased rate of glycolysis in bone after administration of parathyroid hormone, and suggested that *lactate* (rather than citrate) is the agent responsible for bone decalcification. A further modification of the general concept states that it is the *reduction of pH* in bone tissue (rather than the accumulation of a specific anion) which promotes bone solubilization. Carbonic acid has also been proposed to contribute to acidity changes.

Phosphatase Enzymes. Bone is rich in alkaline phosphatases. It was once believed that the enzymes catalyze hydrolysis of organic phosphates arising during glycolysis, and that localized increases in phosphate concentration directly promote bone mineralization.

Since PTH reduces phosphatase activity, the concept arose that PTH prevents the accumulation of phosphate required for mineralization; therefore, the calcium which would otherwise have gone into bone mineral is sent into the plasma.

The concept is no longer held for several reasons. The most important is that alkaline phosphatase activity is now known to be more closely associated with processes of *matrix synthesis* than with mineralization. It has also been pointed out that many tissues which exhibit no tendency to calcify are rich in phosphatase enzymes.

A more recent idea states that organic pyrophosphates act as "crystal poisons," interfering

with both mineralization and demineralization by forming coatings at mineralization "nucleation sites." Pyrophosphatas are therefore necessary for exposure of surfaces before bone can take up calcium; however, no relationships between distribution of pyrophosphatas (or enzymes affecting them) and mineralization processes have been demonstrated.

Halisteresis vs Collagen Destruction

Mechanisms. The concepts presented suggest that PTH maintains plasma calcium concentrations primarily by promoting halisteresis (mineral dissolution without effects on bone matrix). However, PTH increases the urinary *excretion of hydroxyproline*. Since the amino acid is hydroxylated *after synthesis* of procollagen (p. 161) and the hydroxylases do not catalyze actions on free amino acids, it is reasonable to conclude that hydroxyproline excretion reflects *collagen degradation*. (The degradation could theoretically involve collagens from skin or other tissues, but there is no evidence that PTH affects them.)

Proposed Influences of PTH on Collagenolytic Enzymes. For a time there was a tendency to discard the halisteresis concepts in favor of the belief that PTH directly promotes destruction of bone matrix, and that mineral is released as a secondary consequence. This would agree with the knowledge that chronic high PTH levels lead to reduction of bone matrix as well as bone mineral content.

The finding that actinomycin D can block hypercalcemic actions of the hormone without interfering with effects on phosphate excretion led to suggestions that PTH induces synthesis of proteolytic enzymes and especially of collagenases in bone.

Histological data consistent with increased bone cell lysosome activity tend to support the concept. It has also been suggested that PTH promotes release of acid hydrolases, or that it interferes with procollagen utilization.

Halisteresis Reexamined.⁶ Some observers were surprised to learn that PTH promotes an early *increase* in calcium uptake, associated with early *fall* in plasma concentrations of the ion which precede the more sustained rise. The effect is attributed to a direct PTH influence on the plasma membrane (which can be elicited in thyroparathyroidectomized animals).

The resulting elevation of cytosol cal-

cium ion concentrations has both immediate and long range effects. *Citrate accumulates* because of inhibition of isocitric dehydrogenase activity; glycolysis and *lactate formation increase as carbon dioxide production is reduced; and cell pH is lowered.* Changes can be detected within 2 min after PTH administration.

It is reasonable to consider that *localized increases in citrate, lactate, and hydrogen ion can contribute substantially to solubilization of calcium and transfer of calcium from bone to extracellular fluids even when insufficient quantities of those substances enter the plasma to measurably increase their concentrations.*

High cytosol calcium also seems to activate "calcium pumps" on the membrane. If osteocytes have polarity, then calcium solubilization on the bone side combined with operation of pumps on the extracellular fluid side should result in transfer of calcium from bone to plasma. In a sense, this fits the halisteresis concept, but it involves the additional idea that bone cells participate actively in the calcium transfer.

The preceding supports the rapidly growing belief that *moment-to-moment regulation of plasma calcium concentrations can be accomplished with little matrix destruction; it does not conflict with evidence that long range PTH influences are very different.*

PTH promotes elevation of cytosol calcium concentrations in *kidney* and other tissues, and this, too, may contribute substantially to effects on the whole animal.

Concepts of PTH Action Based on Examination of Bone Histology. Bone Cells. When PTH is administered to young animals, and the bone is examined after a suitable time interval, it can be seen that the number of *osteoclasts is greater than the number found in controls not receiving the hormone, and the relative number of osteoblasts is reduced in those receiving PTH.*

Osteoclasts are characteristically found with their "ruffled borders" closely applied to sites of active bone resorption. It seems reasonable to infer that PTH promotes conversion of osteoblasts (bone-formers) to osteoclasts (bone resorbers), and thereby reduces calcium use for new

mineralization while releasing calcium from older bone. However, the idea is not supported by certain other observations. The time needed to demonstrate a measurable increase in osteoclast number far exceeds the time for substantial elevation of plasma calcium levels. (At least 2-4 hr after PTH administration are required for the mouse, 6-12 hr for the rat, and a full 24 hr for the rabbit.)

Osteoclasts probably do not directly initiate bone resorption, but (like other macrophages) they seem to be called in secondarily to mop up the debris; recent work implicates *osteocytes* in early phases of bone resorption and mineral release, while osteoclasts probably function in the much delayed, sustained release.

Studies utilizing tritium-labeled thymidine have failed to confirm an influence of PTH on conversion of osteoblasts to osteoclasts.*

When bone tissue is exposed briefly to radiatively labeled thymidine, the label is taken up rapidly by mesenchymal cells (the only bone cells believed to be capable of mitotic division). The label appears in osteoclasts some 12 hr later; this is consistent with conversion of newly formed cells to osteoclasts. Maximal labeling of osteoclasts has been described 48 hr after initial exposure, but labeling of osteoblasts was not observed to begin before 36 hr and to peak at 120 hr.

The findings are consistent with either conversion of *osteoclasts to osteoblasts*, or, alternatively, with *delayed conversion of mesenchymal cells to osteoblasts*. They are not consistent with conversion of *osteoblasts to osteoclasts*.

Studies of this kind cannot rule out the possibility that osteoblasts present before introduction of the tritiated thymidine did, in fact, undergo such a conversion. Additional reasons for believing that osteoclasts can be converted to osteoblasts, especially during times of bone remodeling, are presented below.

The best available evidence supports a stimulatory influence of PTH on mitosis of mesenchymal cells, leading to an increase in numbers of osteoblasts as well as osteoclasts, with effects of the hormone on functions and modulation of all cell types.

Ground Substance. About 1% of the organic phase of bone is present in the form of a "ground substance" which con-

tains mucopolysaccharides, some noncollagen protein, and small quantities of lipids. Both the mucopolysaccharides and the phospholipids are capable of binding large quantities of calcium. The functions of the ground substance have not been established.

Very early changes in staining properties (which precede changes in cell populations) are consistent with a rapid stimulation by PTH of *depolymerization of the mucopolysaccharides*. It has been proposed that this action "exposes" bone surfaces to proteolytic enzymes within the matrix, and possibly also to substances (citrate?) which facilitate transfer of calcium from bone to blood plasma.

While the possibility that some effects of PTH are mediated in this way has not been ruled out, there are other ideas concerning functions of the ground substance. One is that it provides a "protective coating" which must be locally removed before *mineralization* can proceed. It has been pointed out that whereas collagen components in the various tissues are similar, the quantity of ground substance in bone is small compared with amounts found in skin and other collagenous tissues which do not normally mineralize. A decrease in bone mucopolysaccharide, protein, and lipid content of ground substance just prior to mineralization has been described, and it has been shown that collagen derived from nonosseous sources can calcify when ground substance is removed.

A related concept is that since ground substance binds calcium, it makes the mineral unavailable to the bone; therefore, depolymerization should facilitate calcification. This is difficult to reconcile with another hypothesis that ground substance concentrates minerals for the purpose of orderly presentation to mineralizing surfaces.

Probably the most serious objection to the idea that depolymerization facilitates bone destruction and calcium transfer is that the collagenases needed to act on matrix seem to be located intracellularly.

RECENT WORK ON MECHANISM OF ACTION OF PARATHYROID HORMONE

Actions on Cell Membranes

There is no reason to suspect that PTH provides an exception to the rule that peptide hormones exert their primary actions on cell surfaces. The hormone has

been demonstrated to influence cell coats and aggregation properties as well as properties of the plasma membrane.

Evidence is rapidly accumulating for a direct stimulation of calcium uptake from extracellular fluids, not only by previously recognized "target organs" but also by (among others) cells of the thymus gland and bone marrow, chondrocytes, mammary gland epithelium, skeletal muscle, smooth muscles of the arterial system (and especially of vessels of liver and kidney), tumor cells in tissue culture, and possibly even neurons and cornea. The presence of physiological concentrations of phosphate in the medium increases the amount of calcium taken up, but PTH is effective in its absence.

In many cell types, PTH activates adenylate cyclase. cAMP does not directly stimulate calcium uptake, but it does further elevate cytosol calcium by promoting extrusion of calcium and phosphate from mitochondria, and in some cases by promoting inward movement of plasma membrane-associated calcium.

Calcium efflux from cells is enhanced both PTH and cAMP (or its analogs). This has been attributed to increased passive movement of the ion as well as activation of "calcium pumps"; both effects are dependent on high cytosol calcium levels. In cells possessing polarity, increased influx at one surface and increased efflux at another can result in net calcium transport.

cAMP has been implicated in a variety of processes in addition to influences on cytosol concentration. Very high cytosol concentrations of calcium inhibit adenylate cyclase. Maintenance of the cAMP concentration therefore depends on an adequate rate of calcium efflux. On the other hand, cAMP influences on mitochondrial extrusion of calcium and phosphate can be continued only when sufficient calcium uptake supports the supply to the mitochondria. Usually, administration of PTH or of cAMP (or both) results in some depletion of mitochondrial calcium content.

The problem of predicting the net effect of cAMP on cytosol concentrations is complicated by the fact that the nucleotide may also enhance microsomal calcium uptake.

Cytosol calcium can also be raised by addition of calcium salts to extracellular fluids, and some PTH influences can be

mimicked in this way. Either high calcium, or PTH and normal calcium concentrations, can stimulate proliferation of thymus, bone marrow, and regenerating liver cells. Some PTH effects require cAMP, while others may not. The hormone stimulates adenylate cyclase of thymocytes but does not increase cAMP content of the liver cells.

When PTH stimulates adenylate cyclase, the effects of elevated cAMP concentrations depend on the nature of the cell affected. Some confusion was introduced by the observation that actinomycin D can be used, under specified conditions, to block the hypercalcemic actions of PTH while leaving the phosphaturic action unimpaired. It was suggested that the initial actions of PTH on the two target tissues are qualitatively different, but it is now recognized that a very early effect on both tissues is activation of adenylate cyclase.

Effects of PTH on Bone^{6, 8}

Influences of PTH on bone cells are very difficult to study because the rigid extracellular matrix and scant fluid provide serious technical problems for measurements of extracellular ion concentrations and of rapid metabolic changes within cells. Moreover, bone contains many different kinds of cells (at least from the standpoint of function if not origin), and it is necessary to examine each type separately. Methods have not yet been perfected for complete separation, but it has been shown that very superficial scraping of bone surfaces yields predominantly mesenchymal and osteoblast cells, while deeper grindings yield preparations of mostly osteocytes. Some insight into PTH action can also be obtained from study of soft tissues.

Mesenchymal Cells. PTH promotes RNA and DNA synthesis and *stimulates mitosis*. It is likely that the action is mediated by effects of the hormone on cytosol calcium concentrations, but the importance of elevated cAMP levels and effects of the nucleotide on microtubular function, activation of protein kinases, and on gene expression have not been fully evaluated.

In studies of bone (in which cell separation was not attempted), it was shown PTH stimulated both DNA and RNA synthesis, but high calcium concentrations of the medium stimulated only RNA synthesis. This suggests that

either PTH influences cell proliferation in bone by some mechanism other than elevation of cytosol calcium, or else that intracellular calcium is not sufficiently elevated in the absence of PTH even when the medium is rich in calcium.

There are conflicting ideas concerning the sequence of changes taking place during maturation of the mesenchymal cells. Much of the available data is consistent with the concept that mesenchymal cells are converted to osteoclasts (passing through a stage which has been called preosteoclast), with subsequent modulation to osteoblasts.

The labeled thymidine studies (p. 166) could be interpreted in this way. Moreover, it is known that PTH promotes not only bone resorption, but also bone remodeling, and that the remodeling process seems to involve first osteoclastic resorption followed by new bone formation. (Reduction of bone mass has been attributed to an imbalance in which the rate of resorption exceeds the rate of new formation.) An effect of PTH-stimulated formation of osteoclasts, with later transformation (modulation) to osteoblasts is also compatible with observations of decreased remodeling in chronic parathyroid hormone deficiency.

Administration of PTH leads to an increase in osteoclast number and a decrease in relative osteoblast number. This effect could be explained on the basis of stimulation by the hormone of rapid osteoclast formation, with delayed modulation to osteoblasts.

Another possibility is that there are two (or more) types of mesenchymal cells, and that PTH preferentially stimulates mitosis of mesenchymal osteoclast precursors but exerts an inhibitory or delayed influence on mesenchymal osteoblast precursors.

Since mesenchymal cells contain less calcium than the other bone cell types, and since appearance of daughter cells requires many hours, it is unlikely that effects of PTH on mesenchymal cells contribute substantially (if at all) to moment-to-moment regulation of calcium homeostasis.

Osteoclasts. PTH seems to act more rapidly on existing osteoclasts than on formation of new ones. It promotes synthesis of both RNA and protein.

Effects on incorporation of labeled uridine into RNA and of leucine into protein and glucosamine into mucoprotein are observable within 2-3 hr after administration of the hormone and persist for at least 24 hr.

Increased density of *mitochondrial granules*, and evidence of increased *lysosomal* and *phagocytic* activity have been described. (It is known that high calcium concentrations can also enhance phagocytosis and lysosome disruption.)

It is likely that the described influences on osteoclasts are directly related to (*delayed*) *stimulation of osteoclastic bone resorption* which follows administration of PTH. Since the bone surface on which osteoclasts can act is relatively large, the total quantity of calcium transfer from bone to plasma theoretically resulting from PTH influences on osteoclasts could be very great, and there is probably a close association between the quantities of calcium transferred and the amount of matrix destroyed (by lysosomal enzymes and by associated uptake and intracellular digestion of collagen).

It is unlikely, however, that *rapid* rises in plasma calcium observed following administration of PTH are dependent upon the described influences of the hormone on osteoclast metabolism.

There is also evidence that *PTH prolongs the life of existing osteoclasts and delays their modulation to other bone cell types.*

Osteocytes. These cells exhibit the most rapid responses and are affected by small amounts of PTH. Since they are also the most numerous cells in mature bone, they probably play a major role in calcium homeostasis. Within minutes after hormone administration they increase *lactate* and *citrate* production. Early increases in sizes of both the cells and the surrounding spaces are consistent with *perilacunar demineralization* and with increased density of mitochondrial granules with calcium transport. Histochemistry indicates rapid depolymerization of matrix mucopolysaccharides, followed soon afterward by enhanced lysosomal activity. Osteolytic osteocytes probably account for the hydroxyproline excretion seen soon after administration of PTH (but delayed effects require participation of the osteoclasts).

It is generally agreed that osteocytes are

formed by modulation of osteoblasts, and there are suggestions that PTH increases the rate at which modulation is accomplished.

During the normal course of their existence, osteocytes seem to function first as bone formers and later as bone resorbers, and they may pass reversibly from one form to the other in response to changes in calcium concentrations and PTH levels.

A mechanism for participation of bone-forming osteocytes in mineralization has been proposed which involves uptake of calcium salts by the endoplasmic reticulum and incorporation of the salts into protein-containing vesicle membranes. The vesicles are then said to be extruded at the mineralization front; phosphatase enzymes released from the vesicles contributes to the mineralization process. (cAMP enhances microsomal calcium uptake.)

Osteoblasts. *PTH inhibits RNA and collagen synthesis* and reduces the quantity of matrix synthesized per cell. Decreased alkaline phosphatase activity and decreased rate of incorporation of glycine and proline into collagen have been found after PTH administration. Inhibition of isocitric dehydrogenase activity probably depletes α -ketoglutarate needed for collagen synthesis (p. 161). PTH also seems to promote conversion of osteoblasts to osteocytes. All of the preceding should reduce the amounts of calcium taken up by bone.

PTH promotes a *delayed increase* in the number of osteoblasts, but the quantity of matrix synthesized per cell remains small. The fact that this occurs later than the increase in osteoclast number, combined with the thymidine studies mentioned above, points to formation of osteoblasts from osteoclasts. (There have been suggestions that only certain osteoclasts can be modulated in this way, while others arising from macrophages cannot.)

Participation of Additional Cell Types. Histological studies support the concept that *endothelial cells* of capillaries supplying bone canaliculi participate in transfer of calcium from bone to plasma. There have been additional suggestions that blood vessel cells can, when appropriately stimulated, give rise to true bone cells.

The number of *mast cells* in bone rises after administration of PTH or a calcium-deficient diet to young rats with intact parathyroid glands.¹ Mast cell granules are rich in histamine

and heparin, and high concentrations of these substances may be released locally. Both can enhance phagocytic activity in connective tissues. Heparin has been implicated as a cofactor in osteoclastic bone resorption; it can also bind large quantities of calcium.

It is known that patients receiving long term heparin treatment (for prevention of intravascular clotting) tend to develop osteoporosis. On the other hand, heparin has been implicated by some investigators in polymerization and maturation of collagen.

Actions of PTH on the Kidney

PTH elevates cAMP concentrations in the renal cortex, and promotes increases in urinary cAMP. This can be observed even earlier than phosphaturia.

Neither PTH nor cAMP is capable of promoting characteristic effects on the kidney in a calcium-free medium, and it is likely that the hormonal influences on the kidney are related to elevation of cytosol calcium concentrations in the cells of the renal tubule.

The *phosphaturic* effect is direct, and can be demonstrated on isolated perfused renal tubules. It is also rapid; significant increases in phosphate excretion are apparent within minutes after administration of the hormone. The timing of the response, plus the fact that it is not inhibited by administration of either actinomycin D or puromycin, makes it unlikely that synthesis of new protein is required.

In most species, increased phosphate excretion seems to be attributable entirely to inhibition of phosphate reabsorption in the proximal tubule, and this is accompanied by reduced reabsorption of sodium. However, in a few species (e.g., the chicken), it has been shown that more phosphate is excreted than filtered, and an additional effect on the distal tubule seems to contribute to the overall effect.

According to one hypothesis, peritubular uptake of phosphate leads to increased intracellular phosphate concentrations, and this influences phosphate reabsorption.

PTH also increases the excretion of amino acids, potassium, and bicarbonate. Details of the mechanism are not completely clear, but it is likely that the effect on amino acids is closely related to mechanisms for inhibition of sodium reabsorption in the proximal tubule, while effects on potassium and bicarbonate may be related to a reduced rate of sodium delivery to the

distal tubule. An effect on *calcium reabsorption* was noted above.

PTH elevation of renal cytosol calcium profoundly influences carbohydrate metabolism (and the actions can be mimicked by addition of calcium to the extracellular fluids or by administration of cAMP analogs). Increased activity of phosphoenol-pyruvate carboxykinase and inhibition of pyruvate kinase lead to glucose formation. Lowering of cytosol pH (secondary to calcium and phosphate extrusion from mitochondria) contributes to the effect, and this can be shown by artificially raising cytosol hydrogen ion. The connecting links between effects on carbohydrate metabolism and contribution to calcium and phosphate homeostasis have not been established.

Actions of PTH on the Intestine

The kidney is the major (if not the sole) site for formation of metabolites of vitamin D which regulate intestinal absorption of calcium (Chapter 15). PTH plays a role in regulation of activity of the enzymes involved. Actions of PTH on the intestine seem to result primarily from such influences.

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(see also References 7, 8, and 11, p. 178)

15. Vitamin D

Vitamin D is more properly classified as a hormone than as an essential food (Chapter 1). It can be totally synthesized endogenously, and it travels through the bloodstream. Minute quantities exert actions on "target organs" which resemble those of "typical" hormones.

Vitamin D metabolites promote absorption of calcium from the intestine, exert direct influences on bone, and support many PTH functions.

SOURCES AND CHEMISTRY OF THE D VITAMINS

Endogenous Synthesis

Cholesterol is synthesized in the liver from the ubiquitous acetyl coenzyme A. It travels with the bile to the small intestine, in which a dehydrogenase enzyme catalyzes its conversion to *7-dehydrocholesterol* or *provitamin D* (Fig. 15-1, A and B).

The provitamin is then carried by the circulation to the skin. In the presence of appropriate ultraviolet radiation (sunlight), a photochemical reaction transforms it into *vitamin D₃*, or *cholecalciferol* (Fig. 15-1C). Sunlight acting on the skin also promotes formation of a plasma protein which binds a vitamin D metabolite, *25-hydroxycholecalciferol* (see below).

Sufficient cholecalciferol may be synthesized in this way to meet metabolic needs. Skin pigments (including those acquired during suntanning) protect against hypervitaminosis.

Dietary Sources

Most animals synthesize and store sufficient D₃ during the summer months to supply year-round needs. But humans living indoors and covering the skin with clothing require some vitamin in the diet; this is especially true of pregnant women and growing infants and children. Since the vitamin is stored in the liver, preparations of fish liver oils (and their concentrates) are widely used. Cholecalciferol can also be prepared synthetically by irradiation of precursor molecules.

Plants synthesize the provitamin *ergosterol* (Fig. 15-1E), which can be artificially

irradiated to yield *vitamin D₂* (calciferol, ergocalciferol, viosterol, Fig. 15-1F). Most species, including humans, effectively utilize vitamin D₂, since enzymes acting on D₃ and its metabolites also act on D₂; but a few (chickens and some of the monkeys) do not respond to D₂.

The term "D₁" is no longer seen in the literature; preparations designated by this name were shown to contain a mixture of D₂ and lumisterol, an intermediate in the conversion of ergosterol to D₂.

Absorption of vitamin D requires bile salts, and is enhanced by simultaneous absorption of other lipids. The vitamin is taken up by the lacteals, and has been identified in chylomicrons.

Hepatic storage of vitamin D is especially important for animals whose habitat provides the necessary ultraviolet radiation during only restricted times of the year. Fairly large quantities are avidly bound to a plasma lipoprotein; high concentrations have been found 6 months after administration of a single massive dose. Additional storage takes place in adipose and other lipid-rich tissues. Small amounts are excreted with the bile and lost through the feces, and still smaller amounts appear in the urine.

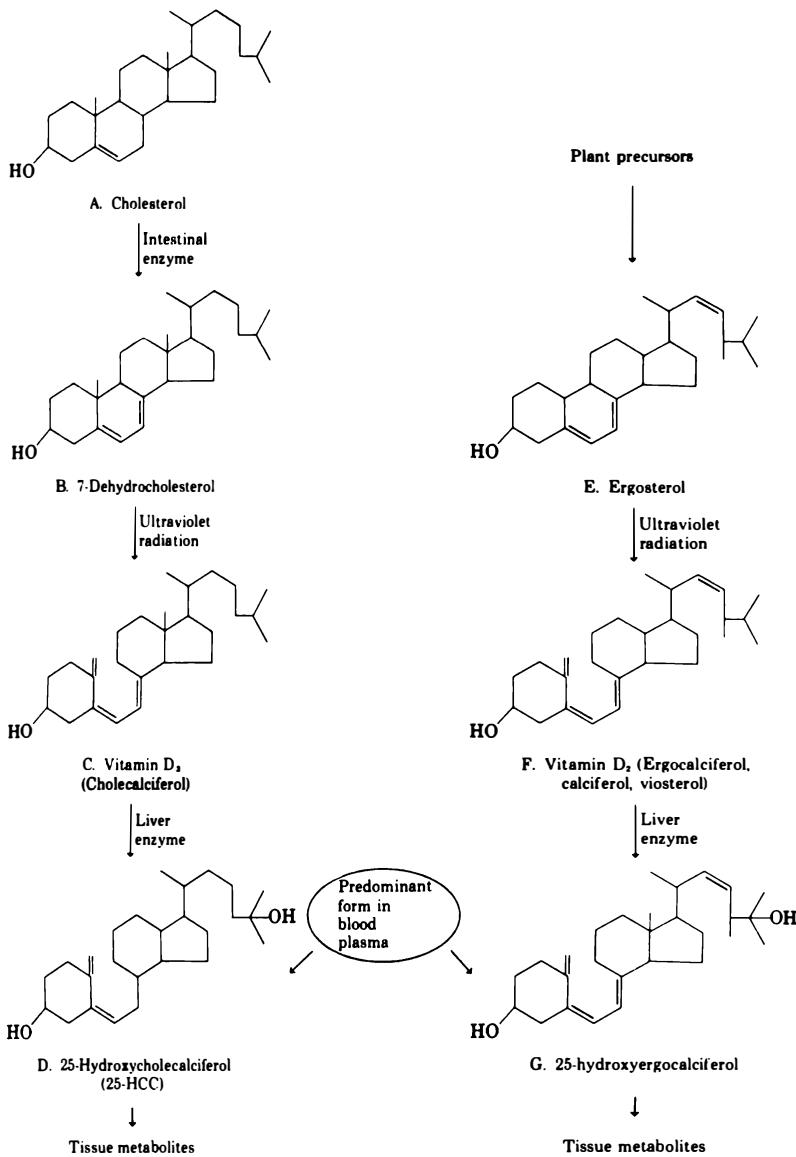
Formation of Active Principles^{6,10}

The vitamin must be activated before it can act. Massive doses are ineffective in preparations (bone cultures, perfused isolated intestine) lacking enzymes needed for the metabolic conversions.

Hydroxylation at Carbon 25

A hepatic D₃ 25-hydroxylase system catalyzes formation of *25-hydroxycholecalciferol* (25-OH-cholecalciferol, 25-OH-D₃, 25-HCC, Fig. 15-1D). In most species it also promotes formation of 25-OH-ergocalciferol (25-OH-D₂).

The system includes a mitochondrial enzyme and a cytoplasmic protein, and utilizes molecular oxygen and NADP. Activity is influenced by other processes within the liver. (For example, chronic ingestion of barbiturates and certain

FIG. 15.1 Vitamin D₃ precursors, and metabolites in blood.

other drugs affects both formation and utilization of 25-HCC.)

Formation of the 25-OH metabolite is *self-limiting* when D₃ intake does not drastically exceed physiological requirements, because the product of the reaction exerts *negative feedback control* over enzyme activity. Two purposes are served: protection is afforded against deleterious effects of too much 25-HCC (extending at least partially

even to injudicious ingestors of tablets containing 100 times the dietary requirements), while a mechanism is provided for prolonging the presence of the intermittently available vitamin.

Inhibition of 25-hydroxylation does not protect against ingestion of massive doses of D₃. The total quantity of 25-HCC formed can be very large. Accumulation in the *blood plasma* does *not* influence enzymatic processes within liver cells.

Failure to appreciate the capacity of certain animals to store D_3 and 25-HCC has led to unfortunate poisoning of Arctic explorers eating polar bear liver. It has been suggested that some forms of D_3 sensitivity can be attributed to defects in ability to inhibit the 25-hydroxylase system.

Most of the 25-HCC formed in the liver enters the circulating blood and binds to a specific protein (different from the one that binds D_3); 25-HCC is the major D metabolite of the plasma. Some is stored in the liver. The presence of the binding protein in many other tissues suggests that small amounts are widely distributed throughout the body. Small quantities of 25-HCC are formed extrahepatically, and the necessary enzymes have been identified in the kidney.

When given to intact animals, 25-HCC acts more rapidly than D_3 to promote calcium absorption from the intestine and mobilization from bone. It is also far more potent for treatment of *pseudo-vitamin D deficiency* (attributed in part to inability to activate the vitamin).

Formation of 1,25-Dihydroxycholecalciferol (1,25-HCC)

Only massive doses of 25-HCC are active *in vitro*, and they are relatively ineffective in patients with severe *kidney* damage. The kidney contains a *1-hydroxylase system* which catalyzes conversion of 25-HCC to 1,25-dihydroxycholecalciferol (1,25-HCC, Fig. 15-2C). The latter is the major metabolite found in the kidney, and it is the form which promotes intestinal calcium absorption.

Unlike D_3 and 25-HCC, it is highly effective *in vitro*. Stimulation of calcium absorption is seen in intact animals within 2 hr (compared with more than 8 required when 25-HCC is given). The dihydroxy-metabolite is also found in *bone*, and it promotes calcium mobilization both *in vivo* and *in vitro*.

Hydroxylation at the 1-carbon seems to be the *rate-limiting* step for vitamin D action on the intestine.

Regulation of Synthesis of 1,25-HCC

Calcium. High calcium ion concentrations inhibit the 1-hydroxylase, and thereby

provide the major control over intestinal absorption of the ion.

Parathyroid Hormone. When intact animals are maintained on calcium-deficient diets, the rate of 1,25-HCC formation is rapid. However, *parathyroidectomized* animal synthesize very little of the metabolite even when dietary and plasma calcium are low. The ability to synthesize 1,25-HCC can be restored with 24 hr after administration of PTH. This suggests that PTH may be needed for synthesis of the 1-hydroxylase enzyme, and that low calcium diets act in two ways: one directly on the enzyme activity, and the other on promotion of PTH secretion. (Calcium concentrations also affect calcitonin secretion and availability of inorganic phosphate; see below.)

Phosphate. Low phosphate diets increase the rate of formation of 1,25-HCC even when calcium content is high. Phosphate ions may directly inhibit the 1-hydroxylase enzyme, but studies comparing effects of varying ion concentrations suggest that the calcium:phosphate ratio may be more important than the absolute concentration of phosphate.

Calcitonin. This hormone is described in Chapter 16. It exerts inhibitory actions on the 1-hydroxylase enzyme.

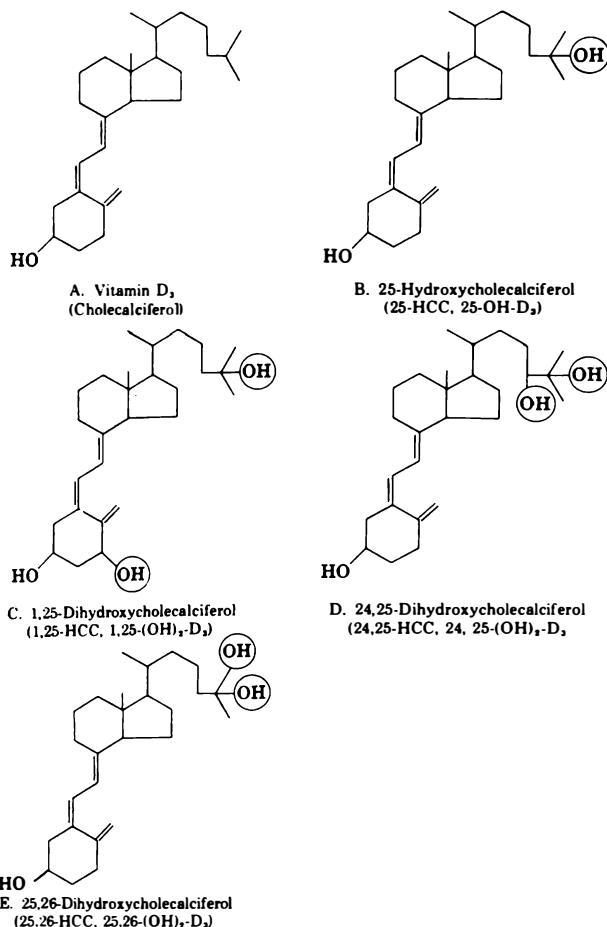
cAMP. PTH activates renal adenylate cyclase, and influences of the hormone on the 1-hydroxylase system seem to be mediated via cAMP. Very high calcium concentrations inhibit adenylate cyclase.

Inhibitory influences of *very high doses* of either cAMP or PTH have not been explained; the actions may be purely pharmacological.

Other Vitamin D Metabolites

Under conditions when 1-hydroxylase activity is low, measurable amounts of 24,25-dihydroxycholecalciferol (24,25-HCC) can be detected in blood plasma. Relatively large quantities have been found in calcium-deficient and in parathyroidectomized rats. The source has not been established, but a prevailing hypothesis states that a 24-hydroxylase system of the kidney becomes active when the 1-hydroxylase system is inhibited.

24,25-HCC has little influence on intestinal absorption of calcium, but it is almost as effective as 1,25-HCC for mobilization of bone calcium. It has been suggested that the 24-hydroxylase system provides an agent which supports PTH actions on bone during periods of

FIG. 15-2. Vitamin D₃ and metabolites found in blood and tissues.

high calcium intake. On the other hand, very little of the metabolite has been found in bone; it may exert primary actions on the kidney. An inhibitory influence on secretion of PTH has been described.¹¹

25,26-HCC is another metabolite found in plasma and in low concentrations in bone. No functions have been found for this steroid, which seems to be synthesized in the liver.

In Figure 15-1, the structures of D₃ and of 25-HCC are presented in a form which permits recognition of the chemical relationship to cholesterol. In Figure 15-2, the "open" form of the molecule is shown since there is a trend toward representation of the molecules in this way in the recent literature. (Neither of the convenient rep-

resentations is intended to convey information on the three-dimensional structure of the molecules.)

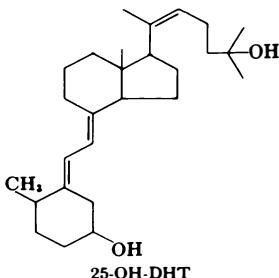
Synthetic Clinically Useful Vitamin D Analogs

Vitamin D is used in moderate dosage to promote calcium absorption in vitamin deficiency diseases (rickets in children, osteomalacia in adults). Since very large doses exert PTH-like actions on bone, they have been used to maintain plasma calcium levels of patients with hypoparathyroidism.

A synthetic steroid, *dihydrotachysterol* (DHT, AT₁₀) has been found more effective for treatment of hypoparathyroidism (but less useful than the natural vitamin in management of rickets). Greater effectiveness of the synthetic

agent for bone mobilization has been attributed to its rapid conversion to a 25-OH metabolite, and to the fact that the product does not exert inhibitory control over the hepatic hydroxylase system.¹

It is believed that 25-OH-DHT acts directly on bone, without need for additional hydroxylation in position 1. If this is true, the effectiveness of AT₁₀ in patients with kidney disorders affecting the 1-hydroxylase system is understandable. 25-OH-AT₁₀ is now available for clinical use.



Procedures have not yet been developed for production of large quantities of 1,25-HCC for use in patients with kidney disease, but 1-OH-D₃¹⁰, 3-deoxy-1-OH-D₃¹⁰, and other synthetic analogs have been used with good results.

Mechanisms for Intestinal Absorption of Calcium

Calcium is absorbed from the mucosal surface by cells possessing a well developed

ACTIONS OF VITAMIN D METABOLITES ON THE INTESTINE

So much emphasis is placed on the need for adequate nutrition that the importance of protection against excess dietary constituents often goes unrecognized. In the case of calcium, biological safeguards are numerous. Calcium absorption is a complicated process which does not proceed to any measurable extent in the absence of 1,25-HCC. Since much of the research in this area has utilized vitamin D rather than its metabolites, the term "D" is used in the following discussion.

"brush border" (system of microvilli) and extracellular glycocalyx. Intermingled with these are small numbers of mucus-secreting goblet cells.

Calcium enters the cytoplasm and is extruded at the serosal surface for passage into extracellular fluids in contact with

blood capillaries. Passage may be down a concentration gradient at the mucosal side (especially when the diet is rich in calcium), but it goes against the gradient at the opposite side. There are debates about whether mucosal uptake is passive. Most investigators favor the concept of facilitated diffusion requiring a specific carrier, but no carrier has been identified.

The fact that ATP is required, the presence of a calcium-activated ATPase at the brush border surface, and evidence that D stimulates ATPase activity (possibly through induction of specific proteins) all point to an active process. However, it is possible that ATP functions in calcium utilization (e.g., incorporation of cytosol calcium into organelles), and that the rate of calcium uptake is limited by what happens afterward.

Direct Actions of D on the Brush Border Membrane

One hypothesis states that D acts directly on the brush border membrane to increase permeability and therefore passive uptake. However, no influence of 1,25-HCC has been observed earlier than 1½ to 2 hr after administration of the hormone.

It has been reported that administration of D induces changes in the phospholipid and fatty acid content of the membrane. Rates of ³²P uptake, relative content of long chain polyunsaturated fatty acids, and phospholipid:cholesterol ratio are increased. Therefore, a slowly developing structural change affecting permeability is possible.

Influences on Carrier Proteins^{3, 5}

A second hypothesis states that D induces formation of calcium carrier proteins. A mechanism of action similar to that of other steroid hormones has been proposed. Evidence has been presented for association of 1,25-HCC with a cytoplasmic receptor, translocation of a steroid-receptor complex to the nucleus, association with chromatin, and induction of a messenger which codes for synthesis of a specific protein.⁶

A calcium-binding protein (CaBP) has been found in the soluble fraction from mucus cells and on the glycocalyx of the brush border cells after administration of

D. The protein is deficient in quantity or undetectable in chronic vitamin deficiency states.

An inverse relationship between the amount of extractable CaBP and the calcium content of the diet has been described, and this suggests dependence on the quantity of 1,25-HCC formed. However, it has been pointed out that influences of D on lipid components of cells can affect the quantities of proteins that are extractable.

Addition of purified CaBP to intestinal sacs *in vitro* increases the rate of calcium transport. However, the role of CaBP remains to be established.

Kinetic studies have cast doubt on participation of CaBP in an essential step in calcium uptake. D enhances calcium uptake before increases in CaBP are detectable, and it is possible that elevation of cytosol calcium promotes conversion of a pro-CaBP to CaBP. Glucocorticoids reduce the rate of calcium uptake, but may increase CaBP formation. (An effect of glucocorticoids on CaBP utilization has not been ruled out.)

Actinomycin D can inhibit formation of CaBP without influencing D stimulation of calcium transport; but the antibiotic may influence calcium movements in unnatural ways. Filipin affects the structure of the brush border membranes, and simultaneously promotes calcium uptake, but it has not been demonstrated that this agent mimics D actions.

Conflicting interpretations have been attached to unrelated observations that both actinomycin D and cycloheximide can, under some conditions, interfere with D stimulation. One proposal is that the inhibitors interfere with conversion of 25-HCC to 1,25-HCC; another is that sufficient antibiotic was given to exert nonspecific toxicity.

A calcium-binding protein has been found in kidney. Its origin and functions are unknown. Transport of calcium in bone does not seem to involve a CaBP, and none has been found in bone.

Influences on the Cell Nucleus

The possibility that 1,25-HCC acts like "typical" steroid hormones to promote formation of messengers coding for specific proteins was mentioned above. However, unlike the other steroids, D metabolites have been found associated with nuclear membranes as well as with the chromatin,⁴ and 1,25-HCC is effective when RNA synthesis is inhibited.

DNA and RNA synthesis are increased following exposure of intestinal cells to D. The possibility that the effects are mediated via changes in cytosol calcium concentrations has not been excluded. Moreover, relatively early increases in adenylate cyclase activity have been found *in vitro* following addition of 1,25-HCC; therefore, participation of cAMP should also be considered.

Mitochondria

It is likely that calcium entering at the brush border passes through mitochondria before being extruded at the serosal surface. The process may involve migration of calcium-laden organelles from the brush border to the opposite cell surface, with return of depleted ones. An effect of D on mitochondria is known, and it has been reported that CaBP affects mitochondrial uptake of calcium.

Calcium-rich, electron-dense granules have been found within the microvilli of brush border cells in vitamin D-deficient animals; after D administration, the number of granules in the microvilli decreases, while those in the mitochondria increase.

ATPase

It is agreed that extrusion of calcium at the serosal surface is an energy-utilizing process, and it has been proposed that D activates a calcium-dependent ATPase. But sodium is required at the serosal surface, and there is evidence in favor of coupling of calcium to the sodium-potassium pump. Energy is used to actively export sodium; energy derived from reentry of sodium down a concentration gradient is then utilized for extrusion of calcium. (High levels of sodium at the mucosal surface may actually slow calcium entry.) An older concept that D promotes calcium absorption by inhibiting passage of calcium from the blood back across the serosal surface has been discarded.

Vitamin D and Intestinal Transport of Phosphate

D stimulation of calcium transport does not seem to depend on the presence of

inorganic phosphate, but high phosphate concentrations can accelerate calcium uptake by *mechanisms independent of the hormone*. They can also secondarily induce inhibition of calcium uptake through interference with formation of 1,25-HCC from 25-HCC.

An early stimulation of phosphate transport (preceding changes in calcium uptake) has been described after addition of 1,25-HCC to media surrounding intestinal cell explants.

It has been frequently stated that intestinal absorption of phosphate is directly dependent upon the presence of calcium, but maximal uptake of the two ions takes place in different parts of the small intestine. Evidently, additional information is needed to clarify the interrelationships.

ACTIONS OF VITAMIN D METABOLITES ON BONE

The functions of 1,25-HCC and of PTH are closely interrelated. Two early influences of PTH are increased cellular uptake of calcium and activation of adenylate cyclase. Both PTH and cAMP promote transfer of calcium from mitochondria to the cytosol. All of the preceding lead to elevation of cytosol calcium ion concentration.

In vitamin D deficiency states, PTH still promotes calcium uptake from the medium; however, impaired intestinal absorption of calcium reduces its availability in extracellular fluids. PTH also stimulates adenylate cyclase. (cAMP levels may be especially high because inhibitory influences of high calcium concentrations on the cyclase are not operative.) Both PTH and cAMP can still promote transfer of calcium from mitochondria to the cytosol, but the *mitochondrial stores* of calcium are low. Therefore the rise in cytosol calcium ion concentration is sharply limited. Early influences of PTH on osteocytes can occur to some extent, but osteoblasts secrete inappropriate quantities of calcifiable matrix, and calcium which might otherwise have been transferred to the plasma is taken up by the matrix.

Vitamin D metabolites mobilize bone calcium. Physiological concentrations transfer the ion from old, resorbing bone to new sites of bone formation. Very large

doses stimulate osteoclastic bone resorption to the extent that sufficient calcium is mobilized to markedly elevate plasma calcium concentrations; therefore such doses are useful for maintaining plasma calcium concentrations in parathyroid hormone deficiency states.

Toxic doses promote bone destruction, excessive elevation of plasma calcium concentrations, and metastatic calcification. This has occurred in infants accidentally overdosed by parents unaware of the high potency of some commercial concentrates, and in patients given massive doses for conditions unrelated to calcium imbalance. (Fortunately, the practice of using such preparations without apparent rationale in the treatment of arthritis is no longer prevalent.)

Calcium mobilizing and bone-resorbing actions of *small to moderate* doses of D vitamins seem to depend on increasing the sensitivity to actions of PTH; they are ineffective in parathyroidectomized animals. There are some indications that vitamin D may also exert a direct influence on the parathyroid gland, promoting conversion of pro-PTH to PTH.

It is possible that extremely large doses of vitamin D and its analogs act on bone in a manner different from that of more physiological quantities. Toxic doses are effective in parathyroidectomized animals.

ACTIONS OF D VITAMINS ON THE KIDNEY

While the major actions of D seem to be exerted on intestine and bone, it has been clearly demonstrated that physiological doses promote a rapid stimulation of calcium, phosphate, and sodium reabsorption by the proximal tubule, and these actions are of sufficient magnitude to supplement the effects of the hormone on bone. Toxic doses promote phosphaturia, and this supports the belief that pharmacological effects are qualitatively different from physiological ones.

In vitamin D-deficient animals, PTH exerts expected promotion of phosphaturia, but the action cannot be sustained. This is probably because the phosphaturic action depends upon ability of PTH to elevate renal cytosol calcium ion concentrations.

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16. Calcitonin and Other Hormones Regulating Calcium Metabolism

SOURCES OF CALCITONIN^{4, 12}

The parathyroid glands of most mammalian species are closely associated with or embedded within the thyroid glands. Since they are also small, sometimes difficult to visualize, and often variable in size and number, "parathyroidectomy" has in many cases involved removal of the entire thyroid gland. It was once believed that administration of thyroxine could compensate for loss of the thyroid gland.

The discovery that thyroparathyroidectomized animals given calcium infusions have difficulty restoring plasma calcium concentrations led to a search for a hormone which lowers blood calcium. Evidence for the existence of the hormone was soon obtained, and it was named *calcitonin*.

It was assumed at first that calcitonin (CT) is secreted by the parathyroid glands. But the gradual unfolding of an endocrinological detective story culminated in the realization that most of the calcium-lowering principle derives from the thyroid gland, and specifically from the *parafollicular* (interfollicular), "light" or "C" cells. These can be readily distinguished on the basis of location and histological appearance from the follicular cells which secrete

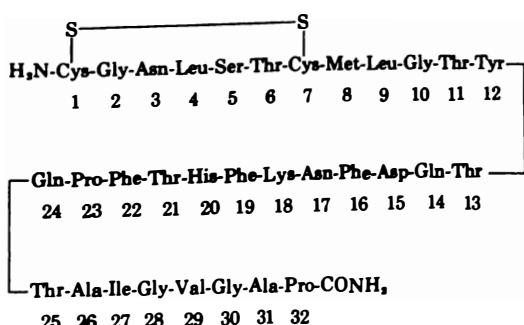
thyroxine and triiodothyronine. The name thyrocalcitonin (TCT) was adopted briefly, but more recently the name calcitonin has gained general favor.

The cells which secrete calcitonin are now known to originate in the neural crests, and to migrate from there to the pharyngeal pouches of vertebrate embryos. In fishes, amphibians, reptiles, and birds they become organized into the *ultrabranchial bodies* (see also Chapter 25).

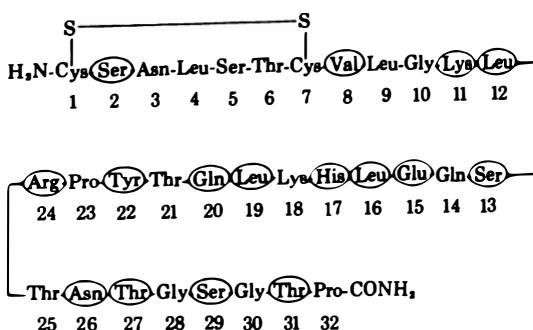
CHEMICAL NATURE OF THE CALCITONINS^{4, 12, 14}

All of the known calcitonins are single chain polypeptides containing 32 amino acid residues, with a disulfide bridge connecting cysteines at positions 1 and 7, and a C-terminal proline. The structures of human and salmon calcitonins are compared in Figure 16-1.

Some of the fish hormones appear to be more potent than mammalian preparations when bioassayed in mammals. Differences have been related to variations in amino acid composition which affect (among other things) the rate of hormone removal and degradation by the liver and kidney. Synthetic calcitonins are now available for investigative and therapeutic use.



A. Human thyrocalcitonin.



B. Salmon thyrocalcitonin.

FIG. 16-1. Comparison of structures of human and salmon calcitonins.

FUNCTIONS OF THE CALCITONINS^{2, 8, 13}

A heated debate is raging over whether CT performs functions in mammals, or whether its presence represents nothing more than an evolutionary relic still with us because it confers no special disadvantage. (The hormones have been identified in every vertebrate class from fishes to man, but not so far in the cyclostomes.)

The vestigial nature of calcitonin-secreting tissue is suggested by the contrast between the scattered C cells of the mammalian thyroid gland and the highly organized, innervated ultimobranchial glands of other vertebrates.

Protection Against Hypercalcemia

The function most widely attributed to CT is that of lowering plasma calcium ion concentrations when they rise excessively;

this may be accomplished largely through inhibition of bone resorption.

Arguments against a Calcium-lowering Function in Mammals. It has been stated that the normal mammal does not require protection against hypercalcemia, since bone avidly takes up calcium and continuous secretion of PTH is needed to keep this under control. If the calcium content of the blood rises just slightly above normal values, PTH secretion is rapidly inhibited.

By contrast, salt-water fishes are surrounded by a medium rich in calcium against which they require protection, while birds must cope with both sustained high PTH secretion which keeps the bones light enough for flight, and with accommodations to egg-laying processes.

It has also been stated that effects of CT on plasma calcium concentrations of normal mammals are of little consequence,

and that no physiological significance should be attached to observations on animals infused with supraphysiological quantities of calcium salts. Thyroparathyroidectomized animals bearing parathyroid implants maintain calcium within normal limits, and patients with CT-secreting medullary carcinomas of the thyroid gland do not suffer from hypocalcemia.

Arguments in favor of a Calcium-lowering Function. There is good reason to question the concept that mammals do not require hormonal safeguards against development of hypercalcemia. An obvious problem is that calcium inhibition of PTH secretion cannot rapidly lower elevated plasma calcium concentrations since this does not provide for destruction of *preexisting* PTH in plasma and tissues or for antagonism of long range influences of previously secreted hormone.

Mammals limit the rate of calcium entry into the blood in many ways (Chapter 15), and there are several routes whereby excess plasma calcium is removed (Chapters 14 and 15). Additional influences are described in this chapter. Multiple mechanisms rarely exist for trivial functions.

The argument that patients with CT-secreting tumors do not suffer from hypocalcemia is weakened by observations that they usually have hyperplastic parathyroid glands.

An interesting explanation has been offered for the failure of administered CT to induce hypocalcemia when PTH secretion is limited. CT seems to affect bone resorption *only when calcium concentrations are elevated*.

Hypercalcemia can be induced in experimental animals by feeding a calcium-rich meal after a period of fasting.¹⁴ CT secretion is stimulated at such times, and this seems to be triggered by both the presence of calcium in the gastrointestinal tract and by gut hormones released during digestion. It has been proposed that CT not only protects against hypercalcemia under such circumstances, but that it also promotes conservation of that portion of the dietary calcium which would otherwise be lost in the urine. The effectiveness of CT under these conditions can be appreciated by comparing plasma and urinary calcium concentrations of intact animals with those of animals deprived of thyroid glands.

The failure of endogenous CT to provide adequate protection against chronic administration of massive doses of PTH or of vitamin D can be interpreted in several ways. The most obvious include (1) CT is not important; (2) conditions would be even worse without CT; and (3) toxicity is encountered only when the CT response is inadequate or when effects of highly potent agents overwhelm CT influences.

Some Unanswered Questions. Younger animals are consistently more responsive than older ones to administration of CT. This has been attributed to the existence in growing animals of a greater osteoid surface for mineralization. But if the primary function of CT is protection against hypercalcemia, should it not be more important in older individuals in whom calcium balance is usually zero or negative, and in whose tissues the danger of metastatic calcification seems to be greater for a given plasma concentration of calcium?

Another question is, if CT acts primarily to inhibit bone resorption, is its basic function very different in mammals from its role in cartilaginous fishes?

CT and Phosphate Metabolism

CT lowers plasma concentrations of inorganic phosphate. Hypophosphatemia could be attributed to inhibition of bone resorption. But CT also promotes renal excretion of phosphate (and the effect can be demonstrated in parathyroidectomized animals). A primary influence on phosphate metabolism which secondarily affects calcium has been proposed.

Influences of CT on Other Electrolytes¹⁵

CT is so potent for promotion of sodium excretion that some investigators believe it is *the* natriuretic hormone (Chapter 13). CT also significantly increases chloride excretion, and large doses may so greatly increase urine volume that circulating blood volume is diminished. CT also reduces the hydrochloric acid content of gastric juice; a direct influence on chloride transport is suspected. (No consistent effect of CT on hydrogen transport in gut or kidney has been found.)

Direct regulation of chloride transport is common among the fishes (Chapter 12); but movements of this anion in mammals

usually result passively from changes in sodium transport.

CT and Bone Remodeling

Influences of CT on bone cells described below, the greater effectiveness of CT in younger animals, and skeletal disturbances in thyroxine-treated thyroidecomized animals raise the possibility that CT exerts its major role on bone remodeling.

glucagon) are known activators of adenylate cyclase in other cell types.

Thyrocalcitonin Release Factor

It was proposed several years ago that the parathyroid gland secretes a "thyrocalcitonin release factor" which acts on the neighboring thyroid. The concept is esthetically appealing, but it has not been supported by later experiments.

REGULATION OF CT SECRETION^{12, 13}

Plasma Calcium Ion Concentrations

Ten to 100-fold increases in CT secretion have been elicited by intravenous injection of calcium salts; and prolonged maintenance of hypercalcemia (by dietary or other means) can induce hypertrophy of the CT-secreting cells.

Very high magnesium concentrations are also effective, but it is not believed that magnesium plays a physiological role in regulation of CT secretion.

Gastrointestinal Hormones

Gastrin, pancreozymin, and enteroglucagon released during digestion of food (or the same hormones injected) promote increased CT secretion. Some authors have suggested that the CT released during normal digestive processes actually results from absorption of sufficient calcium to elevate plasma calcium levels and provide the stimulus for CT secretion even when the plasma calcium elevation cannot be detected by the usual methods. But the fact that the hormones can promote CT release directly suggests a far more interesting concept, *i.e.*, that the gastrointestinal hormones provide for an "anticipatory" rise in CT secretion which favors efficient utilization of dietary calcium as soon as it is absorbed, thereby favoring more complete calcium conservation by blocking postprandial hypercalcemia. (Animals lacking CT excrete more calcium in their urine during the postabsorptive period.)

Adenylate Cyclase

D-cAMP and β -adrenergic agonists (Chapter 9) promote release of CT, and several of the hormones known to stimulate CT secretion (gastrointestinal hormones,

MECHANISM OF ACTION OF CALCITONIN

Effect on Cytosol Calcium

It was noted (Chapter 14) that many actions of PTH seem to depend directly upon ability of the hormone to promote a prompt rise in cytosol calcium ion concentrations, and that this is associated with a transient reduction of plasma calcium concentrations.

CT administration leads to a transient rise in plasma calcium ion concentrations which precedes a more sustained lowering. It is believed that this hormone *promotes calcium efflux* and thereby lowers cytosol calcium. The site of action has not been defined; direct influences on plasma membranes, and activation of a calcium-dependent ATPase have both been proposed.

Effects on Adenylate Cyclase

Since CT lowers cytosol calcium concentrations in some cells responding to PTH with calcium elevation, it was considered likely that CT might lower cAMP. But it was soon established that CT *raises* cAMP, and levels of the nucleotide may go higher when PTH and CT are administered together. It was then suggested that the two hormones could be acting on different adenylate cyclase enzymes. But more recent thinking favors the concept that cAMP elevation in bone cells results from removal by CT of the calcium inhibition of adenylate cyclase. Effects of PTH on bone cells can be mimicked by administration of cAMP, its analogs, and phosphodiesterase inhibitors; but CT actions cannot.

On the other hand, both PTH and CT promote phosphate excretion by the kidneys; this effect seems to be mediated via

cAMP, and it is possible that the two hormones act on common receptors in this tissue.

Specific Effects of CT on Bone^{10, 13}

Osteoclasts. One of the earliest effects of CT is exerted on osteoclasts. Within minutes after administration of the hormone, the histological appearance is altered, and a reduction of osteoclast number can be detected within 15 min. The rapid reduction of osteoclast number has been attributed to stimulation of conversion of osteoclasts to osteoblasts. (The relative number of osteoblasts is increased 1 hr after hormone administration.) The sustained reduction of osteoclast count has been attributed to reduced formation of new osteoclasts from osteoprogenitor (mesenchymal) cells.

Osteocytes. Influences of PTH on osteocytes described in Chapter 14 were related to effects of the hormone on elevation of cytosol calcium concentrations. CT is believed to reduce cytosol calcium and to promote changes opposite in direction. After CT, osteolytic activity is reduced, lactate and citrate production are lowered, and histological changes visible within minutes after administration of CT suggest that solubilization of matrix is decreased (or mineralization is increased). Effects on osteocytes probably contribute substantially to early calcium-lowering effects of CT, while influences on osteoclasts and on osteoblasts sustain the effect on plasma concentrations.

Osteoblasts. Although osteoblast *number* declines, collagen-synthesizing activity is increased. Formation of new matrix provides sites for acceptance of calcium and phosphate from plasma.

Mesenchymal Cells. Mitosis is inhibited. It is believed that an early effect is reduced formation of osteoclasts from undifferentiated cells. But preferential conversion of the latter to osteoblasts is also consistent with available data.

Relationship to the Calciferols. Unlike PTH influences, most actions of CT can be elicited in vitamin D-deficient animals.

Over-all Effects of CT Administration on Bone. Actions of CT on bone are compared with those of PTH in Table 16-1.

Early influences on mesenchymal cells

reduce the number of osteoclasts, and early influences on osteocytes reduce participation of these cells in bone resorption, while activity of the osteoblasts is stimulated. Therefore bone formation proceeds more rapidly than bone resorption.

Delayed influences on mesenchymal cells lead to a reduction in formation of new osteoblasts. Therefore the early increase in bone formation cannot be sustained.

CT has been used with some success for treatment of Paget's disease, in which there is pathological bone resorption. But it has been effective only for a very limited time in conditions associated with defective bone formation; this seems reasonable in the light of the above.

It has been suggested that *intermittent* treatment with CT to stimulate existing osteoblasts, interspersed with administration of sufficient PTH to promote mitosis of osteoprogenitor cells, should be more beneficial.¹⁴

No mention was made in Table 16-1 of actions of the hormones on mitochondrial storage of calcium and phosphate. It was noted that effects of PTH and of cAMP on mitochondrial extrusion of calcium contribute importantly to maintenance of high cytosol calcium. Relatively little information is available on direct influences of CT. One hypothesis states that CT promotes formation of an as yet unidentified "second messenger" (not cAMP) which promotes mitochondrial uptake or inhibits mitochondrial extrusion of calcium, thus contributing to the maintenance of low cytosol calcium. A related idea is that CT stimulates a phosphatase and thereby increases the concentration of inorganic phosphate in bone; as a consequence, more phosphate enters the cells and this favors mitochondrial uptake of calcium, while more extracellular phosphate facilitates bone mineralization. It has not been demonstrated, however, that effects of CT on ejection of calcium from the cell are inadequate for maintaining the reduced cytosol calcium.

Effects of CT on the Kidney^{8, 13}

Although CT reduces plasma concentrations of both calcium and phosphate, and therefore the quantity presented to the kidney glomeruli, it increases the excretion of both ions. If one accepts the concept that

TABLE 16-1
Comparison of Known or Proposed Effects of PTH on Bone with Those of CT

<i>Parameter</i>	<i>Parathyroid Hormone</i>	<i>Calcitonin</i>
A. Earliest observed effect on plasma Ca^{++}	Decrease	Increase
B. Effect on plasma Ca^{++} after several minutes	Increase	Decrease
C. Sustained delayed effect on plasma Ca^{++}	Increase	Decrease
D. Calcium uptake by cells	Increase	?
E. Cytosol calcium content	Increase	Decrease
F. cAMP concentration	Increased because of stimulation by PTH	May be elevated because of low cytosol Ca^{++}
G. Calcium efflux from cells	Increased because of increased cytosol calcium	Increased directly;? effect on "calcium pump"
H. Consequence of G	? Unidirectional transfer of calcium to plasma	? Unidirectional transfer of calcium to mineralization surface
I. Vitamin D	Required for sustained effect on plasma Ca^{++}	Not required for effect on plasma Ca^{++}
J. Bone cell citrate, lactate, H^+	Increase	Decrease
K. Proposed effect of J on labile bone mineral	Increased mineral solubility; transfer of calcium to plasma	Decreased solubility; increased precipitation of available calcium and phosphate
L. Effect on osteoprogenitor cells	Mitosis increased	Mitosis suppressed
M. Moderately early effects of L	Increased number of osteoclasts, increased bone resorption	Reduced number of osteoclasts, reduced resorption
N. Late effects of L	Increased later appearance of more osteoblasts; increased rate of bone remodeling	Decreased later appearance of osteoblasts; decreased bone remodeling
O. Effect on osteoclasts	Increased phagocytic, lysosomal activity Prolongation of function	Decreased activity More rapid conversion to other bone cells

TABLE 16-1—Continued

Parameter	Parathyroid Hormone	Calcitonin
P. Effects of O	Increased bone resorption, calcium release, hydroxyproline excretion	Decreased bone resorption, calcium release, hydroxyproline excretion
Q. Effects on osteocytes	Decreased bone-forming functions; increased osteolysis	Decreased osteolysis (histological evidence of change within minutes)
R. Effects on osteoblasts	Delayed increase in number because of L; inhibition of matrix synthesis; ?more rapid conversion to osteocytes	Delayed decrease in number because of L; stimulation of existing cells, increased matrix synthesis
S. Effects of R	Increased rate of bone remodeling, but less matrix formed per cell, less calcium taken up by new matrix	Decreased rate of bone remodeling; more matrix formed per cell and more calcium taken up, but eventually reduced bone formation

CT functions to protect against excessive concentrations of calcium and phosphate in the blood plasma, then the renal effects can be regarded as contributing to the function. On the other hand, the renal effects oppose CT stimulation of bone formation and mineralization.

As noted above, CT also increases the urinary excretion of sodium, chloride, and water, and tends to reduce the volume of extracellular fluid.

At least part of the influence of CT on urinary excretion is exerted directly on the kidney. It can be demonstrated in renal perfusion studies and does not depend on alterations in calcium content of the surrounding fluids. CT seems to directly inhibit proximal tubular reabsorption of sodium, chloride, and calcium; effects on phosphate may be secondary to those on sodium.

Effects of CT on renal excretion are compared with those of PTH in Table 16-2.

Effects of CT on the Gastrointestinal Tract

CT decreases calcium uptake from the gastrointestinal tract since it blocks formation of 1,25-HCC (Chapter 15). It also reduces gastric acidity.

GLUCOCORTICOIDS AND CALCIUM METABOLISM^{10, 11}

Osteoporosis is a common finding in experimental animals overdosed with glucocorticoids, in patients taking pharmacological doses of steroids over long time periods, and in patients suffering from Cushing's disease. *Protein catabolic actions* of the hormones exerted on the collagenous matrix (Chapter 7) account only in part for the finding. Glucocorticoids *depress calcium uptake* by the intestine and *inhibit its reabsorption by the kidney*. Both actions induce *hypocalcemia* which leads secondarily to *stimulation of the parathyroid glands*. They also exert *direct actions on bone cells*.

Effects on the intestine may be exerted at multiple sites. *CaBP* content is actually *elevated* rather than depressed. Some actions of vitamin D are indirectly antagonized, but glucocorticoids can further depress the low calcium uptake of isolated intestinal sacs taken from vitamin D deficient animals. They block the ability of the vitamin metabolites to elevate citrate production (but the metabolites can promote some calcium absorption when citrate production is blocked in other ways). D metabolites promote calcium and phosphate uptake by mitochondria; steroid-treated animals have *reduced numbers of mitochondria* in responsive

TABLE 16-2
Comparison of Effects of CT with Those of PTH on Urinary Excretion

Component	CT	PTH
Sodium	Increased	Increased
Chloride	Increased	May be slightly decreased
Water	Increased	May be slightly increased
Phosphate	Increased	Increased
Calcium	Increased directly (but reduction of plasma concentrations can lead to decrease)	Decreased (but elevation of plasma concentrations can lead to increase)
cAMP	Increased	Increased
Bicarbonate	Little or no effect	Increased
Hydrogen	Little or no effect	Increased

cells, and the mitochondria exhibit impaired ability to take up calcium. Glucocorticoids also seem to exert inhibitory influences on the hepatic 25-hydroxylase system needed to form active vitamin D metabolites.

Tissue culture studies indicate that glucocorticoids directly inhibit mitosis of osteoprogenitor cells and their conversion first to osteoclasts and later to osteoblasts. RNA and protein synthesis are reduced in collagen-synthesizing cells, and this creates an imbalance in which bone destruction exceeds the rate of new bone formation. The high levels of PTH resulting from glucocorticoid-induced hypocalcemia antagonize glucocorticoid suppression of mitosis, but PTH increases osteoclast activity and contributes to osteoblast inhibition.

SOMATOTROPHIN AND CALCIUM METABOLISM^{8, 10}

STH stimulation of bone growth results largely from increased mitosis of the osteoprogenitor cells and more rapid modulation to osteoblasts. RNA and protein synthesis are increased, and new bone formation proceeds more rapidly than bone destruction. The rate of bone remodeling is also increased.

STH (via somatomedin) directly stimu-

lates growth of cartilage, including that formed at sites which will later be replaced by bone. It seems to antagonize PTH actions on the kidney since it promotes phosphate retention and calcium excretion. The phosphate retention may contribute to influences on bone formation, but phosphate is also shunted into non-osseous growth processes. Calcium absorption from the intestine is increased, but influences on plasma calcium concentrations are inconsistent.

ESTROGENS^{8, 9, 10}

Estrogens exert numerous complex, poorly understood influences on calcium and phosphate metabolism and on bone which involve interactions with several other hormones.

Mammalian endocrinologists have studied effects of estrogens on calcium metabolism and bone formation in birds because actions in this class of vertebrates are reproducible and obviously related to biological needs. The hope has been to obtain insight into more generalized aspects of estrogen functions in calcium homeostasis.

Large quantities of calcium and phosphorus are stored during the period preceding egg laying since these are needed for

both shell and yolk formation. Osteoblastic activity is tremendously increased at this time, and the large quantities of spongy bone formed provide a storage site for the calcium and phosphorus.

Although accumulation of spongy bone in immature rodents can be induced by estrogen administration, pharmacological doses of the steroid are required. There is no evidence for accumulation of spongy bone associated with reproductive processes in mammals.

Estrogens inhibit cartilage growth in immature mammals, and they promote premature calcification of the epiphyses of long bones. Similarly, hypersecretion of estrogens in children leads to shortening of stature. It is suspected that such actions are related to influences of estrogens on gene transcription.

Many actions of estrogens seem antagonistic to those of PTH. The steroids tend to reduce intestinal absorption of calcium, mitosis of osteoprogenitor cells, activity of osteoclasts, and the rate of bone remodeling; they may also stimulate activity of existing osteoblasts, and perhaps hasten conversion of osteoclasts to osteoblasts. The actions find useful application in elderly patients with bone fractures since they promote more rapid healing.

The term *postmenopausal osteoporosis* describes the characteristic reduction of bone mass seen in women when the aging ovary decreases its secretion of estrogens. It seems to be related in part to increased effectiveness of endogenous parathyroid hormone (but age-related changes in bone itself must surely contribute). Short range benefits from estrogen administration have been described; these could be related to ability of estrogens to stimulate activity of existing osteoblasts and accelerate conversion of osteoclasts to osteoblasts. Estrogens are not useful if administered over long periods of time; probably this is because of the depression of mesenchymal cell mitosis, and the ultimate reduction of the number of osteoblasts.

When given over short periods of time, estrogens have been reported to promote a "positive calcium balance," as measured by comparison of the combined urinary and fecal calcium loss with the dietary calcium intake. Some authors have suggested that estrogens increase intestinal

absorption of calcium (since relatively less is lost in the feces). This would be difficult to reconcile with the anti-PTH concept of estrogen action. But others have pointed out that estrogens seem to decrease absorption of dietary calcium, and have given evidence that the positive balance results from decreased intestinal secretion of endogenous calcium.

Estrogens tend to decrease the plasma concentrations of calcium, and they may in this way decrease urinary loss of calcium. An antagonism of PTH effects on the kidney may also play a role.

In addition, estrogens seem to alter the metabolism of D vitamins, possibly by retarding the rate of formation of 25-HCC. (Estrogens are known to exert a variety of influence on hepatic enzymes, including those involved in steroid metabolism.)

ANDROGENS

Androgens are strongly anabolic. The adolescent "growth spurt" in males seems to result largely from direct testosterone stimulation of RNA and protein synthesis by osteoblasts, and also from indirect effects. The hormone promotes growth and strengthening of skeletal muscle, and thereby exerts mechanical effects on bone (see below); it also sharpens the appetite.

As with estrogens, premature secretion of androgens leads to early closure of the epiphyses of the long bones, and interferes with attainment of full stature (while deficiency during the adolescent period often leads to elongation of the bones). Androgens are known to affect gene transcription in target tissues, and synthesis of new proteins may be involved in maturation effects on bone.

Androgens have been used with some good results to promote bone healing after injury in aging patients, and can exert short term (but not long range) influences on retardation of senile osteoporosis. Stimulatory influences on skeletal muscle probably contribute to beneficial effects on bone. Rapid induction of virilism limits the use in females, but the androgens are directly effective in males (and have been given in combination with estrogens in females). Unfortunately, many patients develop jaundice and other signs of hepatic dysfunction even after short term administration.

Androgens promote retention of sodium, potassium, chloride, nitrogen, and sulfur as well as of phosphorus, associated with stimulation of protein synthesis. They may promote short term retention of calcium. Large doses used in the treatment of some neoplastic diseases have been reported to elevate plasma calcium concentrations, but this effect is not seen in normal individuals given moderate doses.

THYROID HORMONES^{1, 2, 8}

Thyroid hormones (thyroxine and triiodothyronine) synergize with STH in supporting skeletal growth. STH is only moderately effective in young, hypophysectomized animals, but normal rates of bone elongation can be obtained when it is combined with either thyroxine or thyroid-stimulating hormone. Thyroid hormones also act on the hypothalamus and pituitary gland to increase STH secretion.

Unlike STH, the thyroid hormones also promote bone maturation. Full bone maturation requires additional influences of reproductive hormones, and their secretion is indirectly increased under the influence of the thyroid.

Excessive doses of thyroxine lead to development of a negative balance of both calcium and phosphate. (But magnesium is retained; plasma levels are reduced as greater amounts are taken up by cells.)

Calcium absorption from the intestine is impaired, and there is increased calcium loss through the feces, urine, and sweat. Osteoclastic and osteocytic osteolysis are enhanced, hydroxyproline excretion increases, and thinning of the bones can be demonstrated by x-ray examination. Bone remodeling is accelerated.

Some actions of thyroid hormones have been attributed to increase sensitivity to influences of both PTH and vitamin D metabolites. (Doses of PTH which stimulate bone remodeling in intact animals are ineffective in thyroidectomized animals.) But other thyroid hormone influences are exerted directly on bone and kidney and can be elicited in PTH deficiency states. (There are also indications that PTH secretion tends to be depressed in hyperthyroidism.)

The negative phosphate balance has been attributed to a direct influence of

thyroid hormones on the kidney, and to associated actions of the hormone on extracorporeal phosphate metabolism. Glomerular filtration of phosphate is increased (and part of this effect may be related to somewhat elevated plasma concentrations often but not consistently found in hyperthyroidism). Unlike PTH, thyroid hormones increase tubular reabsorption of phosphate. But effects on glomerular filtration are quantitatively more important, and usually there is phosphaturia. Some authors have suggested that increased phosphate reabsorption is related (at least in part) to reduced plasma concentrations of PTH often found in hyperthyroid animals.

THE THYMUS GLAND

There are many reasons for suspecting that the thymus gland participates in important ways in regulation of calcium and phosphate metabolism. The topic is discussed in Chapter 25.

The thymus gland is large (relative to body weight) during periods of active skeletal growth, long after major influences of the thymus on the immune system have been accomplished, and there is evidence that the thymus influences STH secretion. Thymus weight is reduced in poorly nourished animals which do not grow normally. Thymectomy of very young animals can lead to growth retardation, but it is ineffective during later stages of skeletal development. PTH and STH tend to elevate plasma calcium concentrations and both stimulate thymus gland growth; glucocorticoids and estrogens tend to reduce plasma calcium and they are potent thyromolitics.

The older literature contains numerous descriptions of thymus-parathyroid antagonism. More recent work has demonstrated the existence in thymus glands of "calcium-lowering" principles. Some have been identified with CT, but others seem to be clearly different. The thymus gland has been shown to provide some protection against experimentally induced hyperparathyroidism. High-affinity calcitonin receptors have been identified in thymocytes.¹³

There are indications that the thymus gland may secrete a heparin-like substance, and heparin has been implicated in

bone mineralization and resorption.³ The thymus has also been shown to influence renal excretion of several electrolytes.

MECHANICAL INFLUENCES ON BONE^{4, 10}

Mechanical stress and physical exercise induce electronegative changes in bone leading to increased osteoblastic activity. Inactivity, bed rest, and weightlessness induce reduction of bone mass; this may be related to observations that electropositivity enhances osteoclast functions, and that PTH stimulation of osteoclasts is associated with membrane depolarization.

The effects of mechanical pressure seem to be direct (*i.e.*, not mediated via nerves or hormone release). But the resulting effects on plasma calcium concentrations can secondarily affect the endocrine system.

The tendency for loss of bone mass during periods of inactivity can be counteracted to some extent by administration of either CT or phosphates.

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V

HORMONAL REGULATION OF METABOLIC RATE, FOOD INTAKE, AND BODY COMPOSITION

17. Thyroid Hormones

Chapter 17 describes functions of thyroxine and of triiodothyronine. Although calcitonin is also produced in the thyroid gland, it is discussed in Section IV.

BASAL METABOLIC RATE

The basal metabolic rate (BMR) of an intact organism is a measure of the energy expended in the postabsorptive condition while at rest in a room of comfortable temperature. Energy is utilized for maintaining vital functions such as the blood circulation and respiration; but the standardized procedures eliminate increments that would be required for voluntary movement, adjustments to hot or cold environments, and those associated with utilization of foods.

BMR is usually expressed in calories per square meter of body surface per hour (or per day); but it is most frequently measured as a function of the associated oxygen consumption. It varies with such factors as age, sex, development of the skeletal musculature, and levels of several hormones.

THYROID HORMONES AND BMR: GENERAL NATURE OF THE RELATIONSHIP

A physiologically important role of thyroid hormones in regulation of metabolic rate has long been known. Until recently BMR determinations provided an important tool for clinical assessment of thyroid function; it was always recognized, however, that the method was of limited value because so many extraneous factors influ-

ence the data obtained. With development of procedures for determination of thyroid hormone concentrations in the blood plasma and for estimation of thyroid and pituitary functions, the BMR has been relegated to a more appropriate (and therefore less prominent) place in differential diagnosis.

Effects of thyroidectomy and of administration of thyroid hormones on metabolic rate can be readily demonstrated in adult mammals, and in immature ones which have acquired the ability to regulate body temperature. Thyroid hormones do not seem to participate importantly in regulation of energy consumption of poikilothermic vertebrates when in their usual habitats, and they do not affect the metabolic rates of the very immature newborns of certain mammalian species. (An effect on poikilotherms maintained at artificially high temperatures can be demonstrated; but this may have nothing to do with physiology.)

Regulation of heat production is one of the mechanisms utilized to maintain body temperature within the narrow range consistent with optimal enzyme activity. Some of the problems associated with deficiencies or excesses of thyroid hormones can be attributed in part to inability to maintain optimal temperatures; but thyroid hormones perform many other functions.

EFFECTS OF THYROID HORMONES ON WHOLE ORGANISMS²

Effects of long term deficiencies or excesses on the whole organism are obvious.

The patient suffering from hyperthyroidism (too much thyroid hormone) is restless, hyperactive, irritable, has a short attention span, and usually suffers from insomnia. Movements are quick, reflexes are exaggerated, and often there are fine tremors. Cardiac rate is rapid, there are characteristic findings in the electrocardiogram, and the blood pressure may be elevated. The cardiac force may be increased; but a serious difficulty in thyrotoxicosis is that the heart can be accelerated to the point where diastolic filling becomes inadequate.

The body temperature tends to be high, the skin is warm and flushed, and sweating occurs at environmental temperatures considered comfortable by euthyroid persons.

Although appetite and food intake are increased, there is usually weight loss in severe hyperthyroidism. Gastrointestinal activity is increased, and this may be associated with bouts of diarrhea.

Weakness of skeletal muscles has been attributed to destruction of protein, defective creatine metabolism, and effects of the hormone on neuromuscular transmission.

By contrast, the severely hypothyroid (thyroid-deficient) subject is apathetic, sluggish and somnolent, and suffers from sensory impairment. The body temperature may be low, especially in cool environments. The skin is usually dry and cold, the hair sparse and coarse, and the activity of sweat and sebaceous glands depressed. The term "myxedema" describes the accumulation under the skin of fluids and mucoproteins, which give the individual a "puffy" appearance and thickening of the tongue; but defects in electrolyte metabolism (Chapter 13) also lead to water retention.

The heart is usually enlarged and dilated. Cardiac rate is reduced and blood pressure may be low. Appetite is usually poor, and depressed gastrointestinal activity may be associated with constipation. Although skeletal muscles tend to be enlarged, they are flabby and weak.

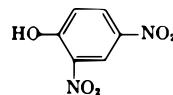
Severe hypothyroidism in the adult can be reversed by administration of thyroid hormones. But thyroid deficiency very early in life leads to the condition of *cretinism*, characterized by severe impairment of growth and development. The skeletal system remains immature, body proportions are infantile, stature is se-

verely shortened, and no development of reproductive structures occurs. Thyroid hormones are required during a specific phase of development of the central nervous system. The most critical time in humans seems to occur between the late prenatal period and the first 2 years of postnatal life.

When human thyroid insufficiency in infants is not corrected soon after birth, damage to the brain is permanent. It is possible to promote growth of skeletal structures and to induce reproductive maturation at a later time, but mental retardation does not respond to administration of thyroid hormones once the critical period is over.

CALORIGENIC ACTIONS OF THYROID HORMONES^{1, 2}

At one time it was believed that all actions of thyroid hormones in homeotherms are directly dependent on the calorigenic actions. There is now good reason to believe that this is not the case. Agents are known, e.g., dinitrophenol (DNP), which elevate metabolic rate, but which are unable to mimic actions of thyroid hormones on growth, development, maturation, and a large number of metabolic reactions.



2,4-Dinitrophenol (DNP)

The Latent Period and its Significance

If control values of basal metabolic rates are established for intact experimental animals, and the animals are then thyroidecomized, *no immediate influence* of thyroid deprivation on metabolic rate can be demonstrated when the conventional methods of measurement are employed.

In the laboratory rat, 2 weeks or longer may elapse before significant reduction of the BMR can be demonstrated. (This interval is the "absolute latent period" for the response.) Usually, the BMR continues to fall over the next 2-4 weeks before a new steady state is reached. (The interval during which such changes occur is often referred to as the "relative latent period".) In humans, earliest changes may become

apparent 3 or 4 weeks after removal of the thyroid gland, withdrawal of thyroid therapy in a hypothyroid subject, or administration of antithyroid drugs; a steady state may require 6–8 weeks for full development.

The latent period is far longer than the time required to deplete the organism of preexisting hormone.

When the steady state is attained, the metabolic rate is reduced to 60–70% of the presurgery value. Under regulated conditions, animals can survive for long periods of time without therapy.

If thyroid hormones are administered to animals with reduced BMRs, a second latent period is observed between the time of administration of the hormones and the first detectable rise in metabolic rate. The results of a typical study are diagrammed in Figure 17-1.

Tissues Directly Involved in Calorigenic Actions of Thyroid Hormones

Skeletal muscle accounts for a very large fraction of total body oxygen consumption. It is not surprising to find, therefore, that influences of thyroidectomy or of administration of thyroid hormones will be visible in muscle taken from pretreated animals. Oxygen consumptions of cardiac muscle, liver, and kidney (and also gastric mucosa) are affected; but no change in metabolic rates of spleen, reproductive tissues, or nervous tissue can be detected in this way (Table 17.1). Yet, effects of thyroid hormones on development of the nervous sys-

tem, on mental activities and reflex responses, and on maturation and function of the reproductive system are well known and obvious. The study is instructive because it demonstrates that *tissues exhibiting strong responses to thyroid hormones do not necessarily show associated increases in oxygen uptake*. It appears that while oxygen utilization may be rate-limiting for some responses, it cannot be for others.

In Vitro vs In Vivo Effects

When tissues are removed from a previously untreated animal and "physiological" quantities of thyroxine are then incubated with the tissues for several hours before measurement of oxygen consumption, no influence of the hormone can be demonstrated (see Table 17.2). The experiment suggests that the latent period cannot be explained on the basis of delayed presentation of thyroxine to the tissues when the hormone is administered to the whole animal. (Implicit is the assumption, however, that presentation of the hormone directly to the tissues is equivalent to presentation of the *active form* of the hormone in a manner which permits rapid access to receptor sites.) The findings also suggest that the influence of thyroxine on metabolic rate (as measured in this way) does not result from *direct* stimulation or activation of existing enzymes which catalyze oxidative reactions.

The described pattern of response is by no means unique for thyroxine. Many dif-

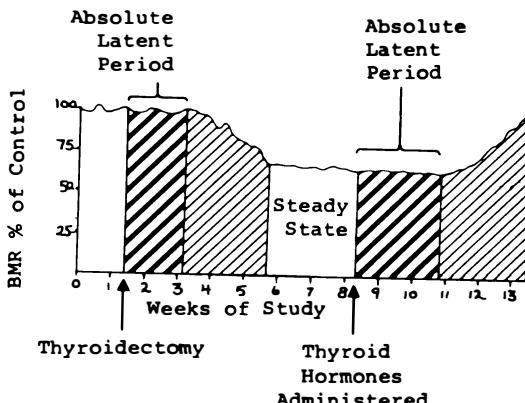


FIG. 17-1. Effects of thyroidectomy and administration of thyroid hormones on basal metabolic rate.

TABLE 17-1

Effects of Thyroidectomy and of Administration of Thyroxine on Oxygen Consumption of Tissues Removed After the Whole Animal Has Exhibited Changes in BMR

Tissue	Effect of Thyroidectomy	Effect of Thyroxine
Skeletal muscle	↓	↑
Cardiac muscle	↓	↑
Liver	↓	↑
Kidney	↓	↑
Spleen	No change	No change
Gonads	No change	No change
Nervous tissue	No change	No change

TABLE 17-2

Effect of In Vitro Administration of Thyroxine to Excised Tissues of Intact Animal

Tissue	Effect of Thyroxine
Skeletal muscle	No change
Cardiac muscle	No change
Liver	No change
Kidney	No change
Spleen	No change
Gonads	No change
Nervous tissue	No change

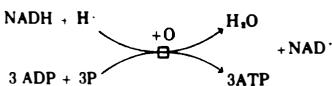
ferent kinds of hormone effects can be demonstrated when tissues are excised from animals previously subjected to hormone administration or deprivation; when the effects are indirect, the hormonal changes cannot be reproduced by *in vitro* administration of hormones to excised tissues. In many cases it can also be demonstrated that (1) the hormone does not require metabolic transformation into an active form before it can affect the tissues examined, (2) no other hormones or other plasma factors are needed to permit the hormone to exert its actions, and (3) studies with inhibitors of protein synthesis suggest that new proteins must be produced to elicit delayed actions.

Influences of Thyroid Hormones on Mitochondrial Oxidative Phosphorylation

Since about 90% of oxygen utilization depends directly on mitochondrial func-

tions, these organelles provide a logical site for investigation of thyroid hormone actions.

"Uncoupling" of Oxidative Phosphorylation. When hydrogen derived from metabolism of food is transported to the mitochondria by the dehydrogenase enzymes utilizing NAD as a cofactor, the hydrogen eventually unites with molecular oxygen to form water, and much of the energy liberated is utilized for synthesis of ATP. Under theoretically optimal conditions, 3 molecules of ATP are formed from ADP for each molecule of water:



Agents are known (e.g., DNP) which "uncouple oxidative phosphorylation"; i.e., oxidation is permitted to proceed, but some of the energy which would otherwise be used for ATP synthesis is dissipated as heat. Therefore, to obtain sufficient ATP, much larger quantities of food materials must be oxidized.

At one time it was thought that DNP might be used to promote rapid burning of excess fat stores, and therefore be useful in the treatment of obesity. But temperatures rose as high as 106°F in patients taking the drug, and as might be expected, toxic effects were encountered.

If very high (unphysiological) concentrations of thyroid hormones are incubated with appropriate tissues, a similar uncoupling can be demonstrated, and it was suggested that the calorigenic action of thyroid hormones might be explained on this basis. The concept in its original form is no longer seriously considered for many reasons: (1) extremely high concentrations of thyroxine are required; (2) there is no correlation between the ability of a variety of compounds to uncouple oxidative phosphorylation and ability to mimic known actions of the hormones (*D*-thyroxine is as effective as *L*-thyroxine for uncoupling, but has little thyroxine-like biological activity); (3) uncoupling and therefore reduced ATP production cannot explain effects of thyroid hormones on growth and development; (4) the total amount of ATP formed is usually increased after thyroxine administration. The possibility remains that some effects of toxic doses of thyroid hormones (fever, excessive

tissue destruction) may be related to such actions. But attempts to demonstrate uncoupling in mitochondria prepared from skeletal muscle of animals strongly overdosed with thyroid hormones have yielded largely negative data.

"Loose" Coupling. Oxidative functions of mitochondria are normally regulated ("tightly controlled") by the availability of ADP. There are indications that thyroid hormones can "loosen" control, i.e., they can increase oxidation of substrate beyond rates usually pertaining at a given level of ADP availability, without impairing phosphorylation. In this way more heat is generated (and more oxygen consumed) without reduction of ATP formation.

In addition, thyroid hormones seem to sharpen the "sensitivity" of mitochondria to other agents (e.g., DNP) which enhance oxygen utilization; they may also increase the rate of transport of ADP into the mitochondria.

It is likely that some influence on the structure of the mitochondrial membranes and alignment of respiratory assemblies is involved. Mitochondrial swelling associated with increased uptake of water (but to a lesser degree than observed after pharmacological doses) has been demonstrated, and observations that the organelles of hyperthyroid animals "leak" pyridine nucleotides at an increased rate point in the same direction. Induction by the hormones of a substance that enhances mitochondrial swelling has also been considered.

Thyroid hormones are also known to increase the activity of plasma membrane ATPases. But it is not certain if an action on the membrane stimulates ATPase activity and thereby increases the supply of ADP to the mitochondria, or if mitochondrial uptake of ADP favors a secondary increase in ATPase activity.

The described influences on mitochondria develop rapidly, are small in magnitude, and are of short duration. They can be demonstrated in liver, but not in skeletal muscle preparations. Some role in early hormone actions, and in mediation of catecholamine actions which cannot be elicited in thyroid-deficient animals seems likely. But they do not seem to be involved in the delayed, sustained elevation of metabolic rate induced by thyroid hormones.

Long Range Influences of Thyroid Hormones on Mitochondria. Delayed, sustained actions are clearly dependent upon RNA-directed synthesis of new proteins and can be totally blocked by admin-

istration of the usual inhibitors. Prolonged hormone action leads to striking increases in the size and number of mitochondria, and in elevated content of several enzymes including cytochrome oxidase, cytochrome c, and succinoxidase.

Information on the existence and functions of specific cellular components which bind thyroid hormones is rapidly accumulating. But the "primary event" has yet to be defined. Binding to mitochondria has been demonstrated, and one hypothesis presented is that thyroid hormones promote release of a "translation acceleration factor" which acts on ribosomes to increase protein synthesis. Another is that there is direct stimulation of mitochondrial synthesis of a small quantity of specific protein which mediates hormone action. A third idea is that mitochondria release something which travels to the nucleus to affect RNA synthesis.

In one type of *in vitro* study it was shown that addition of thyroid hormones to preparations containing both mitochondria and ribosomes leads to an accelerated rate of protein synthesis, whereas addition of the hormone to mitochondria-free preparations does not. But the data have been interpreted in conflicting ways, and it has been suggested by some that the experimental conditions employed are too artificial to shed light on physiological mechanisms.

THYROID HORMONES AND PROTEIN SYNTHESIS^{1, 2, 14}

Specific hormone receptors have been found in the nucleus, and a significant increase in rapidly labeled RNA can be demonstrated in liver cells within 4-6 hr after administration of the hormone to intact animals. Some hours after that, increased RNA polymerase activity is evident.

The RNA changes can be blocked by administration of very small amounts of actinomycin D which preferentially blocks ribosomal RNA synthesis; but this does not rule out formation of small amounts of new messenger RNA. Many hours later there is a great increase in the rate of cytoplasmic protein synthesis. It is possible, therefore, to visualize a sequence of events in which stimulation of messenger formation leads next to appearance of new protein; however, the facts are more consistent with indirect actions of the hormone.

RNA changes have not been found when the hormone is added directly to preparations of isolated nuclei. Target cells contain cytoplas-

mic hormone receptors which have been implicated in binding the hormone for later transfer to active sites, but they may do something more. Actions must be different from those of steroid hormone cytoplasmic receptors because it has not been possible to demonstrate transfer of large amounts of cytoplasmic receptor to the nucleus.

Between the time of appearance of RNA stimulation and the burst of cytoplasmic protein synthesis, there is a period of rapid formation of new phospholipid which is incorporated into microsomal membranes. There is good evidence that thyroid hormone stimulation of protein synthesis depends heavily upon the formation of new populations of ribosomes which differ from preexisting ones in their tighter attachment to membranes and their tendency to become parts of large ribosomal aggregates; their functions may also depend upon sequestration within specialized parts of the cytoplasm. Formation of new mitochondrial membranes also seems to precede by many hours the increase in respiratory chain enzymes. Thyroid hormones may also affect protein synthesis through preferential acceleration of transport of nuclear components to the cytoplasm.

While there is no evidence for direct thyroid hormone stimulation of adenylate cyclase, some protein synthesis effects may be related to cAMP. Thyroid hormones can induce formation of new enzyme, and enhance ability of other hormones to activate existing enzyme.

It is obvious that calorigenic actions are related to production of increased quantities of respiratory chain enzymes, and that growth promotion requires accelerated synthesis of proteins already present in lesser amounts. But influences of the hormone on cell proliferation may be at least as important in immature animals. Developmental functions evidently involve promoting formation of new species of proteins; regulatory influences of thyroid hormones on the pituitary gland and on the hypothalamus involve very specific proteins.

Thyroid hormones also perform important degradative functions. During amphibian metamorphosis, thyroid hormones increase activity of lysosomal enzymes needed for destruction of larval structures not required by the adult. Inhibitors of protein synthesis block ability of the hor-

mones to promote tail resorption and other changes in tadpoles.

While low hormone dosages augment growth, high doses induce a negative nitrogen balance and impair growth. It is likely that hormonal influences on degradative enzymes predominate under such circumstances. Ability of thyroid hormones to lower plasma cholesterol concentrations are also related to greater stimulation of steroid degradation than steroid synthesis.

INDIRECT INFLUENCES OF THYROID HORMONES

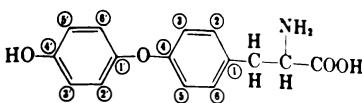
Thyroid hormones "create an environment" in which other hormones can more effectively influence metabolic processes. Hyperthyroid animals are extremely sensitive to actions of catecholamines which depend upon cAMP, including cardiac stimulation and lipolysis. Animals chronically overdosed with thyroxine succumb to about 3% of the dose of epinephrine required to seriously affect euthyroid ones.

Metabolites arising from actions of other hormones can also provide substrates for thyroid hormone functions. Thyroid hormones also increase availability of oxygen through influences on the circulatory and respiratory systems, and on erythrocytes.

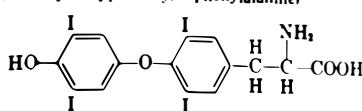
SYNTHESIS OF THYROID HORMONES^{1, 2}

The mammalian thyroid gland is comprised of large numbers of microscopic sized follicles closely associated with capillaries. Each follicle consists of a single layer of epithelial cells surrounding a central cavity or lumen which contains amorphous matter, the colloid. The height of the epithelium varies in different locations, and also within the same follicle at different times. Rat thyroid glands have about 100,000 of these small units, human glands about 1 million. In many species, the gland is organized into two distinct lobes joined by a narrow isthmus.

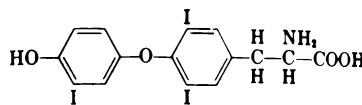
The follicles synthesize, store, and secrete the two kinds of molecules usually known by the term "thyroid hormones": thyroxine (T4) or L-3,5,3',5'-tetraiodothyronine (Fig. 17-2B); and triiodothyronine (T3) or L-3,5,3'-triiodothyronine (Fig. 17-2C). The conventional numbering system for the carbon atoms of the thyronine molecule is shown in Figure 17-2A.



A. L-Thyronine
(4-(4'-hydroxyphenoxy)-L-phenylalanine)



B. Thyroxine
(T4; L-Thyroxine; L-T4; 3,5,3',5'-Tetraiodothyronine)



C. T3
(Triiodothyronine; 3,5,3'-Triiodothyronine; L-T3)

FIG. 17-2. Structures of thyronine, T4 and T3.

Concentration of Iodide

Dietary iodine is absorbed from the intestine in the form of iodide and carried by the plasma to the thyroid gland. The follicles actively take up the iodide against an electrochemical gradient and concentrate it. Under normal conditions the relative amounts of the anion in thyroid, as compared with the serum (T:S ratio) or plasma (T:P ratio), are in the range of 20–25:1; but the ratio can go as high as 300:1 in iodine deficiency states or when, for other reasons, the secretion of thyroid-stimulating hormone (TSH) is increased.

The concentrating process utilizes ATP energy, requires a specific carrier (which seems to be a lecithin), and is associated with uptake of sodium ions. It is affected by high concentrations of chloride and a variety of other anions, and may be rate-limiting for T3 and T4 synthesis.

Individuals with genetic defects that specifically impair transport mechanisms can synthesize normal amounts of thyroid hormones if plasma iodide concentrations are markedly raised.

The ability to concentrate iodide is not unique to the thyroid gland. Salivary glands, skin, gastric mucosa, and other epithelial structures take up considerable quantities, and such uptake may contribute to iodide conservation. The use of preparations containing potassium iodide for "loosening" a dry cough probably depends upon pharyngeal iodide transport

and the associated movements of water molecules.

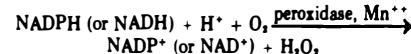
The thyroid gland is special in the extent to which the concentrating ability is developed, in the use of the iodide for synthesis of hormone, and in its susceptibility to control by TSH. (But synthesis of small quantities of T4 outside the gland has been detected in thyroidectomized animals.)

Oxidation of Iodide¹⁵

The iodide must be oxidized before incorporation into organic molecules. The "activated" form may be elemental iodine, iodinium (I^+), hypoiodite (OI^-), or a free radical.

A heme-containing peroxidase enzyme system in the thyroid gland has been isolated and characterized. It has been implicated in iodination of tyrosyl units and in the coupling reaction (see below) as well as in the oxidation process.

Reduced pyridine nucleotides derived from glucose metabolism are utilized, and hydrogen peroxide is generated. According to one proposed sequence, the reduced pyridine nucleotides are utilized directly.



It is possible, however, that the enzyme catalyzing peroxide formation is not the same as the enzyme promoting utilization of H_2O_2 .

The support for indirect participation of reduced pyridine nucleotides (via reduction of a flavoprotein which, along with a vitamin K cofactor reacts with oxygen) may be stronger. Another hypothesis suggests that H_2O_2 formation involves intermediate oxidation of tyramine by thyroid amine oxidase.

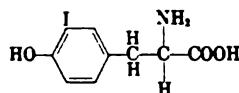
High concentrations of iodide interfere with the oxidation and use of iodide (and large amounts of a mixture of iodine and potassium iodide are used in control of hyperthyroidism). According to one proposal, the enzyme acting on the iodide is also needed to oxidize the protein with which the anion combines, and excess iodide interferes with preparation of the protein.

Iodination of Tyrosyl Components of Thyroglobulin

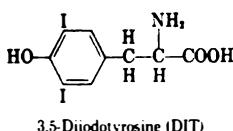
Follicular cells synthesize a large glycoprotein, *thyroglobulin*. Tyrosyl compo-

nents of the protein are iodinated. Free amino acids do not seem to be directly acted upon in the thyroid gland, but iodinated derivates of the thyroglobulin molecule may be hydrolyzed off and released.

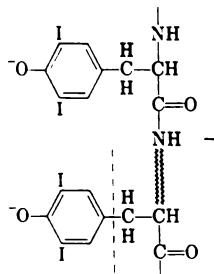
nine residue at the site formerly occupied by one diiodotyrosyl moiety.



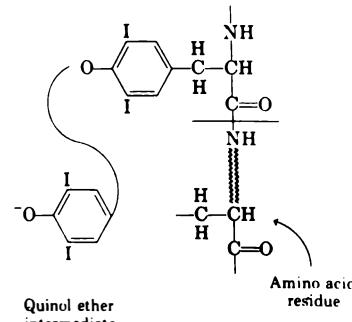
3-Moniodotyrosine (MIT)



3,5-Diiodotyrosine (DIT)



Iodotyrosyl free radicals



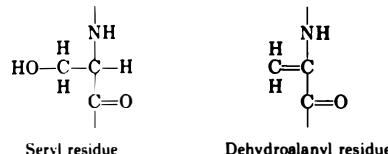
Condensation of Iodotyrosyl Units

Iodotyrosyl components of the thyroglobulin molecule unite to form the thyroid hormone precursors. The condensation may utilize the same peroxidase mentioned above.

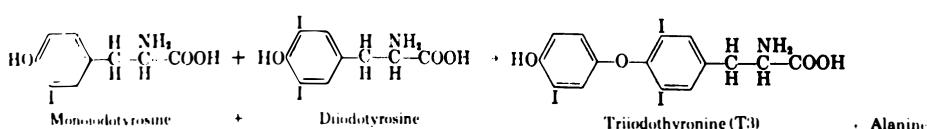
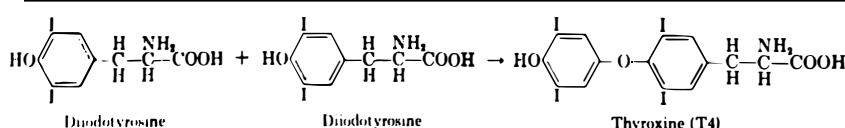
Relationships between MIT, DIT, and the thyroid hormones can be shown in the equations at the bottom of the page which do *not* represent reactions actually taking place in the thyroid gland.

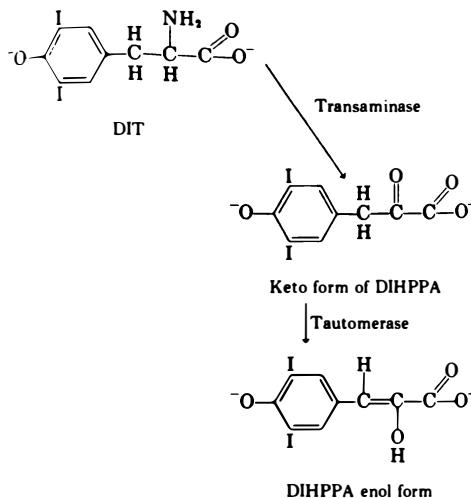
One hypothesis for the coupling reaction proposes that peroxidase catalyzes generation of diiodotyrosyl free radicals which combine to form a quinol ether intermediate that is subsequently hydrolyzed.

Splitting of the quinol ether intermediate would then yield either a serine or dehydroalanine residue.

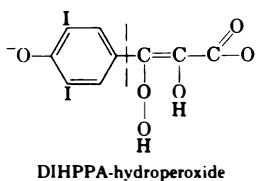


An alternate hypothesis is that a diiodotyrosyl residue is first deaminated to yield 4-hydroxy-3,5-diiodophenyl pyruvate (DIHPPA) which is then tautomerized to the enol form.





The peroxidase may then catalyze oxidation of DIHPA to DIHPA-hydroperoxide, and it is proposed that the latter combines (nonenzymatically) with a diiodotyrosyl moiety of thyroglobulin to yield a thyroxyl residue.



By-products of the reaction would then be ammonia (incorporated into other amino acids) and pyruvate.

Formation of T₃ presumably follows a similar pathway but involves combination of one monoiodinated with one diiodinated component.

Very small amounts of DIHPA (and of its monoiodinated analog, MIHPA) have been detected in thyroid tissue.

It is unlikely that thyronine formation ever precedes iodination, since no uniodinated thyronine has ever been found in the gland.

Storage of Thyroid Hormone

T₄ and T₃ residues, still attached to the thyroglobulin, are stored within the lumen as part of the colloid. Little or no thyroglobulin leaves the normal gland to enter the capillary circulation, but small amounts may be taken up by the lymph.

The thyroid stores more hormone than any other endocrine gland, and can supply needs for at least 2 weeks. This may be

especially important during periods when dietary iodine is in short supply.

Proteolysis of Thyroglobulin

Thyroglobulin is taken up from the lumen by a phagocytic process called *endocytosis*. Microvilli and pseudopods appear at the luminal surface of the follicular cells, and lysosomes migrate towards them. The thyroglobulin soon becomes visible within the cells as "colloid droplets" or vesicles, and *phagolysosomes* are formed by fusion of the vesicles with the lysosomes.

The processes of lysosome migration and vesicle formation are separable; actinomycin D permits the first to proceed while blocking colloid droplet formation. Microtubules have been implicated in both processes; hormone secretion is impaired by administration of agents (colchicine, vinblastine) which affect microtubules, but there are conflicting interpretations concerning action of those drugs.

Reduction of disulfide bonds of the thyroglobulin molecule by glutathione may be required to render the molecule susceptible to actions of hydrolase enzymes.

Proteolysis is accomplished within the phagolysosomes, and free T₄ and T₃ are liberated for diffusion into the capillaries. Usually more T₄ is released, and the most recently synthesized iodothyronines seem to be liberated first.

Deiodination in the Thyroid Gland

Very little MIT and DIT leave the thyroid gland. A deiodinase catalyzes removal of the iodine atoms and both these and the amino acids are recycled. Since the iodinated amino acids have no hormone potency, an important conservation function is performed.

The presence of T₃ moieties in the colloid indicate some is directly synthesized. But the T₃:T₄ ratio in thyroid gland effluent is higher than in the colloid, and this has been related to a second deiodinase enzyme which acts on free T₄. In times of iodine deficiency, the T₃:T₄ ratio can be increased 30-fold. The T₃ molecule has greater biological activity, and such conservation contributes substantially to

maintenance of thyroid hormone functions.

Synthesis of Thyroglobulin

Thyroglobulin is the major macromolecule of the thyroid gland lumen. It is a glycoprotein with a molecular weight of about 660,000. Differences in amino acid composition among the mammalian species seem to be small (except for guinea pigs, which also have unusual insulin and somatotrophin).

Polypeptides are synthesized on follicular cell ribosomes. Although the existence of "giant polysomes" has been described, synthesis may require participation of several different polysomes. Small (3-8S) proteins consistently found within thyroid glands are joined to form first 12S and later 17-18S molecules known as *prethyroglobulins*. (Inhibitors of protein synthesis impair formation of the small proteins, but not their combination.)

Thyroglobulins contain 8-15% carbohydrate which is added in a series of steps after completion of polypeptide synthesis.

Mannose seems to be incorporated first when the protein is associated with the endoplasmic reticulum, *galactose* and *fucose* are added when the product travels to the Golgi region, and *sialic acid* is added last. (Inhibitors of protein synthesis do not block addition of the sugar components.)

The 17-18S glycoprotein is secreted into the lumen. Iodination and maturation occur mostly or exclusively extracellularly. During maturation, the 19S iodinated glycoprotein is formed, but even larger (27S and 32S) molecules appear within the colloid. The completed thyroglobulin contains more than 200 cysteine components, and S-S bonds are formed in association with iodination reactions.

While the 17S prethyroglobulin seems to be linear, the 19S and larger forms are folded and more resistant to hydrolysis. (Newly formed iodinated thyroglobulin is preferentially utilized when TSH is administered; it is not known if this is attributable mainly to the greater susceptibility to degradation by proteolytic enzymes, to anatomical location within the lumina, or to both.)

The 19S form seems to contain four polypeptide chains and about 120 tyrosyl units. Only a relatively small fraction of the tyrosyl residues is iodinated when iodide supplies are adequate; it is believed, therefore, that these amino acid groups are contained largely within the interior of the molecule.

Attempts have been made to determine whether patients with congenital hypothyroidism produce abnormal thyroglobulins. In most cases, the evidence points to a defective rate of synthesis of a thyroglobulin of normal composition, and this may be associated with increased iodination of other thyroid proteins.

BINDING OF THYROID HORMONES TO PLASMA AND TISSUE PROTEINS

Thyroid hormones leaving the gland enter the blood plasma and form reversible combinations with plasma proteins. Since it is believed that only the "free" or unbound fraction is active, protein-linked components could provide inert but readily recruitable reserves. The binding functions in the circulatory transport and prolongs the biological life of the hormone by protecting it against loss through the kidney glomeruli and by tissue degradation. Relative binding affinities of plasma vs tissue proteins may play a role in regulation of the quantity of hormone delivered. It has been estimated that no more than 2 ng of T4 per 100 ml of plasma (or about 0.03% of the total T4) is present in the free form.

A number of pharmacological and physiological substances are known to affect binding affinity. Salicylates reduce linking to plasma proteins, while heparin reduces binding to both plasma and tissue proteins.

About 75% of the circulating T4 travels with a neuraminic acid-rich glycoprotein (molecular weight of about 60,000), which has one binding site for thyronines per molecule. The protein was formerly known as *thyroxine-binding globulin (TBG)*; since it is now known to also hold some T3, the name has been changed to *thyronine-binding globulin*, with the initials retained.

An additional 10-15% of circulating T4 is attached to *thyroxine-binding prealbumin (TBPA)*, a protein of approximately 50,000

molecular weight, which exists in plasma as a complex with vitamin A-binding protein. Binding of T₄ to TBPA may assume greater importance when the plasma content of TBG is reduced. Like TBG, TBPA seems to have a single thyronine-binding site.

Small amounts of T₄ also adhere to the plasma albumin fraction.

The concentration of T₃ in plasma is very much lower than the concentration of T₄ (estimated at about 100–160 nanograms per 100 ml as compared with 3.3–6.6 µg of T₄). T₃ binds to TBG, but much more weakly than does T₄, and it hardly binds at all to TBPA. By contrast with T₄, T₃ is attached more tightly to tissue than to plasma proteins, and the greater ease with which it enters tissues probably contributes to its greater biological potency.

The plasma binding proteins are synthesized in and secreted by the liver, and conditions affecting hepatic protein synthesis influence the quantity of these substances found in the blood plasma.

The binding capacity of TBPA is lower in children than in adults, and it increases during adolescence. Androgens decrease synthesis of TBG but increase that of TBPA. Effects on TBG may be of greater physiological significance, since androgens tend to increase the quantity of free T₄. Stress, severe illness, and administration of glucocorticoid hormones tend to reduce both TBG and TBPA.

During pregnancy and use of estrogen-containing oral contraceptives, TBG synthesis may be markedly increased. Then, an increase in total plasma content of T₄ is not necessarily associated with elevated concentrations of active hormone.

METABOLISM OF THYROID HORMONES

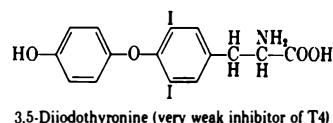
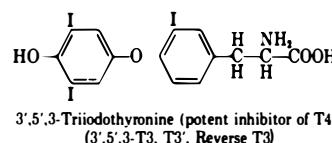
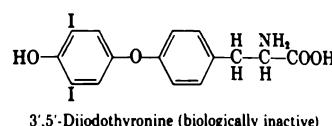
In Target Organs

Deiodinases catalyzing conversion of T₄ to T₃ are present in many cell types. A small portion of the liberated iodine ends up in the urine, but most is recycled.

There is growing evidence that T₃ is the active form in certain target organs. But T₄ receptors (distinct from T₃ receptors) have been identified, and it is possible that the two forms of the hormone carry out different functions. (The situation is reminiscent

of that seen for male sex hormones (Section VI) in which testosterone seems to act directly on certain parts of the brain, while conversion to dihydrotestosterone is necessary in some other structures.)

Other deiodination reactions yield thyronines which seem to be devoid of biological potency, and a few that are inhibitors of T₄ actions.



Such reactions may be involved in physiological termination of hormone activity.

In the Liver

The liver takes up T₃ and T₄ from the plasma; some is secreted into the bile and subsequently reabsorbed. "Enterohepatic circulation" is affected by diet; greater amounts of hormone escape absorption and are lost in the feces when bile formation and movement are stimulated by diets high in bulk and fat content. In rats (but perhaps not in humans) the system is activated by higher plasma thyroxine concentrations. Some fecal hormone arises directly from intestinal secretion. The liver also conjugates the hormones with *glucuronic acid*, and to a lesser extent with *sulfate*, and double conjugates (sulfo-glucuronides) have been found in sheep. Some of the conjugates appear in the bile; they are more readily reabsorbed if they are hydrolyzed by intestinal enzymes. Small quantities of conjugated hormone

also appear in the plasma; since they bind less strongly to the plasma proteins, they are more rapidly excreted.

Metabolic degradation reactions include deamination, decarboxylation, and combinations of the processes with deiodination.

There are unanswered questions regarding possible physiological functions of certain metabolic products. Some confusion regarding their potency has arisen from failure to consider effects of molecular alterations on absorption. For example, it was once believed that a deamination product of T₄ metabolism possessed greater potency for induction of amphibian metamorphosis; however, it was subsequently shown that the difference was accounted for by greater rate of uptake of the product than of T₄ from the surrounding fluids.

Some of the degradation products are shown in Figure 17-3.

THYROID-STIMULATING HORMONE

Source and Chemistry^{2, 9}

Thyroid-stimulating hormone (TSH, thyrotrophin, thyrotropin) is synthesized and secreted by thyrotroph cells of the adenohypophysis which are described in Section VII. It is a glycoprotein containing 7-8% carbohydrate, with a molecular weight of about 28,000. There are small species differences in amino acid composition. Within the same species, differences in carbohydrate components have been reported. It has not been established whether carbohydrate differences have functional significance or are attributable to artifacts associated with extraction procedures.

In common with some other pituitary hormones, TSH is made up of α - and β -subunits

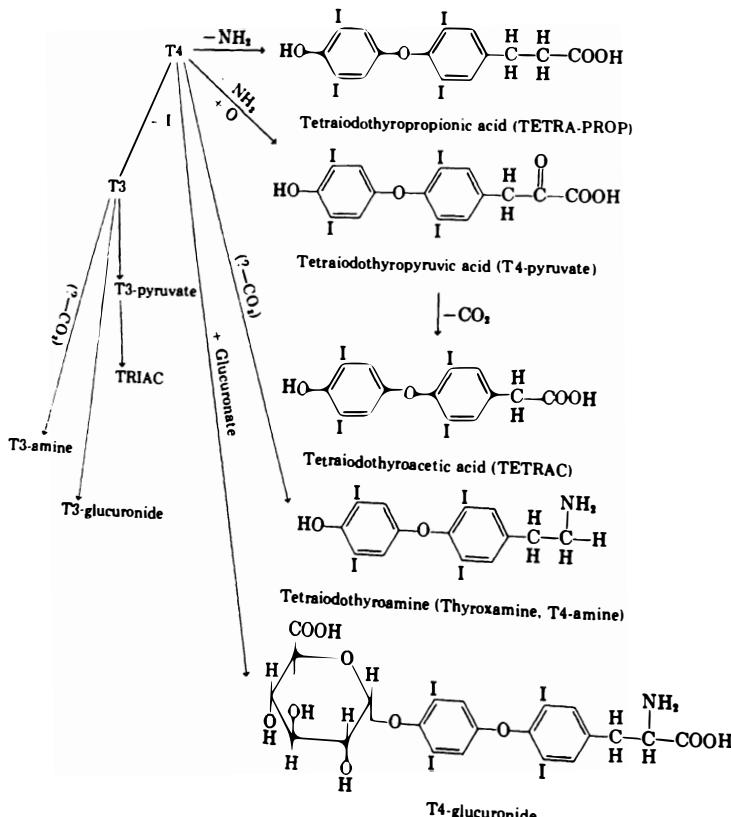


FIG. 17-3. Some metabolites of thyroxine.

linked together by noncovalent bonding. The α -subunit (MW approximately 13,600) contains five disulfide bonds and is similar in composition to the α chains of follicle-stimulating and luteinizing hormones, and shows little species specificity. When separated, the α -unit seems to be devoid of biological potency, but may function in binding of TSH to its receptor; small amounts have been found in the plasma.

The β -subunit (MW about 14,400) contains six disulfide bonds and seems to carry the active sites for hormone action.

Functions^{1, 2, 7}

TSH exerts a *tonic trophic action* on the thyroid gland, maintaining its *structure and promoting blood flow* to the gland. The thyroid atrophies when no TSH is present, and undergoes hypertrophy and hyperplasia when excessive TSH is administered or secreted. The actions are evidently mediated via cAMP, since TSH is a powerful stimulant of thyroid adenylate cyclase. All of the known TSH actions have been mimicked by administration of D-cAMP (and can be potentiated by caffeine, theophylline, and aminophylline).

In high dosage, TSH can stimulate adenylate cyclase in other cell types. It promotes lipolysis in adipose tissue, but it is not known if this represents a physiological function.

Iodide Uptake. TSH provides the major control for iodide uptake. In untreated hypophysectomized animals, the T:S ratio may fall well below 8; and in iodine deficiency states, the very high values depend directly upon increased TSH secretion.

TSH depolarizes the thyroid cell membrane, and other agents which depolarize can also enhance iodide uptake. Both sodium and potassium are required in the extracellular fluids, and sodium is known to enter the cell. TSH enhances ATPase activity, and inhibitors of Na-K-ATPase (e.g., ouabain) depress iodide uptake. Actions of TSH are indirect; there is early iodide efflux, but accelerated uptake does not develop for many hours and seems to require new protein synthesis. (A latent period of 10 hr has been described in hypophysectomized rats, with full development of response to a single large TSH dose in about 30 h.)

It has been proposed that TSH promotes synthesis of either new "iodide pumps" on the basal membrane or something which increases activity of existing pumps. How-

ever, the hormone also promotes incorporation of phosphate into membrane lipids (and may induce formation of iodide carrier). A mechanism has been proposed which involves interaction of iodide with the phosphate components. TSH also promotes glucose uptake and utilization by thyroid gland cells; this may be important for provision of ATP energy for iodide uptake and other functions.

Other Influences on T3 and T4

Synthesis. TSH seems to exert influences on iodide oxidation, iodination of tyrosyl moieties of thyroglobulin, and coupling of iodotyrosyl units, which are separable from effects on iodide uptake. It also enhances the rate of amino acid incorporation into thyroid gland proteins, and thyroglobulin synthesis.

Influences on Secretion of T3 and T4.

TSH promotes endocytosis of thyroglobulin, migration of lysosomes, proteolysis of thyroglobulin, and release of the hormones into the capillaries.

Influences on secretion of preformed hormone are among the first measurable effects. It is likely that hormone exit secondarily affects other processes influenced by TSH. Within minutes after TSH administration, changes in staining properties of the colloid can be demonstrated, and vacuolization soon follows. Prolonged TSH action may lead to an increase in the T3:T4 ratio of the thyroid effluent.

Regulation of TSH Secretion^{2, 4, 8, 13}

Thyroid Hormones. It was noted above that thyroid hormones increase oxygen consumption of a large number of cell types, but exert no obvious influence on oxygen consumption of a few others. The thyrotrophs of the pituitary gland are unique in that thyroid hormones depress their metabolism. The effect requires at least 1 hr to develop, and seems to depend upon formation of a specific regulatory protein. (Thyroid hormone depression of TSH secretion can be blocked with inhibitors of RNA or protein synthesis.)

The question of whether T3 or T4 is the prime regulator of TSH secretion is difficult to resolve, since administration of either can be effective, and pituitary cells can convert T4 to T3.

Thyrotrophin Release Factor (TRF)

^{8, 9, 10} TSH-secreting cells require a hypothalamic hormone for maintenance of morphology and functions. The structure of the thyrotrophin release factor (TRF, thyrotrophin releasing-hormone, TRH) is shown in Figure 17-4. A nonribosomal TRH-synthetase system catalyzing its formation has been identified.

TRF has been found in many parts of the brain, and it undoubtedly performs functions in addition to regulation of thyrotroph cells. It promotes release of prolactin, and exerts influences on cells secreting STH. It is described in greater detail in Section VII.

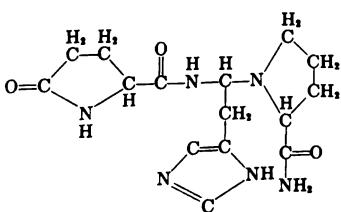
Thyroid hormones have been reported to induce formation of the TRH-synthetase system, and thereby exert positive feedback control over T₃ and T₄ synthesis. But they also exert negative control by decreasing sensitivity of the thyrotrophs to TRF influences.

LONG ACTING THYROID STIMULATOR (LATS) AND CLINICAL HYPERTHYROIDISM^{1, 2}

It was once thought that clinical hyperthyroidism (Grave's disease) could be explained on the basis of insensitivity of the thyrotrophs to thyroid hormone inhibition. However, it is now known that the overwhelming majority of patients have very low plasma levels of TSH.

An immune globulin of the IgG type has been found in the serum of at least three-quarters of such patients. It was named long acting thyroid stimulator (LATS) because it exerts influences on the thyroid gland qualitatively similar to those of TSH, but slower in onset and longer in duration.

Very small differences have been noted; e.g.,



Pyroglutamic Acid-Histidine-Prolineamide

FIG. 17-4. Structure of TRF (thyrotrophin release factor, thyrotrophin-releasing hormone, TRH).

both TSH and LATS stimulate phospholipid synthesis by follicular cells but there is relatively more phosphatidylinositol after LATS and more lecithin after TSH.

LATS is believed to be produced by thymus-dependent lymphocytes in persons having a genetic predisposition. Lymphoid follicles have been found in thymus glands of patients with Grave's disease, and it has been proposed by some that LATS is synthesized there. Others believe that the release from the thyroid of proteins normally sequestered, triggers peripheral synthesis of the protein.

While LATS is related to the etiology of Grave's disease, there are unexplained observations. Some patients with the disorder and with low TSH levels do not seem to have LATS in their sera, while the protein has been found in relatives of patients with the disease who do not themselves have hyperthyroidism. Several explanations have been offered: (1) It is the thyroid, rather than the plasma content of LATS, which determines influences on thyroid function. (2) LATS acts indirectly in a "permissive" manner which requires the presence of as yet undiscovered factors. (3) There are LATS antagonists. (4) Hyperthyroidism becomes overt only when thyroid cells are abnormally sensitive to LATS.

There is a very high incidence of thymus gland hyperplasia (and of thymus-related disorders) among patients with Grave's disease. It has not been established whether abnormal thymus function is involved in the etiology, or whether the hyperplasia results from chronic hypersecretion of thyroid hormones (See also Section VIII).

There is a much debated question about the role of *emotional factors* in spontaneous hyperthyroidism. It is common to find in case histories that the thyroid disorder first appeared soon after *removal* of an emotionally stressing condition—a rebound effect. An older concept that emotional stress acts on the hypothalamus to affect TRF release has lost favor in the light of information that TSH levels are depressed; however, glucocorticoids are released during periods of stress and have been shown to influence both TRF secretion and the immune system.

EXOPHTHALMOS^{2, 3}

Clinical hyperthyroidism is commonly associated with bulging of the eyeball (ex-

ophthalmos) which may affect one or both eyes. The orbital contents is increased because of accumulation of fluid and a hydrophyllic connective tissue ground substance rich in hyaluronic acid, increased numbers of connective tissue cells, and increased mass of the extraocular muscles.

Before it was recognized that TSH levels may be very low in hyperthyroidism, it was suspected that TSH might be the factor which induces exophthalmos. The suspicion was strengthened by observations that patients responding to agents which reduce thyroid hormone secretion (and therefore the supposed feedback inhibition of TSH) sometimes exhibited exacerbation of eye problems, and by experimental demonstration of induction of exophthalmos in fish, ducks, and guinea pigs by administration of TSH.

Subsequently, a protein similar to (but distinguishable from) TSH was found in pituitary glands and named *exophthalmos-producing substance* (EPS). It was suspected that conditions leading to increased secretion of TSH also promote secretion of EPS. But a recent study has demonstrated that purified TSH exerts both qualitative and quantitative actions on functions of the Harderian gland (which occupies much of the orbit) in guinea pigs, and that the effects were enhanced by prior thyroidectomy.

In Grave's disease, it is suspected that something other than LATS is largely responsible for effects on the eye.

GOITROGENS^{1, 2}

A goiter is an enlargement of the thyroid gland. It usually results from hypersecretion of TSH. The term goitrogen can be applied to any agent inducing the condition, but it is sometimes used in a more restricted sense to designate agents which interfere with T3 and T4 synthesis.

Agents Affecting Iodide Transport

A number of monovalent ions can compete with iodide for transport mechanisms, and thereby reduce iodide availability for synthesis of T3 and T4. These include thiocyanate, nitrate, perchlorate, and the radioactive pertechnetate (TcO_4^-). They are useful tools for study of thyroid functions because they promote rapid discharge

of inorganic iodide and thereby provide a means for readily distinguishing between "free" and "bound" iodide within the gland.

When the diet is somewhat deficient in iodine, these agents can interfere with thyroid hormone synthesis to the point where normal negative feedback controls on TSH secretion are impaired; they are then goitrogenic. When the diet contains normal amounts of iodine, perchlorate cannot act as a goitrogen, whereas thiocyanate and nitrate (which also affect peroxidase enzymes) are effective.

Agents Affecting Metabolism of Thyroid Hormones

Diets containing excessive quantities of foods which stimulate biliary excretion and impair absorption of thyroid hormones from the intestine can be goitrogenic. Very large amounts of soy beans or walnuts can do this, but quantities normally consumed do not have such action.

Salicylates and some other drugs impair binding of thyroid hormones to plasma proteins and thereby increase the rate of excretion and degradation. Very large doses given for long periods of time can be goitrogenic.

Naturally Occurring Goitrogens Which Affect Hormone Synthesis

Cabbage contains small amounts of thiocyanates, and a large number of plants contain cyanogenic glycosides which are metabolized to thiocyanates. Nitriles in cigarette smoke can also lead to elevation of the thiocyanate content of blood and urine. It is suspected that such plants have negligible direct influences on thyroid function, but can aggravate effects of iodine deficiency.

A large number of vegetables (including cabbage, kale, kohlrabi, rutabaga, cauliflower, and mustard) contain *progoitrin* which is metabolized to *goitrin* (L-5-vinyl-2-thiooxazolidone, Fig. 17-5, A and B). Goitrin acts like antithyroid agents described below, but is of low potency. An irregular occurrence of goiter has been described in rabbits fed a cabbage diet. But it is unlikely that the quantities of progoitrin-containing foods normally in-

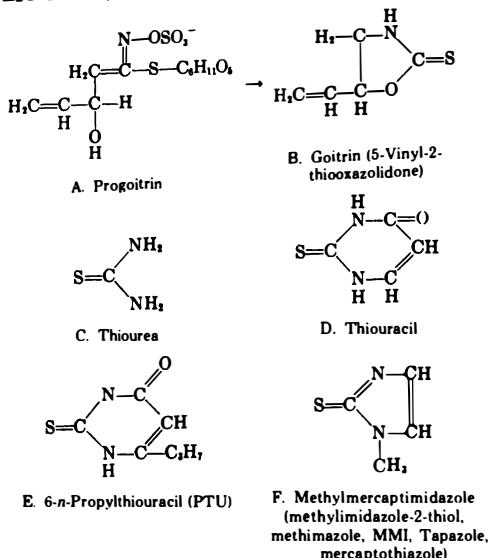


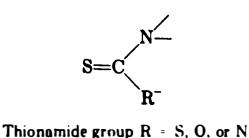
FIG. 17-5. Structures of progoitrin and of some thionamide goitrogens.

gested exert important influences when iodine is present in the diet.

Potent Antithyroid Compounds

Agents which interfere with the peroxidase system of follicular cells are used both clinically and in research. Small doses seem to preferentially block coupling reactions in the thyroid gland, while large amounts interfere with iodination of the tyrosyl components of thyroglobulin. Some (e.g., propylthiouracil, see below) also reduce effectiveness of administered T4 and this has been related to interference with conversion to T4 to T3.

The most potent antithyroid compounds contain the *thionamide* group.



The simplest member of this group is *thiourea*, but it is less potent and less useful than the others. Thiouracil and 6-n-propylthiouracil (PTU) are widely used in the laboratory, and PTU is also administered to patients. *Methylmercaptoprimidazole* (*methimazole*, *methylimidazole-2-thiol*,

2-thiol, *MMI*, *Tapazole*) is highly potent. It is most often used clinically and for some research studies. The structures are shown in Figure 17-5. Some aromatic agents used for other purposes (Fig. 17-6) are also weak goitrogens.

The potent compounds are used to prepare patients with severe hyperthyroidism for thyroid surgery, since they can *reduce surgical risk* by lowering hormone stimulation of the heart and prevent occurrence of "thyroid storm" which could result from release of large quantities of prestored T3 and T4 during manipulation of the gland. They are usually given with large quantities of iodides (see below) to limit the increase in size and vascularity of the thyroid gland which would otherwise occur when goitrogens are administered alone.

Goitrogens are sometimes administered to patients scheduled for radioactive iodide destruction of thyroid tissue; in this case, it is desirable to increase vascularity of the gland.

A certain number of patients are safely treated with the agents alone. Reduced thyroid hormone secretion occurs as early as 24 hr after ingestion of the first dose, and some individuals can be maintained in a relatively euthyroid condition until "spontaneous remission" of the hyperthyroid state ensues.

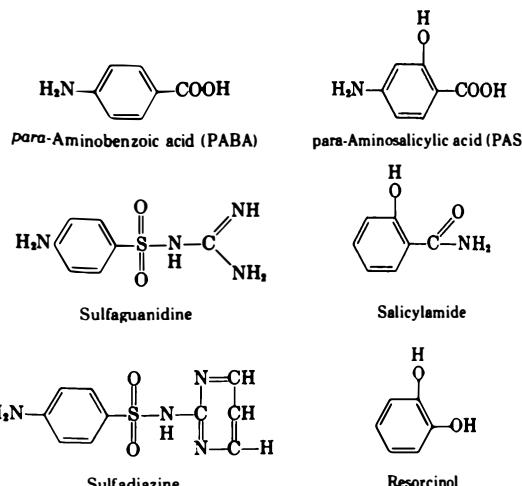


FIG. 17-6. Aromatic compounds with antithyroid activity.

Goitrogens are also widely used in the laboratory for induction of experimental hypothyroidism, and in studies of thyroid gland physiology. The agents are effective when administered orally, and can be mixed with food or placed in drinking fluids.

Hypothyroidism can be induced without the complications of surgery, anesthesia, damage to other structures, or removal of parathyroid glands and calcitonin-secreting cells. Moreover, it is possible to achieve partial depression of thyroid hormone synthesis by using smaller doses, and to permit animals to recover by withdrawing the agents. Ability to block iodide utilization for hormone synthesis without interference with iodide uptake is also useful in certain kinds of studies.

The slow development of thyroid deficiency is of advantage for some kinds of investigation, but a disadvantage for others. "Side effects" of the drugs on nonthyroid tissues sometimes present problems for both clinicians and investigators. Effects on the bone marrow can lead to agranulocytosis. Patients sometimes suffer from skin rashes and nausea.

IODINE-DEFICIENCY GOITER

Iodine-deficiency goiter occurs most commonly in areas in which the water and soil (and therefore the vegetation and animals used for food) are deficient in iodine. The condition is relatively uncommon now

because of the widespread practice of adding iodides to table salt. But it is occasionally seen in patients on salt-restricted diets and under conditions of suboptimal iodide intake complicated by excessive loss of iodide from the body. Iodide loss is increased during pregnancy, in severe kidney conditions (nephrosis), and in persons with genetic defects affecting synthesis of deiodinase enzymes.

In the early stages, iodine-deficiency goiter has all of the characteristics to be expected from increased secretion of TSH. The thyroid cells are enlarged, colloid is depleted, and the capillary supply to the gland is increased. The condition can be reversed by administration of iodine. In later stages, degenerative changes in the gland are irreversible.

EFFECTS OF VERY HIGH IODINE INTAKE¹²

Large doses of iodide exert a number of inhibitory influences on the thyroid gland which are only partially understood. They are of considerable interest because of the information that can be gained on mechanisms of thyroid hormone synthesis and its regulation, and also because large doses of iodide can provoke a rapid and clinically useful (but often transient) decrease in secretion of thyroid hormones in hyperthyroid patients.

When plasma iodide concentrations are very low, they can limit the rate of T3 and

T₄ synthesis, and there is a small range in which increases can be related to increments in hormone synthesis. T:S ratios are very high under these conditions because the feedback inhibition of thyroid hormones on TSH secretion is incomplete.

At considerably higher plasma iodide concentrations, the anion accumulates in the thyroid gland, and this leads to formation of an as yet unidentified molecule within the gland which limits subsequent uptake. (The existence of this *autoregulatory* mechanism within the thyroid gland can be demonstrated in hypophysectomized animals.) T₃ and T₄ synthesis are adequate and the T:S ratio is relatively low.

Very high iodide concentrations saturate the iodide transport system, but the gland content of iodide rises excessively. Inhibitory influences on glucose utilization and on the thyroid peroxidase system become apparent, while efflux of nonhormonal iodide increases. In patients with hyperthyroidism, reduced rate of release of thyroid hormones from the gland can be demonstrated starting about 24 hr after administration of large amounts of iodine and potassium iodide. It has been suggested that nonhormonal organic iodides released from the gland inhibit TSH secretion. Very high iodide concentrations within the gland may also act directly on T₃ and T₄ release by impairing proteolysis of thyroglobulin.

Inhibitory influences of the very high doses of iodides can be demonstrated in patients in which LATS seems to be responsible for the hyperthyroid state, and also in experimental animals treated with propylthiouracil.

OTHER FACTORS AFFECTING THYROID GLAND FUNCTION^{10, 11, 16}

Thyroid-pineal interrelationships are described in Chapter 25. TRF has been found in pineal glands, and it has been proposed that it may participate in regulation of TSH secretion under some conditions. There are conflicting reports concerning influences of the pineal hormone *melatonin*. In most cases in which positive data were obtained, the influences seemed to be inhibitory. Pinealectomy does not lead to serious disruptions of thyroid function, either because the described influences are

physiologically unimportant, or else because stimulatory and inhibitory factors are simultaneously removed.

Vasopressin and *melanocyte-stimulating hormone* have been reported to stimulate the thyroid gland, but the physiological significance of the findings is unknown.

Prostaglandins have been identified in normal thyroid glands and in culture fluids of thyroid medullary carcinoma cells. PGE₁ and PGE₂ stimulate thyroid adenylate cyclase and can elicit TSH-like influences on iodide uptake and on hormone release (but not on phospholipid metabolism); moreover, small quantities of the prostaglandins can synergize with low TSH doses.

TSH promotes prostaglandin synthesis in the thyroid, and the effect can be blocked by administration of inhibitors of prostaglandin synthesis. Agents which interfere with actions of prostaglandins have been reported to block TSH and PGE₁ influences on iodide uptake and hormone release, but not TSH stimulation of thyroid adenylate cyclase.

The findings point to some role of prostaglandins in thyroid hormone physiology, but more information is needed to clarify their functions.

The thyroid gland receives *sympathetic innervation*, and there have been suggestions that stimulation of the nerves leads to rapid increases in blood circulation to the thyroid gland and increased gland activity. The nerves may promote release of *serotonin* which participates in the vascular effects.

RADIOACTIVE IODIDE

Tracer doses of ¹³¹I are used to measure ability of the thyroid to take up and concentrate iodide. The test actually measures TSH rather than thyroid gland function in most cases, but can pick up defects in transport mechanisms when used in conjunction with other tests. Under the usual conditions, glands which rapidly take up iodide also produce large quantities of thyroid hormones. But iodide uptake (and discharge) are accelerated when administration of goitrogens or defects in enzyme systems interfere with iodide utilization. Effects of tracer doses are transient because much of the isotope soon appears in the urine, and the short half-life of

remaining isotope precludes severe damage to the gland.

Tracer doses are also useful for detection of thyroid cells outside the gland, and in diagnosis of some disorders in which malignancy is suspected.

Large doses of ^{131}I are used to deliberately destroy thyroid tissue ("radiothyroidectomy") in experimental animals and in some patients suffering from hyperthyroidism. In addition to avoidance of problems associated with thyroid surgery, there is the added advantage that aberrant thyroid tissue will also be affected.

However, high doses of the radioactive isotope can affect neighboring structures, and there is a suspicion that they can contribute to development of malignancy.

In patients with thyroid cancer there is an obvious danger. Thyroid cells most normal in function will avidly take up the isotope and be destroyed, while cells which have undergone regressive changes have a greater chance of survival.

Longer-lived isotopes of iodine have been used alone and in combination with ^{131}I for other kinds of studies on the thyroid gland. ^{125}I exerts influences that are more localized; this is of advantage for certain procedures.

TESTS OF THYROID FUNCTION²

Since ^{131}I uptake can be influenced by extrathyroidal metabolism of iodide (and especially by renal excretion), a comparison of rate of ^{131}I uptake with plasma concentrations of ^{131}I (the "thyroid iodide clearance rate") is sometimes made. The rate of reduction of radioactivity in the thyroid gland provides a measure of the rate of hormone release (but is not specific for thyroid hormones; it is affected by release of nonhormonal iodine).

Under most conditions, determinations of the *protein bound iodine* concentration of the blood plasma (PBI) give a good indication of the quantity of circulating thyroid hormone. But the PBI also measures nonhormonal iodinated proteins and it is not useful in patients who have taken certain iodine-containing dyes for x-ray diagnosis. Moreover, the PBI does not tell anything about the quantities of "free" or active thyroid hormone in the plasma. The *butanol-extractable iodine* (BEI) eliminates problems associated with high

inorganic iodide concentrations in the plasma but has many of the shortcomings of the PBI procedure.

Recently, more precise methods for measurement of plasma T4 and of T3 have been developed, and these provide a reliable indication of hormone secretion. However, they give no information on ability of the organism to respond to thyroid hormones.

The basal metabolic rate measurement reflects effects of thyroid hormones. But, as noted, it is a cumbersome test to use, is not very sensitive, and is affected by nonthyroidal factors.

Under certain specified conditions, *cardiac rates* are a useful reflection of changes in levels of thyroid hormones. Tests of this kind can only be used under highly controlled conditions when rates have been established before initiation of hormonal manipulations.

Measurements of *serum cholesterol* have been used in diagnosis of hypothyroidism, since the hormone tends to reduce the values. They can be helpful in conjunction with other procedures, and especially when repeated measurements are made within the same individual during the course of therapy. But values vary widely from individual to individual, and in the same person at different times, and are influenced by a large number of factors unrelated to thyroid hormone function.

Recent availability of TRF and procedures for determination of plasma TSH have made it possible to distinguish between hypothalamic and hypophysial factors affecting the thyroid. In most hypothyroid subjects, TSH concentrations and sensitivity to TSH are high, since thyroid hormone feedback influences are low. But in a few, the pituitary gland shows poor responsiveness.

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18. Food Intake, Body Composition, and Hormones

Food provides the building blocks for protoplasm, fuel for present and future use, and substances which participate in the regulation of metabolic functions.

Genetic factors determine to a great extent what *kinds* of macromolecules are formed and how they are distributed. Cows and chickens eating the same amino acid mixture synthesize some very different proteins and arrange them in characteristic ways. But food and hormones affect the *numbers* of macromolecules manufactured, their relative *proportions* and the *rates* at which they are synthesized and degraded. Hormones also influence their distribution and are especially important for determination of what will be stored for future use and what will be preferentially degraded during times of stress, strenuous activity, and food deprivation.

Hormones and diet also affect the quality of body chemistry. They are needed for realization of developmental potential, and can influence the makeup of those biological molecules for which the organism tolerates variation, e.g., the kinds of fatty acids which predominate in adipose tissue.

Highly complex interactions between the nervous, endocrine, and motor systems on the one hand, and environmental factors on the other, enter into the determination of what is eaten when, and how the food is utilized. The state of general health, the condition of the skeletal musculature, functions of the gastrointestinal tract, sensory receptors (gustatory, olfactory, visual, tactile), age, sex, previous dietary history, and psychological conditioning affect responses to availability and palatability of the diet and to the conditions under which it is offered. And the food itself contains components which *indirectly* as well as directly affect what is vaguely known as "appetite."

Singly caged animals of gregarious species often eat less than when they are housed in groups, but members of the group can impose stress or limit access to the food cup. Nocturnal animals respond differently to food offered in bright daylight than they do to food presented in semi-darkness. Animals approaching the time of hibernation may eat much more than they do at other times. Responses to

small quantities of food offered at frequent intervals can be very different from responses to the same food presented once a day. Some seasonal variations can be directly related to environmental temperature, humidity, and daylength, but others cannot.

Studies involving manipulation of the diet pose numerous problems for interpretation. Any change (dilution with non-nutrients, alteration of the carbohydrate: fat ratio, or amino acid content) is invariably accompanied by changes in texture and usually flavor. Underfeeding, force-feeding, requiring animals to "work" for food, brief periods of fasting, and presentation of unfamiliar foods, all constitute forms of stress, as does placement of animals in metabolic cages.

REGULATION OF THE QUANTITY OF FOOD INGESTED: PHYSIOLOGICAL, PSYCHOLOGICAL, OR BOTH?^{1, 2, 20}

Few questions have generated as much heat (and as little light) as those involving the sorting out of factors affecting food intake when food availability is not a limiting factor. One may ask why a healthy laboratory rat confronted with a week's supply of food pellets eats a "reasonable" and predictable quantity each day, why most pet cats eat enough to sustain body weight when more than enough is offered, why dogs of some breedsgulp everything in sight to the point where they become ill, and why *some* cats grow fat.

The Case for Psychology

It is often stated that the higher the development of the brain, the more food intake is regulated by psychological factors; and the concept is most obviously applied to human subjects. Some of the interest in the topic stems from attempts to cope with the high incidence of obesity in modern societies. An obvious advantage to psychological control is that food intake can be "anticipatory"; *i.e.*, food can be taken when it is available and before deficiency problems develop.

It is easy enough to point out psychological influences. One may very well decide to eat because it is "time" for breakfast, because others are eating, because the hostess has worked hard to prepare a spe-

cial (usually highly caloric) delicacy, because mother (mother-in-law, boy friend, etc.) will be offended if the offered food is not eaten, because it is included in the fixed-price dinner, because there are only two chocolates left in the box, because what else is there to do on such a long airplane flight?

Not only the timing but also the *quantity* of food taken is influenced by non-physiological factors. Big and small men, those active and those sedentary, may eat two eggs for breakfast because that is the quantity served. Persons who feel hungry at 10 A.M. and those not particularly interested in food at noon may eat the same size sandwich for lunch (because there it is and nothing else will be available until dinner time). And early conditioning to "clean the plate" can have lifelong consequences.

The problem is not *whether* psychological factors affect food intake, but rather *how important* are they in determining long range relationships between consummatory behavior and regulation of body weight? Individuals exposed to similar external factors react very differently; is this because they differ *physiologically*, or can everything be explained on the basis of what food "means" (psychologically) to the individual?

Some nibble when under tension, others gorge, and still others shun food. Some seek out alcohol and the associated calories are added to the usual supply from food; others drink instead of eating and tend to lose weight. Still others find "oral" outlets in black coffee, chewing gum, or cigarettes.

There are those who eat when weary and feel especially refreshed afterward if the food contains sugars; others claim that eating at such times only adds to the sense of fatigue. Some "feel good" after exercising moderately and take less food than when they are completely sedentary; persons in this category come in all sizes and shapes. But many feel tired and especially hungry after only the mildest of physical exertion.

Individuals also differ widely in their choice of foods. Can this be attributed entirely to conditioning? Or are there different metabolic needs which demand satisfaction?

There are suggestions in the literature

that persons who are grossly overweight or excessively lean suffer from characteristic neuroses or "personality disorders." But there are also indications that some of the psychological factors result from the awareness of being different, and there is also evidence that persons of normal weight suffer from the same kinds of problems as those described among the obese. It has additionally been proposed that psychological disorders found among persons with "severe eating problems" are unrelated to factors which control feeding behavior in "normal" individuals.

The Case for Physiology^{8, 9, 10}

Without negating obvious influences of social factors, psychological conditioning, and sensory stimulation, strong arguments can be made for predominantly physiological control of the relationships between food intake and maintenance of body weight.

Some individuals exhibit very large variations in food intake in response to the factors described above, and still maintain body weight within narrow limits without much conscious effort. Others work successfully at compensation—for example, they may skip meals or skimp on them to the point of feeling genuinely hungry if they have recently been stimulated to "overindulge;" or they may make real efforts to take in more food than feels comfortable if too much weight has been lost. The "compensation" is accomplished without serious interference with daily activities. But there are those who work very hard at attempts to compensate, and endure genuine physical discomfort in the process; yet, they manage over long time periods to acquire gross deviations from "normal" body weight.

From one point of view, the adjustments of the metabolically healthy individual seem remarkable indeed. Human adults having ready access to a varied diet manage to consistently select out the equivalent of about three quarters of a million food calories each year. The nature of the selection varies widely in one locality as compared with another, and from one individual to another. Often it is accomplished with little thought directed at satisfying anything other than appetite. Yet the daily

addition or deletion of the nutrients contained within a single small slice of lightly buttered bread could lead theoretically to a body weight change of about 100 pounds in a decade if no adjustments were made to the change.

Experimental animals of many species readily increase the quantity of food consumed when their diet is diluted with nonnutrients, when they are exposed to cold temperatures, or when they are forced to increase their activity. They readily decrease bulk intake when the diet is concentrated.

If food intake is controlled by the investigator, animals can be made to gain or lose weight. For example, animals can be force-fed or subjected to brief periods of food deprivation. It is also possible to severely disrupt their regulatory mechanisms through injections of hormones or presentation of highly unusual foods. But if the manipulation is discontinued in an animal without irreversible injury, the pre-experimental weight is reestablished during a brief period of "over" or "under" eating. Something "tells" the animal how much it needs. If animals are continually force-fed, they stabilize weight at some high level; they do not continue to gain indefinitely.

Body weight control must involve adjustment of food utilization as well as of food intake. Animals deprived of food usually reduce voluntary activity; but metabolic changes also play a role. Rats given a protein-deficient diet have been observed to ingest enough to satisfy amino acid requirements without gaining the weight that might have been expected from the increased caloric consumption.

In a study of human volunteers who had previously maintained body weight within normal limits without conscious effort,²⁶ food intake was voluntarily increased in an attempt to add 20% additional weight. An increment in food intake far beyond that suggested by conventional methods for calculation was required for the gain. Most of the subjects had great difficulty putting on the weight, and tended to lose it while continuing to ingest the enriched diet. A few could hold the extra poundage only when fat intake was very high. Interestingly, many decreased voluntary activity during the time they had difficulty holding the added weight.

FOOD INTAKE, BODY COMPOSITION, AND HORMONES SET POINTS^{7, 10, 11}

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Some of the observations cited above, and others described below, are consistent with the concept that each individual has some kind of "set point" for body weight which affects (and is affected by) the food intake. Obese persons who consciously restrict food intake and achieve significant weight loss find themselves returning again and again to the same high level when they cease vigilance; excessively lean persons who accomplish slow weight gain through ingestion of more than the appetite dictates find themselves rapidly returning under conditions of stress to the initial low level. The term "yo-yo syndrome" has been applied especially to obese dieters. Animals with lesions that affect food intake tend to stabilize at some new level when given free access to food, and maintain the ability to reestablish that level when subjected to transient manipulations.

The set point seems to vary widely among individuals. In the study described above, human volunteers easily maintained normal body weight on a diet supplying 1800 food calories per square meter of body surface per day; they needed 2700 to maintain a higher weight even after reaching the plateau. By contrast, a group of obese subjects maintained a far greater average weight with 1500 calories per square meter per day, and had to cut this drastically to lose measurable amounts. The set point also seems to vary in the same individual at different times; some humans and other animals are consistently heavier during the winter months. One wonders if accepted standards of "normal" weight really apply to all individuals.

In humans with long-standing obesity and in animals which have grown fat because of genetic factors or lesions, restriction of caloric intake usually leads to relatively greater loss of protein and less of depot fat than in individuals of lower weight who are subjected to the same restrictions.

Persons whose weight is easily maintained within socially acceptable limits often express the opinion that those who "overeat" (as judged by body fat stores) are lacking in "will power." Yet it has been repeatedly demonstrated that those of normal weight are far more distressed by very

limited food deprivation (which does not threaten body function) and will put up with more inconvenience and even social censure to acquire food.

The plethora of advice offered to obese persons, its conflicting nature, and the long range futility of following most of it, suggest that little is really known of the regulatory factors.

REGULATION BY INTERNAL VS EXTERNAL SIGNALS^{4, 20, 21}

It has been proposed that those who respond with little difficulty do so by responding to "internal" cues; they eat when they "feel hungry" and this may involve awareness of gastric contractions, a gut sensation of "emptiness," perhaps headache, and probably a large number of factors which never ascend to the conscious level. By contrast, those with difficulty maintaining weight eat in response to external signals. Presumably, members of the first group also stop eating in response to internal signals.

In support, it has been shown that falsifying the time of day has a far greater effect on obese than on normal weight subjects. When some of the former were placed in rooms with fast-running clocks, they reported feeling hungry at "dinner time" while those of normal weight did not. Similarly, when clocks ran behind, the subjects of normal weight reported hunger at a time when they usually ate and were not influenced by the clock reading, whereas obese subjects reported fewer hunger sensations.

Perhaps the possibility should be considered that obese subjects feel hungry most of the time, and even more so at the usual dinner hour, but that they have trained themselves to ignore internal signals. Therefore, when the clocks ran fast these subjects felt "entitled" to eat, but when the clocks ran behind they worked harder at hiding the sense of hunger.

In another study, obese subjects ate larger numbers of sandwiches than did normal-weight controls, when three were placed nearby on a table; but they ate fewer when one sandwich was placed on the table and others were readily available in a refrigerator near the table. The data

were interpreted as indicative of greater impact of food-related stimuli upon the obese persons. (And unrelated experiments have pointed to more acute perception by overweight individuals of details within the environment.)

Past conditioning could also affect the findings. Obese subjects sensitized to criticism of their eating habits are more likely to suffer inhibitions against opening someone else's refrigerator. Obviously, the obese subject does not hesitate to obtain food from his own refrigerator.

In a third study, a liquid diet was made freely available under conditions in which the subject could not gauge the quantity consumed. Obese subjects tended to take little of the liquid and some lost weight, while the control subjects took enough to maintain body weight. The obese subjects could have been demonstrating poor responses to internal cues; but persons long accustomed to self-denial might be expected to take less interest in food that offers little pleasure.

The distinction between internal and external cues is difficult to reconcile with some commonplace observations. It is much easier for persons of all sizes to touch and smell a single peanut and put it down again than it is to "eat just one." This suggests that some kind of inhibition against eating is released by everyone as soon as pleasing food is tasted or chewed. Obese persons are not necessarily more likely to start eating peanuts, but many will eat more of them. This suggests that the difficulty resides in the "turning off" mechanism rather than with inhibition of meal initiation. Many obese persons eat little during early parts of the day, and consume most of their food during a restricted portion of the evening. The "night-eating syndrome" and "binge" eating could be explained on this basis. So could the ingestion of three sandwiches in rapid succession, with hesitation to eat more than one if the meal must be interrupted by a trip to the refrigerator.

SATIETY

Understanding mechanisms for regulation of food intake would be much facilitated by definition of the conditions under which meals are voluntarily terminated. Few persons who easily regulate body

weight can provide appropriate descriptions. Some say they "feel full," but they approach this point on different days after consuming meals which vary greatly in bulk. Although changes in electrical activity of parts of the brain associated with regulation of food intake have been detected in response to distention of the stomach, it is likely that distention plays only a minor role in satiety. Animals with gastric fistulas (which make gastric distention impossible since excess bulk is extruded) will stop eating in response to the presence of small volumes of food materials placed directly into the intestine. Animals given a diet high in bulk will consume enough of it to obtain the needed calories. Obese subjects often report feeling "unsatisfied" and continue to eat after the first part of a meal has induced gastric distention. And those who have tried cutting down on food intake by ingestion of carboxymethylcellulose and water to distend the gastrointestinal tract before meals, report no long range influences on food intake.

Experimental animals will stop eating for many reasons. It has been suggested that "true" satiety can be recognized by its association with tranquility and grooming behavior.

When hungry animals are injected with sera from overfed ones, the former often refuse food. It has been proposed that they are responding to a humoral satiety factor released in response to eating.

When animals made obese through damage to the hypothalamus are joined parabiotically with normal animals (so that they share a common circulation), the obese partner often continues to gain weight, while the previously normal one becomes very lean. One interpretation is that the lean partner is responding to a satiety factor released by the obese one, while the latter has lost ability to respond to the factor. (In such studies it is difficult to rule out the greater ability of the heavier animal to gain access to the food cup or a greater response of the lean one to stress associated with the parabiotic condition; moreover, not all parabiosis studies have yielded the same findings.)

When force-fed animals are permitted to return to *ad libitum* feeding, they eat smaller meals until they re-establish normal body weight. If there is a humoral fac-

tor, it seems to affect the *quantity* of food ingested rather than meal initiation under these circumstances. It is also possible that separate factors affect short and long range aspects of eating behavior. Chemical influences on feeding behavior are considered later in the chapter.

DO CALORIES "COUNT"?

Among the thousands of suggestions offered to obese persons for loss of body weight, one that has received the most attention in recent years is that the deciding factor in weight control is not the caloric intake, but rather the carbohydrate content of the diet.

Influences of glucose on lipid metabolism were described in Section II. It was noted that glucose uptake by adipose tissue is a limiting factor for fat synthesis, and that when there is insufficient glucose, fat degradation predominates over fat synthesis. The liberated fatty acids are partly converted to ketone bodies and excreted in the urine.

It has therefore been proposed that obese individuals can eat all of the protein and fat-rich foods they desire with total disregard of the caloric intake, and that fat will be rapidly degraded and lost from the body in the form of the ketones. A variation on the theme recommends that very large quantities of fats should be ingested, and especially ones containing a high content of "polyunsaturated" fatty acids, since the latter "promote lipid mobilization."

The concept that it is "permissible" to eat all kinds of foods which persons on calorie-restricted diets formerly ate only with guilt, has obvious appeal. And limitation of carbohydrate intake has indeed proven effective for some persons. However, it has been pointed out that the carbohydrate restriction often leads secondarily to caloric reduction, since some highly concentrated foods (candy, cake, etc.) are eliminated from the diet, and one eats less butter without the bread and less salad dressing when the vegetables are limited.

Problems can arise from high-fat diets. Ketosis might approach dangerous levels if most persons did not, in fact, "cheat" from time to time. Fat does not provide substantial quantities of carbohydrate, but some of the latter is needed to maintain body

functions. If it is not contained in the diet, then body protein catabolism will be accelerated for gluconeogenesis. In some individuals, sufficient protein destruction will result to provide glucose for synthesis of substantial quantities of new body fat. Moreover, there are definite limitations to the calorie loss through ketonuria.

Carefully controlled studies support the concept that the major determinant of fat storage is the caloric intake, and that calorie-restricted diets rich in carbohydrate can be effectively used for treatment of some cases of obesity.

THE ROLE OF THE HYPOTHALAMUS^{6, 8, 12, 14, 18}

Patients with hypothalamic tumors have been known to exhibit unusual eating patterns associated with gross changes in body weight. In some cases the conditions have been corrected by removal of the tumor. It is widely stated that such observations bear no relationship to slowly developing disturbances of body weight regulation seen in persons with no known hypothalamic injury. However, the observations have influenced thinking about control mechanisms.

When large bilateral lesions are placed in the ventromedial region of the hypothalamus of experimental animals, but lateral areas are left intact, a characteristic syndrome follows. Even before effects of the surgical anesthetic wear off, the animals seek out the food cup and begin to eat voraciously. During the early or "dynamic" phase they become remarkably obese. After a time they enter the "static phase" in which body weight tends to stabilize at a level which may be fully twice that of unlesioned control animals.

Hypothalamic obesity has been induced in rats, mice, dogs, cats, pigs, and chickens, and studies involving electrical stimulation of the hypothalamus of ruminant animals suggest that this part of the brain similarly influences feeding behavior.

Relatively large bilateral lesions of the *lateral hypothalamus* lead to very different disturbances. Animals refuse to eat or drink, and will die of starvation and dehydration when both food and water are within easy access, although they retain the motor ability to approach the nutritive

ents, to lick, chew, and swallow food. They turn away from food directly offered, and eject liquids and solids placed directly in the mouth.

The concept was advanced that the lateral hypothalamus functions as a "feeding center." Electrical stimulation of this area in intact animals elicits licking, chewing, salivation, and swallowing, and well fed animals recommence eating. Chronic stimulation can lead to augmentation of daily food intake.

The ventromedial nuclei were said to contain a "satiety center" which receives information on nutritional status and exerts inhibitory control of the lateral hypothalamus. According to the concept, damage to the ventromedial nuclei removes the usual inhibitory control over the lateral hypothalamus; therefore animals become hyperphagic and obese. Stimulation of the ventromedial region of intact animals can lead to abrupt cessation of feeding in previously fasted animals just beginning to eat; the meal will be resumed as soon as stimulation is discontinued.

It has been proposed that the described animals have a disturbance in "set point"; this is supported by observations that the magnitude of hyperphagia is influenced by preoperative treatment. Animals made obese prior to surgery eat less afterward than those receiving ventromedial lesions when of normal weight. It has also been found that animals fasted before being subjected to lesioning of the lateral hypothalamus usually eat for a time before exhibiting food aversion.

Closer scrutiny of effects of hypothalamic injuries demonstrates that much more than a disturbance in set point is involved. Although those with ventromedial lesions readily take in enormous quantities of palatable food that is within easy reach, they are unwilling to work as hard as intact animals to acquire food. They will press bars a few times if this is required; but if the experimental setup demands that bars be pressed many times for delivery of each morsel, that heavy lids be lifted off food cups, or that other effort be expended, the lesioned animals cut their food consumption to the point where they lose weight. It has been stated that they do not suffer from motor impairment since if they are fasted long enough, they will work

much harder; but it is difficult to evaluate the effect of fasting on the motor ability.

Animals with ventromedial lesions have been described as "finicky"; they will not eat sufficient food to sustain weight if the flavor has been altered by addition of quinine or other unpleasant substances, although the food will be accepted by intact animals. Lesioned animals are also more sensitive to appetite-suppressing drugs such as amphetamine than are controls. In addition, they exhibit behavioral changes; they become relatively inactive when left alone, but are more irritable than normal animals and may become vicious.

The animal with ventromedial lesions is very different from an otherwise normal animal that cannot tell when it has had enough food. It takes only a little imagination to see it as an unhappy creature who attempts to utilize food to make up for something that is missing, and as one too depressed to use much energy to acquire food not readily available. The concept is strengthened by studies in which electrodes have been placed in "pleasure regions" of the lateral hypothalamus, and connected with bars that animals can press to activate a circuit. Animals will go to all kinds of trouble to press such bars. In one experimental setup, rats could obtain "self-stimulation" by pressing a bar at one end of a long cage and obtain the next stimulus only by pressing a second one at the extreme other end before returning to the original bar. The rats ran back and forth so often that it became necessary to remove them from the cage for periods of rest and feeding.

Presumably, animals with ventromedial lesions are unable to send the necessary signals for "pleasure" to the lateral hypothalamus. Bar-pressing in intact animals with electrodes in the lateral hypothalamus is transiently increased by damage to ventromedial regions, and can also be influenced by feeding.

Comparisons have been made between animals with ventromedial lesions and patients with resistant obesity, and it has been suggested that both eat for taste and pleasure rather than for calories.

In addition to "psychological problems," animals with ventromedial lesions suffer from metabolic disorders. When food is restricted, they lose more protein and rela-

tively less fat than do food-deprived intact animals. And endocrine changes include subnormal secretion of somatotrophin.

Similarly, animals with lateral hypothalamic lesions are very different from healthy animals that have "lost their appetite." If they are kept alive for several days by intragastric feeding, some will go through a series of recovery stages, starting with acceptance of moist food placed directly in the mouth and reaching the point where they will take water in combination with dry food. (It is claimed that only animals with limited injury can recover.) The animals eventually acquire ability to make some adjustments of food intake to periods of brief deprivation and to changes in environmental temperature. But weight is stabilized at a subnormal level, responses to stimuli such as insulin hypoglycemia are lost, and animals will not drink in response to a strong osmotic stimulus.

Attempts have been made to more precisely define loci within the hypothalamus that affect feeding behavior. While some separation of effects of large lesions has been achieved, this does not mean that feeding in intact animals is ever completely separated from other hypothalamic functions (which include body temperature regulation, responses to dehydration, control over certain autonomic functions, and control over the endocrine system).

Cuts made along the anterior lateral edge of the ventromedial nuclei with no injury to the nuclei themselves produced the obesity syndrome, but more posterior cuts were ineffective, as was isolation of ventromedial and lateral regions from the rest of the brain. Horizontal cuts across the ventromedial nuclei produced maximal overeating, cuts between the ventromedial nuclei and the arcuate nuclei yielded moderate symptoms, while ones still lower (through the median eminence) were ineffective.

If the most medial portions of the ventromedial nuclei are left intact, animals become obese but not "finicky"; the medial portions have been implicated in taste sensitivity.

If the ventromedial nuclei are left uninjured but isolated from the rest of the brain, while lateral nuclei are severely damaged, animals will passively swallow food placed in the mouth (without showing the aversion seen if ventromedial connections are left intact); however, they will never regain the ability to eat spontaneously, and will starve to death if not hand fed.

When a small rim of lateral hypothalamic tissue remains, animals recover ability to eat after several days.

MUSCULAR ACTIVITY, BODY WEIGHT, AND BODY COMPOSITION².

It has been claimed that obese persons (and especially obese children, adolescents, and young female adults) differ markedly from "normal" persons in their activity patterns, and exercise programs have been prescribed for induction of weight loss.

The effects of exercise are far reaching and often difficult to evaluate. Skeletal muscle comprises 40-50 per cent of the metabolic mass in most adults, and it has been shown that the oxygen consumption of muscle can be increased up to 20-fold during bouts of vigorous activity. Presumably therefore, excess fat can be "burned up" to supply the needed fuel, and weight will be lost if a diet which previously just maintained weight is continued.

The amounts of fuel required to sustain muscular work may have been previously underestimated. Charts in physiology texts relating so many minutes of a specified activity (walking briskly, swimming, etc.) to so much extra oxygen consumption have been compiled for most part from measurements taken just before and during the activity, in volunteer subjects who often are young medical or graduate students in good physical condition.

Relatively little information is available on *long-range effects* of the exercise on muscle tone, or on altered energy expenditure of otherwise sedentary and obese subjects. There seems to be little or no interest in determination of differences in energy needs of persons who are "invigorated" by exercise as compared with those who avoid all unnecessary movement and complain bitterly of fatigue when coerced into activity.

The concept that increasing energy expenditure should facilitate reduction of fat stores seems reasonable. Yet many obese individuals are quite consistently active while many lean persons who enjoy food are sedentary.

Exercise does much more than raise metabolic rate. It promotes secretion of catecholamines, glucagon, adrenocortical

hormones, renin, and vasopressin; and significant influences on secretion of STH and of prolactin are seen in some species, while insulin release is inhibited. More fat and less protein seem to be lost than when weight reduction is accomplished through food restriction.

Effects of exercise on hunger and appetite are difficult to assess, since these are affected by hormones, by metabolites arising from the activity, and by psychological reactions to exercise. Some kinds of data suggest that tennis on Sunday morning may have a far greater influence on the amount of dinner eaten on Tuesday than on Sunday. Observations of adolescent girls in summer camps point to short-range decreases in food intake associated with moderate increases in activity, but factors other than muscle movement must surely be involved.

In animal studies, there are data pointing to no significant increase in food intake (and associated small losses of body weight) if activity is slightly increased, and considerable augmentation of food intake with maintenance of body weight if the work load is greater. When the imposed exercise is severe, animals usually lose weight; but the importance of stress must be considered in this case.

Most obese patients subjected to severe caloric restriction reduce their voluntary activity. In some, the change has been related to psychological depression. But food restriction may also bring about metabolic changes that affect the effort required to perform work. Healthy laboratory animals also reduce voluntary activity when food is restricted, and tend to maintain body weight under moderate conditions.

GENETICS AND OBESITY^{2, 9, 11, 28}

The known tendency for obesity to "run in families" has often been attributed to the sharing of common undesirable patterns of food intake. The observation that fat parents tend to have fat children has similarly been related to the tendency for parents who eat too much to give too much food to their children, or to place undue emphasis on the importance of food. Similar interpretations have been attached to statistical evidence that fat infants and children are very likely to become fat adults.

Alternate interpretations are possible. An obvious one is that persons in families in which several members are obese share a common genetic defect. The concept finds support in observations that identical twins separated soon after birth and reared in different households tend to acquire similar body weights, and in the higher correlation between body weights of identical as compared with fraternal twins reared in the same household. Very early onset of obesity could reflect either a more severe form of the disorder or an interplay between heredity and environment.

A role of genetic factors in etiology of obesity has been clearly established in laboratory rodents. Several kinds of defects have been identified in rats and mice in which afflicted individuals not only accumulate more fat than their lean relatives when permitted to eat more, but also show a higher percentage of body fat (and correspondingly less protein) when pair-fed.

In one strain of mice which remains lean on the usual rations, obesity is induced by feeding a high-fat diet. But other strains of mice do not become obese on the same diet. Genetically transmitted obesity is associated with abnormalities of the endocrine system.

ADIPOSE TISSUE CELL NUMBERS VS ADIPOSE TISSUE CELL SIZE^{9, 15, 29, 30}

Adipose tissue cells survive for long periods of time, and can withstand large and repeated alterations of size and fat content. In adult mammals, body weight gains or losses have been attributed almost exclusively to changes in sizes of existing fat cells, with little influence on cell numbers.

Statistical studies on human subjects indicate that adults whose obesity originated during infancy or early childhood tend to have larger numbers of cells than those who are lean or whose obesity first developed during adulthood. Those with "life long" obesity generally experienced great difficulty losing weight, and positive correlations have been found between the severity of their condition and the numbers of fat cells. When such persons succeeded in attaining substantial weight reductions, the average sizes of their fat cells were lower than those of lean individuals.

An upper limit to the amount of fat that can be accumulated by each cell may be

imposed by the gradual decline in insulin sensitivity reported to be associated with increase in cell surface. The possibility has been considered that the number of insulin receptors per unit surface area is diminished.

When neonatal rats are reared in very small litters, they grow faster, deposit more fat, have increased numbers of fat cells, and develop into obese adults. By contrast, slower growing infants of large litters develop fewer fat cells and are more resistant to development or maintenance of obesity when overfed as adults.

On the basis of the described observations on humans and rats, plus statistical indications that "plump" children are more likely than lean ones to develop into obese adults, it has been recommended that parents impose restrictions on food intake of infants and young children to limit formation of adipose tissue cells and thereby protect them against development of resistant adult obesity.

Several things should be seriously considered before such advice is universally accepted.

Relationships between Neonatal Rats and Neonatal Humans

The rat is born, after a gestation period of about 3 weeks, in a highly immature condition. It weighs about 3.5-4 g (compared with 260-550 g for the adult; there are marked strain differences in length and other parameters). The newborn rat's eyes and ears are closed, it has little adipose tissue and no fur or temperature-regulating ability; and it requires much more than food during the early days if it is to survive. The human infant is by comparison far more mature. It has long since passed through the developmental stage of the very young rodent, and its adipose tissue is well developed. Larger human infants may temperature-regulate from the first neonatal day. Therefore, comparisons of influences of external changes on development must be made with the greatest of caution.

When infant rats are reared in small litters, they receive much more than extra food from the mother. They are stimulated in many ways and exhibit greater development of the nervous system, skeletal musculature, etc., and have very different re-

sponses to stress when they are older than do animals reared in very large litters.

Numbers of Adipocytes

The concept that fat cells increase in number only during early developmental stages has been questioned. It is known for certain that numbers can be increased in rodents after the weaning stage if they are exposed to cool environmental temperatures and other external factors. There are also serious questions about identification of fat cells. When depleted of reserve food materials, they may be difficult (or impossible) to recognize. Therefore, those of well fed animals are more easily counted. Recently depleted cells of previously obese subjects may be easier to recognize than undeveloped ones of lean individuals; and markedly enlarged cells of the previously lean persons may make recognition of neighboring undeveloped cells more difficult.

While clear evidence for hormonal stimulation of adipocyte proliferation in adults is not available, it has been shown that both insulin (which is present at high levels in almost all forms of obesity) and STH promote increases in DNA content of the stromal elements of adipose tissue. Moreover decreases in fat cell number have been described in animals placed for extended periods on calorie-restricted diets.

Causal Relationships between Numbers of Adipocytes and Obesity

The statistical data cited refer to average numbers of fat cells detected in obese vs lean or normal weight adults. Individual values vary widely over both ends of the scale. Some adults with lifelong obesity have counts well within the range for those who have always been lean, while some lean persons have values well within the range for the lifelong obese. This may or may not be related to commonplace observations that there are lean adults who were plump children and exceedingly obese adults who were described as scrawny during childhood. Moreover, a remarkable degree of sustained obesity can be achieved by a variety of procedures instituted after attainment of full maturity.

A serious problem with statistical correlations is that they can never provide

evidence for relationships between cause and effect. There is no assurance whatever that persons exhibiting lifelong obesity and high fat cell numbers would not have grown into obese adults if the diet had been restricted during childhood. One possibility is that they suffer from a genetic defect which leads to adipocyte proliferation whether excess food is available or not; another is that the same degree of obesity would have developed if fat cell proliferation could have been restricted.

Not all children are susceptible to overfeeding. It is not at all unusual to find siblings of the same sex and close in age fed together who differ markedly in body weight and fat content; the situation has been encountered with nonidentical twins. Moreover, it has not been established that fat infants and young children do, in fact, eat more than their lean siblings.

Possible long-range psychological and metabolic consequences of keeping infants and children hungry, of installation of a sense of guilt associated with eating, and of pointing up differences between siblings which have connotations of good and bad should also be considered. Obese children may suffer from metabolic defects which are not necessarily benefited by food deprivation.

GLUCOSTATIC REGULATION OF FOOD INTAKE

Hypothalamic Receptors^{8, 9, 10}

The most potent agent known for stimulation of the appetite is insulin. Actions of the hormone depend largely on ability to lower blood glucose concentrations; effects are very much attenuated if glucose is administered along with the insulin.

Receptors for the hypoglycemic stimulus are believed to be located within the *lateral hypothalamus*. Animals with lateral lesions lose ability to respond to both insulin and to hypoglycemia induced by other means. But additional receptors may be located in other parts of the brain; and also in the liver.

Administration of sufficient glucose to elevate plasma concentrations leads to inhibition of feeding behavior, but only under conditions in which the glucose can

be taken up by the receptors. Feeding is not inhibited in animals with large ventromedial hypothalamic lesions; and the preference of such animals for sweet food is probably related to destruction of specific glucose receptors. Glucose is also ineffective in animals that are severely insulin-deficient, and the response can be restored in such animals by administration of the hormone. It has not been determined whether simple glucose entry is all that is required, or whether some glucose metabolite must be formed within the receptors.

When *gold thioglucose* (GTG, aurothioglucose, ATG) is injected into susceptible strains of rats and mice, it is concentrated in the ventromedial hypothalamus. This is soon followed by destruction of cells in the vicinity, and precipitation of a syndrome similar to that described after electrolytic damage. Insulin is evidently needed to promote GTG uptake by cells of the ventromedial nuclei; insulin-deficient animals are not damaged by GTG, but susceptibility is restored by administration of insulin. The hormone is also required for subsequent development of the hyperphagia and obesity.

The uptake of 2-deoxyglucose is also enhanced by insulin; but unlike glucose itself, the deoxy hexose stimulates appetite. It may act by reducing glucose availability to the receptor cells. Since 2-deoxyglucose also competes with glucose for hexokinase, it may interfere with formation of a glucose metabolite that acts on the receptor cells.

It is worth noting that most studies on glucoreceptors have been performed on *mongastric* animals, in whom elevations of blood glucose characteristically follow ingestion of a meal. In ruminants, little glucose is obtained from a meal, and responses to both insulin and glucose administration are different from those observed in rats, cats, dogs, monkeys, humans, and others. Glucose administration may actually increase food intake in sheep and goats.⁷

While evidence for participation of hypothalamic glucoreceptors in acute responses to blood glucose changes in the laboratory is convincing, it is not widely believed that the mechanisms are important for day-to-day regulation of food intake in animals on normal feeding schedules. When animals eat a diet to which they are accustomed, rapid re-

sponses of pancreatic islet cells to changes in plasma glucose concentrations (Section II) keep the sugar concentrations within highly restricted limits. Animals maintained on high protein, low carbohydrate diets show relatively minor fluctuations in blood glucose concentrations, and the cycles that have been found seem to bear no obvious relationship to feeding rhythms or to evidence of hunger. If animals are fasted for a time (so that insulin secretion is low), then sudden ingestion of sugar can raise the plasma glucose concentrations. Under these conditions there is a time lag between presentation of the sugar and activation of the receptors; this could provide for intake of a moderate quantity of sugar before feeding behavior is inhibited. It is difficult to fit the receptors into a picture of long-range adjustments, e.g., reduction of meal size when animals previously force-fed are permitted to return to voluntary eating patterns.

Hepatic Receptors^{2, 23}

There are reasons to believe that very different kinds of sugar receptors are located in the *liver*. It was observed that when carbohydrate reserves accumulate, there is an outward movement of glucose and potassium from hepatic parenchymal cells, leading to hyperpolarization of the cells and signs of "satiety" in animals. Similar electrical changes can be obtained by feeding the animals, or by administration of drugs (e.g., amphetamine) which depress the appetite. Hyperpolarization seems to be related to activation of α -adrenergic receptors (Chapter 9).

When the liver is carbohydrate depleted, the hepatic cells take up glucose and are depolarized, while animals at the same time show signs of hunger. The depolarization is associated with β -adrenergic receptors and generation of cAMP. (It will be recalled that glycogenolytic actions of glucagon at times when blood glucose concentrations fall are also associated with cAMP generation.)

Lean animals were found to have much more responsive liver cells than fattened ones. Perhaps there is some connection between this and observations that lean persons are more highly motivated to seek

out food during very brief periods of deprivation.

LIPOSTATIC REGULATION OF FOOD INTAKE^{16, 18}

Since the quantity of lipids stored changes much more slowly than the blood glucose concentrations, it seems reasonable to implicate lipids in mechanisms for long-range regulation of food intake.

Free Fatty Acids

During limited periods of food deprivation, fat degradation is accelerated and the free fatty acid (FFA) concentration of the blood rises. According to one proposal, hunger is correlated with elevation of FFA, and satiety with lowering. When subjects are deprived of food for long periods of time, the FFA concentration may be lower than in well fed subjects just before a meal, and many food-deprived persons lose their appetite.

One problem with the concept is that FFA concentrations tend to go higher in individuals with larger fat reserves. This could lead to the not readily acceptable conclusions that fatter animals are hungrier after short periods of food deprivation than are their lean counterparts, and that previous eating provides the long range stimulus for more eating.

Ketones and Steroids

A variation on the hypothesis states that hunger is signaled by something linked to lipolysis, while satiety appears when the balance shifts from lipolysis to lipogenesis. Another suggestion is that some product of lipid metabolism signals hunger. Ketone concentrations rise in the blood during fasting, and there is a tendency for relatively greater elevation in lean animals. Ketones are also very high in patients with untreated diabetes mellitus; and such patients often experience compelling hunger. Glucocorticoid hormones and STH promote both ketosis and increased appetite.

A different idea is that long-range control of food intake depends upon some as yet unidentified lipid molecule, perhaps a steroid, which is produced and degraded at

a fixed rate and is partitioned between the aqueous blood plasma phase and the adipose tissue lipid. Elevation of the fat content of adipose tissue would be expected to reduce the concentration of the factor in blood plasma. A hypothalamic site has been proposed for the receptor, but neither the receptor nor the regulatory molecule has been demonstrated to actually exist. If a hypothalamic receptor is present, its function must be supplemented by others, since animals with severe hypothalamic injury retain some capacity to stabilize body weight.

Prostaglandins^{4, 14}

Injection of PGE₁ into some parts of the hypothalamus can lead to inhibition of feeding behavior. Prostaglandins of the E type are synthesized in and released by adipose tissue under conditions in which lipolysis dominates over lipogenesis. Moreover, very small doses of prostaglandins promote lipolysis and elevation of plasma FFA concentrations.

The problem with trying to fit the preceding into a concept for long-range control of feeding behavior is that eating would be inhibited at the time when fat stores are being mobilized. It seems more likely that if the observations have physiological meaning, they could indicate that prostaglandins participate in feeding inhibition in response to either diets excessively rich in fats or during exercise. The last also fits with the finding that prostaglandin-stimulation of fat hydrolysis seems to be indirect and mediated by sympathetic nerves and catecholamine release (which also occur during exercise) and depressed by insulin (whose secretion is inhibited by epinephrine). It has been suggested that small amounts of prostaglandins might find use in treatment of obesity in humans.

By contrast with small doses, large doses of prostaglandins act directly on adipose tissue to oppose lipolysis. Large doses are most effective during insulin deficiency. An unidentified lipid molecule (which could be a prostaglandin) has been detected in the blood of animals with hypothalamic obesity; it inhibits lipolysis, and it has been proposed that this agent is responsible for defective lipid mobilization seen in such animals.

Triglycerides

When animals are fed high-fat diets, they usually decrease their total food intake. In some cases this is because the flavor and texture are unappealing; it is often difficult to avoid traces of rancid odors since deterioration of the diet occurs during the time it is left at room temperature in food cups and more slowly when it is stored in a refrigerator. When the food is sweetened to increase palatability, animals may take more, but there is no way to separate effects of the sweetener from effects of the higher fat intake.

High concentrations of fat can irritate the gastrointestinal tract, especially if even limited oxidation of fatty acids occurs. They can also reduce appetite because they promote release of inhibitory gastrointestinal hormones (see below).

Animals often gain weight on high-fat diets because so many calories are ingested with a small bulk, and because less energy is required to convert dietary fat to depot fat than to form the latter from carbohydrate or protein foods. The tendency toward development of obesity on high-fat rations is largely under genetic control. Some strains of rats and mice consistently become obese while others eating the same food do not. Some individuals within a cage become obese while their siblings do not.

AMINO ACIDS AND FOOD INTAKE²²

Diets excessively high in protein tend to depress the appetite; food containing unbalanced mixtures of amino acids is even more effective. These diets also tend to elevate body temperature because of the high specific dynamic action (SDA) of proteins.

SDA is the increment in metabolic rate attributable to metabolism of foods. It can be demonstrated when influences related to ingestion, mastication, digestion, etc. are eliminated, e.g., by fitting animals with indwelling venous catheters and comparing oxygen consumption before and during the period immediately following administration of amino acids, sugars, etc. Maximum SDA for carbohydrates and fats is equivalent to about 4-6% of the basal metabolic rate; but it can go as high as 30%

for amino acids. The high SDA for proteins is attributed to the "inefficiency" of deamination and associated reactions, in which considerable quantities of energy are lost in the form of heat rather than conserved in the coupling of oxidation to ATP phosphorylation.

Use of proteins as an energy source has been described as "metabolically wasteful" since relatively more of the potential food energy is lost in the form of heat than when isocaloric quantities of glucose or fatty acids are utilized. (Such "waste" may be considered advantageous by those on "reducing" regimens. It must also be planned for by nutritionists designing menus that will meet caloric needs.)

When the amino acid makeup of the diet is highly unbalanced, the food is not readily utilized for protein synthesis. (An extreme case is one in which certain amino acids are given with an early feeding and different ones at feedings many hours later; if the spacing is wide enough, animals may not be able to incorporate any of the food into new protein.) Under these circumstances, deamination reactions are accelerated, and most of the food is used as a direct energy source or for synthesis of reserve carbohydrate and lipids. The resulting elevations of body temperature can, of themselves, contribute to appetite depression. Deamination reactions also increase ammonia production in the liver, and it has been proposed that liver receptors thereby activated exert inhibitory influences on feeding behavior. (Appetite is notoriously poor in patients and experimental animals with liver disorders and uremia.)

Glycine and some other amino acids are avidly taken up by certain neurons, and this leads to depolarization. It is possible that the cells function as receptors involved in regulation of food intake. Animals with ventromedial lesions of the hypothalamus exhibit appetite depression when fed the imbalanced diets. This could mean that receptors are not located in the ventromedial nuclei; but it could also mean that animals are exhibiting one more aspect of their "finicky" behavior.

Destruction of the olfactory bulbs does not affect the responses to amino acid imbalances. But animals with lesions of the prepyriform cortex and medial amygdala

(both of which project to the lateral hypothalamus) may actually show a preference for the imbalanced diets. (They do, however, show appetite depression in response to high concentrations of balanced proteins.)

When food intake is impaired in response to amino acid imbalances, it can be corrected by administration of missing essential amino acids.

INTERNAL AND ENVIRONMENTAL TEMPERATURES AND FOOD INTAKE^{16, 22}

Homeothermic animals increase food intake and metabolic rate in response to a fall in environmental temperature. When external conditions are mild, body weight is maintained or may be even slightly increased.

Animals that have "recovered" from lesions of the lateral hypothalamus exhibit regulatory responses to temperature changes. But those with ventromedial damage do not cope well with the cold, and also show heat aversion. Lesions of the anteromedial hypothalamus abolish temperature responses but do not necessarily affect food intake in usual environments.

In healthy animals (uninjured except for implantation of measuring devices), localized temperature elevations in the preoptic hypothalamic region have been recorded during the first 10–15 min of feeding. With small meals, the rise continues after food intake ceases; but with large meals, the temperature begins to fall while the animals are still feeding.

Temperature elevations associated with feeding are more reproducible and of greater magnitude when the food requires chewing than when it is presented in liquid form; and warm food is more effective than cold food.

It has been proposed that localized elevation of temperature in the preoptic part of the hypothalamus (an area involved in body temperature regulatory mechanisms not directly related to feeding) provides a satiety signal for meal termination. One is tempted to speculate in this connection, on relationships between the observations and the following reactions of some humans: (1) temporary alleviation of feelings of hunger between meals afforded by chewing gum,

(2) greater satiety after hot than after cold meals, (3) cravings for very crunchy snacks, (4) tendencies to take less total food when the meal is eaten slowly, and reduced appetite if the meal is interrupted in the early stages and then resumed, (5) reduced interest in food for an extended period of time after vigorous exercise has elevated the body temperature, and (6) preferences for cold desserts.

The signal may be a *rise* in temperature rather than the absolute level. If food is taken at the low point of the diurnal cycle, eating is inhibited when preoptic temperatures have risen but not to the point they would otherwise reach at another time of the day. Moreover, animals deprived of food for several hours will eat at the height of the diurnal temperature cycle.

That elevation of preoptic temperature is neither an essential nor a sufficient condition for satiety is suggested by ability of animals to adjust feeding to caloric needs when offered diets warm or cold enough to significantly affect preoptic neurons.

In most homeotherms, reactions to very warm environments do not elicit obvious responses (vasodilation, sweating, etc.) until a small rise has occurred in the temperature of the blood within the anterior hypothalamus. It is likely that feeding responses also await the appearance of the elevation. On the other hand, cold stimuli peripherally perceived elicit regulatory responses when no reduction of hypothalamic temperature has occurred. Hormonal responses to cold stimuli are relatively prompt, and these probably play a role in feeding reactions to the cold temperatures.

GASTROINTESTINAL HORMONES AND FOOD INTAKE^{6, 27}

It has been known for half a century that digestion of fatty foods leads to release of some humoral factor given the name *enterogastrone*. The structure has not been determined.

More recently it has been shown that entry of fats or fatty acids in absorbable form into the small intestine leads to release of a peptide which inhibits gastric motility and induces behavioral changes associated with "satiety." Animals with

gastric fistulas exhibit similar reactions if small quantities of liquid food are placed directly into the intestine, even if they are unable to taste or swallow the food.

The peptide is evidently unrelated to enterogastrone but may be identical with *cholecystokinin* (named for its ability to promote contraction of gall bladder muscle). Similar effects have been elicited with a synthetic peptide which is the same as the COOH-terminal octapeptide of cholecystokinin. (Some authors have questioned the description of effects of administration of the peptide and have suggested that the animals stop eating because they are nauseated.)

Gastrointestinal hormones probably play some kind of role in decisions about termination of a meal in progress. But it seems unlikely that they function in long-term adjustment of food intake to metabolic needs.

ADRENERGIC RECEPTORS AND FOOD INTAKE^{6, 8, 14}

α - and β -adrenergic receptors, agonists, and blocking agents were described in Chapter 9.

Intrahypothalamic administration of norepinephrine and related α -agonists can promote resumption of eating in "satiated" animals; and the effects can be antagonized with α -blocking agents. Isoproterenol (a β -stimulator) can inhibit feeding in fasted rats, and this action is antagonized by β -blockers.

The simplest interpretation of such findings is that there are α "hunger cells" promoting eating behavior which can be stimulated by norepinephrine, and β "satiety cells" which can be stimulated by isoproterenol. Presumably, endogenous epinephrine, which exhibits both α - and β -stimulating properties, can act on both receptors; the end result depends on prevailing conditions, the dosage, and possibly the associated presence of norepinephrine which exhibits primarily α -stimulating actions. The described conclusions are shown in Table 18-1 under the heading "Interpretation I."

Since the ventromedial nuclei have been associated with "satiety" (and their destruction leads to hyperphagia and obesity), the simplest

TABLE 18-1

Observed Effects of Intracerebral Administration of Agents Affecting Adrenergic Receptors in Rats Offered Food Pellets, and Two Possible Interpretations

Agent Administered	Observed Effect	Interpretation I	Interpretation II
α -adrenergic agonist, e.g., norepinephrine	Satiated animals eat	Stimulation of α -“hunger receptors” of lateral hypothalamus	Inhibition of α -satiety receptors” of ventromedial hypothalamus which would otherwise inhibit lateral “hunger receptors”
α -antagonist, e.g., phentolamine	Blocks effects of α -agonist	α -“hunger receptors” blocked	α -“satiety receptors” actively inhibit lateral “hunger receptors”
β -agonist, e.g., isoproterenol	Fasted animals refuse food	Stimulation of “satiety receptors” of ventromedial hypothalamus	Inhibition of β -“hunger receptors” directly
β -antagonist, e.g., propanolol	Blocks effects of β -agonist	β -“satiety receptors” blocked	β -“hunger receptors” active
α -antagonist + β -agonist	“Supersatiety”	Hunger receptors blocked, satiety receptors stimulated	β -hunger receptors inhibited directly and indirectly

concept visualizes the existence of the β satiety cells in the ventromedial nucleus, with inhibitory pathways to the “eating center” in the lateral hypothalamus. The same scheme visualizes the presence of α “hunger cells” in the lateral hypothalamus; activation of the α -receptors should therefore directly stimulate eating behavior.

One problem with this relatively simple interpretation is that the *medial* hypothalamus is most sensitive to α -stimulation. If α -stimulation in the medial hypothalamus leads to feeding behavior, this could be explained by the concept that the medial “satiety receptors” usually inhibit the lateral “feeding cells” and that α -stimulants act on *inhibitory* receptors; therefore, α -agonists act to inhibit satiety cells, leaving the activity of hunger cells unopposed. This alternate explanation is shown in Table 18-1 in the column marked “Interpretation II.” Similarly, the lateral hypothalamus which has been designated a “feeding center” is sensitive to β -stimulators, and such stimulation inhibits eating. Therefore, the finding can be explained if the lateral hypothalamic “hunger cells” received β inhibition. This, too is shown in the last column of Table 18-1.

Probably both interpretations represent

oversimplifications, since other kinds of studies (measurements of localized electrical activity at various sites within the hypothalamus after application of norepinephrine, with and without atropine) indicate the presence of at least two different kinds of cells responsive to norepinephrine. The lateral hypothalamus may contain some cells which exert inhibitory control over other cells; and there is good reason to believe that the ventromedial and lateral hypothalamic areas are reciprocally innervated.

Some of the studies described above were performed on rats offered standard laboratory food pellets and water. The whole concept of hypothalamic control of feeding behavior was made more fascinating when the same kinds of agents were administered to fasted and fed animals offered a choice of sweetened milk or milk made bitter by addition of quinine.

Administration of isoproterenol (which inhibited pellet-eating in fasted rats) caused animals to refuse the bitter milk, but the animals overate when sweet milk was offered. The rats might be said to have eaten for taste rather than for calories and to have become “finicky.” In this sense, isoproterenol-treated or β -stimulated rats resembled animals with ventromedial lesions. The results are shown in Table 18-2.

TABLE 18-2

Effects of Administration of Agents Affecting Adrenergic Receptors to Rats Offered Sweetened and Bitter Milk

Agent Administered	Observed Effect	Possible Interpretations
β -stimulator or α -blocker	Refusal of bitter milk; overconsumption of sweet milk	Inhibition of ventromedial hypothalamus leads to hyperphagia and finickiness; decreased satiety; heightened taste sensitivity; eating for flavor but not calories
α -stimulator or β -blocker	Limited consumption; ready acceptance of bitter milk	Stimulation of ventromedial hypothalamus; increased satiety; decreased taste sensitivity; eating for calories but not flavor

Predictably, norepinephrine or α -stimulated animals showed the opposite reactions. They reduced total consumption, but were willing to readily accept bitter milk. They seemed to be eating for calories rather than for flavor without "finickiness," and to be responsive to satiety signals. In this sense, they acted like hungry rats with intact hypothalami. One could of course attach different interpretations, including heightened taste sensitivity after β -stimulation and reduced taste sensitivity after α -stimulation.

Amphetamine and related agents effectively suppress appetite for limited periods of time in humans and other mammals, and are believed to function as β -agonists. If interpretations of Table 18-1 are accepted, the action is explained by stimulation of satiety receptors, inhibition of hunger receptors, or both. However, another hypothesis states that these compounds are α -agonists; this would fit with Table 18-2, but no explanation is provided for the sensitivity of animals with ventromedial lesions to these agents. A third hypothesis states that amphetamine-like drugs act via elevations of hypothalamic temperature.

Another aspect of regulation was indicated by studies demonstrating that hypothalamic administration of norepinephrine suppressed feeding behavior if given to rats during darkness (when rats are active and do most of their eating), but that similar treatment during daylight hours facilitated feeding behavior.

Prostaglandin inhibition of eating behavior was mentioned above; the effects seem to be

specific, and can be inhibited by administration of an antagonist, polyphoretic phosphate (PPP).

PGE₁ inhibition is most effective when administered at hypothalamic sites where norepinephrine elicits feeding, and PGE₁ blocks effects of subsequent administration of the catecholamine. PGE₁ seems to function as an α -receptor blocker and PPP as an α -stimulator. (And effects of PPP are antagonized by α -blockers.)

PGE₁ may also act elsewhere as a β -agonist, since injection into hypothalamic sites which stimulate feeding are blocked by β (but not α) blocking agents.

The literature contains numerous descriptions of participation of other parts of the nervous system in regulation of food intake. Influences of acetylcholine (which can be blocked with atropine) and dopaminergic pathways have been demonstrated.

CATECHOLAMINES AND BODY COMPOSITION

Catecholamine participation in carbohydrate metabolism was described in Chapter 9. Adipose tissue is sympathetically innervated, and sympathetic nerves function in mobilization of depot fat. When fat molecules are hydrolyzed, the fatty acids and glycerol liberated can enter the plasma and be utilized elsewhere.

This action of catecholamines can lead to net reduction of body fat. It has been suggested that some obesity problems, and especially the accumulation of fats at localized sites, can be attributed to inadequate function of the sympathetic nerves. Localized defects can be experimentally demonstrated by cutting the nerves.

Fatty acids can also be reutilized for synthesis of new fat molecules. But it has been shown that this can be accomplished only after ATP is utilized to activate the fatty acids (to fatty-acyl coenzyme A) and for formation of the glycerophosphate which combines with the activated fatty acids.

Catecholamine stimulation of the "fatty acid cycle" constitutes a major mechanism for elevation and maintenance of body temperature when the environmental temperature falls. During "nonshivering thermogenesis," the same fatty acids are used again and again, so that the total fat content of adipose tissue may not change. But each time a molecule of fat is broken down and resynthesized, large quantities of ATP are broken down, and this is associated with liberation of substantial amounts of heat. Adrenal demedullated animals do not effectively elevate metabolic rate in response to the cold and may succumb to temperatures well tolerated by intact animals. The catecholamine-stimulated functions are most important during early phases of cold exposure.

When animals are well fed they more effectively engage in nonshivering thermogenesis because of the larger stores of depot fat, and because insulin promotes glucose uptake by adipose tissue and thereby provides the sources of both glycerophosphate and ATP energy.

TSH AND THYROID HORMONES*

High concentrations of TSH activate adipose tissue lipases, at least in certain species including rats, mice, guinea pigs, and dogs. It has not been demonstrated that sufficient TSH is liberated during cold-exposure to exert this action in healthy animals.

More importantly, TSH promotes secretion of T₃ and T₄ which exert "permissive" actions on lipolysis, and build up the mitochondrial machinery for oxidative phosphorylation.

In hyperthyroidism, the rate of lipolysis is greatly enhanced (along with many other catabolic activities); fat stores are usually depleted because the high food intake often does not keep pace with the even higher metabolic rate. Protein and glycogen destruction are also accelerated, but blood glucose levels may be high.

Much has been written and said about use of thyroid hormones in the treatment of obesity. When they are administered to myxedematous patients (Chapter 17), body weight falls rapidly, but this is usually accounted for largely by correction of defects in water, electrolyte, and mucopolysaccharide metabolism, and fluid loss results in part from direct hormonal influences on the circulatory system and kidney.

When small doses of thyroid hormones are given to obese but euthyroid patients, little long-range influence on body weight and composition is effected. Hormonal feedback mechanisms usually lead to compensatory reduction of endogenous secretion, so that plasma levels of thyroid hormones may not change. When somewhat larger doses are given, small effects on metabolic rate are often counter-balanced by stimulation of the appetite, although a few individuals increase their activity to the point where a small negative caloric balance is achieved. (Usually this effect is of short duration.)

The physician understandably hesitates to give sufficient T₃ or T₄ to induce a thyrotoxicosis, since in addition to elevation of metabolic rate he may precipitate onset of tachycardia, diarrhea, restlessness and insomnia, skeletal muscle weakness, and other undesirable sequelae. Moreover, it has been claimed by some that any body weight lost over and above that which can be accomplished by caloric restriction alone, is largely attributable to greater loss of tissue protein than of accumulated fat stores. (But other clinicians claim that the hormones are useful for maintaining body composition in patients that have already achieved weight loss through diet.)

Thyroid hormones usually promote reduction of plasma cholesterol levels, apparently because stimulation of steroid catabolism is greater than the known stimulation of steroid synthesis. There are claims that synthetic analogs of thyroid hormones exert relatively greater influences on cholesterol metabolism and smaller ones on the cardiovascular system than do T₃ and T₄; and separation of other hormone effects seems possible, since actions of the hormones on mitochondria of heart muscle are known to be different from actions on mitochondria elsewhere. This raises the hope that an analog may one day be syn-

thesized which preferentially affects adipose tissue.

ACTH AND GLUCOCORTICOIDS¹⁰

In common with TSH and other peptide hormones, high concentrations of ACTH activate adipose tissue lipases. This could be of some importance during times of stress, but amounts needed for such action are probably not released at other times. ACTH promotes secretion of glucocorticoids which are absolutely essential for realization of lipolytic actions of catecholamines.

Influences of glucocorticoids on mobilization and redistribution of body fat have been described (Chapter 7). The fat content of the liver is usually increased, and there is a mild ketosis. Appetite is also usually increased.

In some forms of genetically transmitted obesity, the obese condition can only develop when the secretion rate of glucocorticoids is high. Glucocorticoid function is often elevated in other forms of obesity, but this seems to be a secondary consequence of insulin hypersecretion.

Patients with Cushing's disease (hyperadrenocorticalism) often give the superficial appearance of obesity because of water retention and preferential accumulation of fat on the cheeks and abdomen; but not all such patients have excess total stores of body fat. Adrenalectomy does not prevent development of hypothalamic obesity if the animals are kept in reasonably good health.

SOMATOTROPHIN¹⁰

Influences of STH on fat mobilization and elevation of FFA concentrations of the plasma were described (Chapter 10), and it was noted that the hormone tends to increase total body protein while decreasing relative fat content.

There are some interesting aspects of STH influences on appetite. STH seems to be more potent in males than in females, whereas prolactin (which has some overlapping metabolic actions) is more potent in females. Perhaps there are connections between such findings and the remarkable capacity of adolescent boys for ingestion of food, and the hunger sometimes experienced by women during lactation.

Effects of STH on food intake may be very different in young, rapidly growing animals than in adults. According to one hypothesis, STH counteracts inhibitory influences of the ventromedial hypothalamus and thereby promotes maximal feeding during the growing period. In weanling rats (in which STH inhibition may be maximal), lesioning of the ventromedial hypothalamus has no discernible influence on food intake or on responses to STH stimulation of the appetite. In older animals, STH may act quite differently; hypothalamic lesions lead to hyperphagia which can be lessened by administration of STH.

ADIPOKINETIC HORMONES^{21, 25}

Peptides present in extracts of pituitary and hypothalamic tissue have been partially purified and separated from known hormones. They are potent stimulators of lipid mobilization. It has not been established that they are of physiological importance, but there is interest in use of such peptides in treatment of resistant obesity.

In some forms of obesity, fats seem to be poorly mobilized during periods of caloric restriction. But studies on isolated adipose tissue have not consistently revealed defective lipolysis in obese subjects. (In fact, some data indicate more rapid lipolysis in the larger, insulin resistant fat cells.) (See also Chapter 24.)

OTHER HORMONES PROMOTING LIPOLYSIS

High concentrations of glucagon can activate adipose tissue lipases (Chapter 6); physiological functions in mammals are doubted, but glucagon may play a role in regulation of lipid metabolism in the liver. Glucagon is also an appetite depressant.

Other hormones shown to have lipolytic action, but for which no physiological role has been established, include melanocyte-stimulating hormones and vasopressin.

INSULIN¹⁰

Insulin secretion is increased in all known forms of obesity. Secretion is stimulated by the increased food ingestion, but insulin also promotes appetite, and is absolutely essential for the development of the obese condition.

In addition to promoting glucose uptake by adipose tissue, insulin activates plasma lipoprotein lipases, and thereby frees fatty acids for uptake by the tissues. It also induces synthesis of enzymes needed for formation of fatty acids. (In some species, e.g., rats, much of the synthesis takes place in adipose tissue; in others, including man most fatty acids are synthesized in the liver and then transported in combination with proteins to sites of triglyceride formation and storage.)

Animals can be made obese if they are repeatedly injected with insulin and given free access to food. The hormone acts both through stimulation of appetite and facilitation of fat synthesis. (Appetite stimulation can be attenuated in monogastric animals by administration of glucose; in ruminants, glucose does not depress the appetite but the animals show a similar increase in food intake and body weight gain.) It is suspected that at least part of the appetite stimulation results from direct hormonal influences on hypothalamic neurons.

ESTROGENS^{10, 18}

Female animals exhibit cyclic variations in food intake which can be directly related to estrous cycles. In mature animals, estrogens depress the appetite, and they also exert actions leading to increased motor activity; therefore body weight tends to diminish. (And ovariectomized animals of some species become obese.)

There is evidence that the ventromedial nuclei of mature animals take up estrogens, and some appetite depression may be related to this. But the hormones are also effective in animals with ventromedial lesions and may therefore act at multiple sites.

Estrogens are not taken up by the ventromedial nuclei of sexually immature animals, and in these there is no influence of moderate doses on food intake.

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VI

HORMONES AND REPRODUCTION

19. Sex Determination and Development of the Reproductive System

WHAT IS SEX?

The term "sex" can have very different meanings when used by geneticists, cytologists, embryologists, endocrinologists, behavioral psychologists, psychiatrists, sociologists, lawyers, clothing designers, and writers of modern fiction. The one aspect which all usages have in common is some kind of association with a type of reproduction. But language patterns have made the association vague; thus, one hears the term "sexy" applied to inanimate objects while high school biology students describe reproductive processes in plants and clams as "not very sexy."

In this chapter distinctions will be made between *genetic* (or *chromosomal*), *phenotypic*, *hormonal*, *behavioral*, and even *legal sex*.

ASEXUAL REPRODUCTION

The term "reproduction" usually means formation of new living entities (cells, organisms) which closely resemble the parent species. Sexual and asexual reproduction differ in the *number of parents* involved in the process.

Many microorganisms, plants, invertebrate animals, and individual cells of higher organisms reproduce asexually. Genetic material contained within a single entity is replicated, and copies are passed on to the progeny. Except when mutations occur, daughter forms contain genetic material identical with that of the parent. Binary fission, budding, fragmentation, and spore formation may be totally asexual, as is mitotic division of cells within the tissues of higher animals and plants.

SEXUAL REPRODUCTION

The concept of *sexual reproduction* implies nothing more (or less) than the union of genetic material from two different sources, followed by formation of offspring which contain a mixture and therefore differ from both parents.

Sexual reproduction takes many forms and may occur in organisms which reproduce asexually at other times. In some unicellular groups (e.g., flagellates), two entire individuals fuse prior to division. In others (e.g., paramecium and plants such as *Spirogyra*), genetic material is passed through a bridge or conjugation tube formed shortly before the reproductive process commences. The two parents are morphologically similar, and therefore there is no basis upon which to apply terms such as male and female. But different "mating types" have been recognized and designated as (+) and (-).

In more complex multicellular organisms, reproductive cells are formed in specialized regions, usually designated as *gonads* in animals (or *gametangia* in plants). Precursors of the sex cells, the *gonocytes*, divide by meiosis to yield haploid *gametes* which later fuse with other gametes by the process of *fertilization* to form diploid *zygotes*. When the gametes appear morphologically identical they are known as *isogametes*.

In higher animals (and also in plants, which will not be further considered), *heterogametes* are formed. The type which is smaller and more motile is usually regarded as the *male type* or *sperm*; the term *male* is also applied to the animal which produces them, and the *gonad* is a *testis*. The larger, usually nonmotile gamete is the

egg cell or ovum produced within the ovary of the female.

HERMAPHRODITISM AND SEX REVERSAL

Earthworms have both ovaries and testes within the same individual, but two animals must engage in the process of *cross-fertilization*. Since earthworms are solitary creatures, perpetuation of the species is favored by the ability of any two that happen to meet to participate in formation of zygotes. It is not necessary for a boy worm to seek out a girl worm since clearly there are no boys or girls in this setup.

Tapeworms also have ovaries and testes within the same individual. Fortunately for the tapeworm, since it is isolated from other members of the species (but unfortunately for future hosts), tapeworms reproduce by *self-fertilization*. Some of the advantages of genetic mixing, and the potential for loss of unfavorable mutations, are sacrificed to provide the only means of perpetuation by sexual processes. In most hermaphroditic species, self-fertilization cannot occur because of anatomic locations of the gonads or because production of mature sperm and egg cells is not synchronized.

Hermaphroditism (possession of male and female reproductive structures within the same individual) is "normal" for some animal types. Disturbances of sex differentiation (considered below) can lead to the condition in species (including humans) in which it does not ordinarily occur. Other disturbances result in *pseudohermaphroditism* in which individuals possess some characteristics of one sex and some of the other.

There are many interesting variations on the basic theme. Oysters, slugs, and some of the fishes and frogs function as males at one stage of the life cycle (producing sperm, engaging in male type behavior, and having the appearance associated with males) and function at different times as females (producing ova and mating with males.) A transitional, intermediate hermaphroditic stage is recognized in some. There are varieties of fishes which can be "true" males or females for indefinite periods, but special circumstances (e.g., segregation of the sexes) can lead to "sex reversal" of one or more individuals. Sex

reversal can also be accomplished experimentally by treatment of individuals at critical stages of development with sex hormones. Thus, relationships between reproductive processes and the concepts of male and female are not constant throughout biological species.

CHROMOSOMAL SEX¹

Although *autosomes* can affect reproductive processes, *sex chromosomes* are directly involved in sex determination. In most of the mammals the female has two similar *X* chromosomes, while the male has one *X* and one smaller *Y* sex chromosome. A similar situation is found in some of the amphibians and fishes.

A different arrangement is common among birds and reptiles. The female has one *W* and one *Z* chromosome, while the male has two of the *Z* type. Yet other arrangements have been described in certain invertebrates; e.g., sex can be determined in some by the relative number of sex as compared with autosomal chromosomes.

MITOSIS AND MEIOSIS

Proliferation of somatic cells (e.g., those of skin and kidney) takes place by the much studied process of *mitosis*, in which genetic material is first duplicated to form "pseudotetraploid" cells. When the division is completed, each daughter cell contains a complete copy of the genetic material originally present in the parent cell, and it therefore resembles both the parent and other daughter cells.

Gametes are produced by *reduction division* or *meiosis*. This too starts with duplication of the genetic material, but duplicated pairs of homologous chromosomes combine to form *tetrads* or *bivalents*. (The process is sometimes called *synapsis*.)

At this stage, "crossing over" may take place; some maternally derived genetic material from one member of a homologous pair exchanges with paternally derived material of the closely apposed member of the pair.

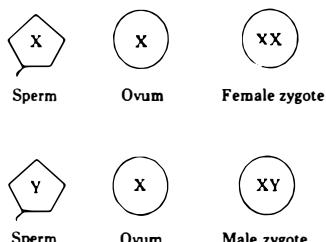
Then, the homologous pairs separate, and one set goes to each daughter cell. The products of the first division therefore contain duplicated single pairs and are said to be "pseudodiploid"; they differ substantially from each other in genetic makeup.

Each daughter cell divides once more to form haploid gametes. The products of this division differ slightly from each other because of changes occurring during the crossing-over process. If all survive, a total of four gametes is derived from a single gonocyte. When egg cells or ova are produced, each contains one X chromosome. Sperm cells may contain either an X or a Y.

Human cells contain 22 pairs of autosomes. (The characteristic number for other vertebrates varies widely.) Maternally and paternally derived genetic material can be passed on to gametes in any combination. Theoretically therefore, 2^{23} different kinds of gametes could be produced by one individual if no crossing-over occurred, and the gametes combine with others of equally variable composition. Since crossing-over further adds to the dissimilarity, the number of different kinds of zygotes that can be produced far exceeds $2^{23} \times 2^{23}$.

FERTILIZATION (SYNGAMY) AND SEX DETERMINATION

Union of two haploid gametes restores the diploid number of chromosomes. *Genetic (chromosomal) sex* is determined at this time by the sperm.



RELATIONSHIPS BETWEEN GENETIC OR CHROMOSOMAL AND PHENOTYPIC SEX

Under most circumstances the fertilized egg or zygote undergoes an orderly sequence of developmental changes. When a Y chromosome is present, a testis and male-type internal and external reproductive structures form, and postpubertal testicular function promotes emergence of male secondary sex characteristic. In zygotes with two X chromosomes, a normal female phenotype develops.

But several kinds of disorders (or experi-

mentally induced conditions) can disrupt the processes, and it is possible for XY zygotes to develop into fetuses (and later adults) with phenotypically female characteristics, while XX zygotes can undergo masculinization. The earlier the conditions are imposed, the more completely can the processes be affected. Examination of the details of normal and abnormal development lead to the conclusion that genotypic and phenotypic sex are not necessarily inseparable.

EARLY DEVELOPMENT OF THE MAMMALIAN REPRODUCTIVE SYSTEM^{1, 2, 8, 9, 10}

Developmental changes in human embryos and fetuses are described below. Except for telescoping of the time scale in species with short gestation periods, the pattern is similar in most placental mammals.

The "Sexually Indifferent" or "Ambisexual" Stage

About 4 weeks after conception, a *genital ridge* becomes visible on each side of the embryo, just medial and ventral to the mesonephros (temporary kidney, Wolffian body) close to the adrenal gland. It is formed from the coelomic epithelium and underlying mesenchyme.

A few days earlier, "primordial germ cells" or *gonocytes* can be seen proliferating by mitosis within the yolk sac. These cells migrate through the hindgut toward the genital ridges by ameboid movement. A humoral substance (telepherion) has been implicated in attraction of the cells to the future gonad.

Developing genital ridges of XX and XY embryos are morphologically indistinguishable up to at least 42 days after conception, and are known as "*indifferent gonads*." (Fig. 19-1A) They possess the potential for differentiation into either testes or ovaries, and the germ cells can become either male or female sex cells. The gonocytes are apparently essential for full development of the ridges, and the gonads are needed for maturation of the gonocytes.

At this early stage, the embryo also possesses two pairs of ducts, the mesonephric or *Wolffian*, and the *Mullerian* ducts. A sexually indifferent *genital tubercle* and

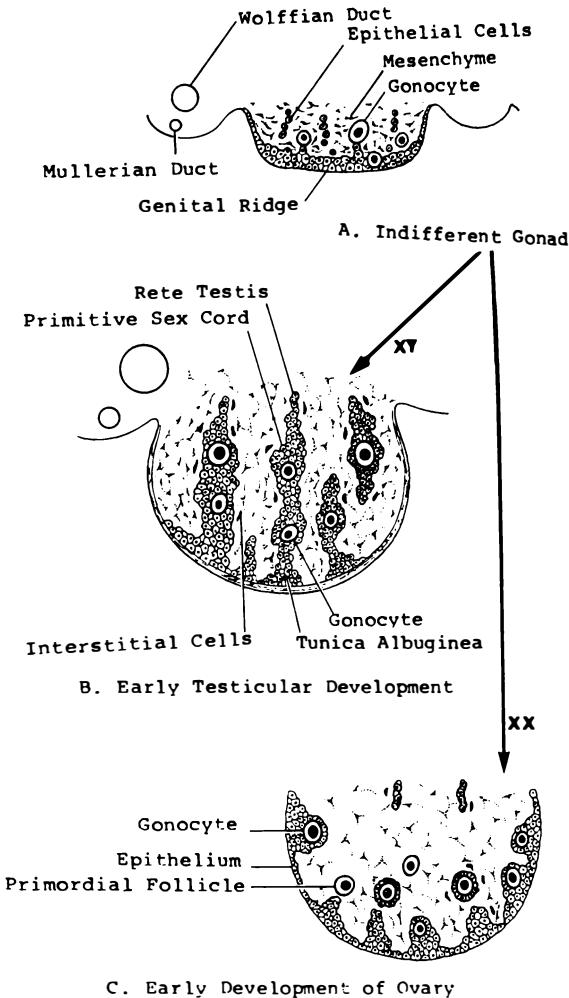


FIG. 19-1. Schematic representation of early development of testis and ovary.

associated cloacal folds are visible on the surface of both XX and XY embryos.

Early Development of Male Embryos and Fetuses

The Testis. The influence of the Y chromosome first becomes apparent about 6½ weeks after conception. The genital ridge grows faster in male embryos. The gonocytes migrate inward toward the medullary portion of the ridge and they soon become closely associated with (surrounded by) epithelial cell forerunners of the future Sertoli cells. Soon afterwards, they cease

proliferation; this has been attributed to inhibitory influences exerted by the epithelial cells. The gonocytes become committed to male-type development and are then known as *spermatogonia*.

The nature of the information imparted by the Y chromosome is debated. According to one hypothesis it induces rapid proliferation of the gonadal epithelium and the latter assumes control over future events.⁴ According to another, it promotes the association between the epithelial cells and the gonocytes.⁵

The epithelial cells and spermatogonia become organized into *primitive sex cords*

which are solid at this stage but eventually develop into seminiferous tubules. They migrate inward, and small strands extending in the direction of the mesonephric tubules form the precursors of the *rete testis* which will later transport sperm to the epididymis. The outer cortical portion of the genital ridge narrows, and a membrane forms between the medulla and the cortex. The membrane becomes thickened with connective tissue and other elements to form the *tunica albuginea* (Fig. 19-1B). The previously indifferent gonad thus becomes transformed over the course of little more than a week into a recognizable testis.

Interstitial cells (between the sex cords) proliferate rapidly during the 3rd month and develop ability to synthesize and secrete steroid hormones.

Most authors believe that the fetal interstitial or Leydig cells originate from the mesenchyme, that they function for a time during fetal life, become quiescent, and reawaken at the time of pubertal development. Others place their origin in the gonadal epithelium, and some believe that fetal Leydig cells die off and are replaced at puberty by an entirely new population.¹⁰

Accessory Reproductive Structures. As the testis develops, the Wolffian ducts start differentiation into the future epididymis, seminal vesicles, vas deferens, ejaculatory duct, etc., and the Mullerian ducts degenerate (atrophy of the latter is evident by the 60th day).

Duct changes clearly depend upon regulators synthesized by the fetal testis.

Removal of testicular anlage in very young embryos prevents both maturation of the Wolffian duct and degeneration of the Mullerian duct. Removal on one side arrests changes on that side only, while transplantation of a developing testis to a very young XX embryo induces "masculinization" on the side of the transplant. (The transplant is not effective, however, if made at a later time.)

Testosterone and a protein with high binding affinity for testosterone have been identified in Wolffian duct preparations; and injections of testosterone can promote maturation of the structures. Therefore, it seems likely that male sex steroid produced by the immature testis acts physiologically on the Wolffian duct.

However, testosterone does not promote atrophy of the Mullerian duct, while implantation of an immature testis does. It seems clear that some other humoral factor is involved, and the names *medullarin* and *Mullerian duct-inhibiting hormone* have been given to the hypothetical substance. There are good reasons for believing that medullarin is a peptide, but other possibilities (e.g., nucleic acids) have not been ruled out.^{2, 12}

Opinion is also divided on whether medullarin should be called an *embryonic inducer* (since it seems to pass from cell to cell without benefit of the circulatory system), or whether it is a "true" hormone. It has also been proposed that medullarin acts as an antibody which combines with surface receptors on the Mullerian duct and initiates an immune response that leads to atrophy.

Those who favor the hormone designation point out that medullarin must travel relatively long distances, or that it may be secreted for long periods of time after fetal development. In one study on frogs it was shown that alternate atrophy and recovery of ovaries can be accomplished by repeated insertion and removal of transplants of adult testis into adult females. Tissue culture studies suggest that the situation in mammals may be different, since fetal but not postnatal mammalian testes were found to release something which inhibits Mullerian duct development.

External Genitalia.¹¹ Formation of a penis from the genital tubercle and of a scrotum from the cloacal folds starts soon after duct development commences, and proceeds rapidly. The embryo becomes externally recognizable as a male within 2 months after conception and external genitalia are well developed 2 weeks later. The process is clearly steroid hormone-dependent. It can be mimicked in XX or XY embryos by injection of testosterone, and prevented with cyproterone which competes with testosterone for receptor sites. Unlike the Wolffian duct, the genital tubercle cells contain an enzyme catalyzing conversion of testosterone to dihydrotestosterone, and such conversion seems to be necessary for hormone action.

The events described above must take place during a restricted period of development. If the testes are removed from young embryos and reinserted later, normal patterns cannot be obtained.

At a later stage testosterone promotes

descent of the testes into the scrotal sacs. This usually occurs shortly before birth in the human and postnatally in some of the smaller animals such as the rat. The timing of this event is evidently not critical; boys with undescended testes often respond satisfactorily to testosterone administration during later childhood.

Early Development of Female Embryos and Fetuses

Ovaries. At the time when formation of the embryonic testis would be proceeding in the male, the female gonad retains its indifferent characteristics; it grows slowly and gonocytes continue their mitotic divisions. (Continued proliferation has been attributed to delay in association of gonocytes with epithelial cells.) The gonocytes enlarge, become committed to formation of egg cell precursors and are then called *oogonia*.

During the 11th to 12th prenatal week some of the oogonia enter into the prophase of the first meiosis and are then called *primary oocytes* (*primary ovocytes*), but division is arrested at that stage until the time of puberty. Other oogonia continue proliferation. It is estimated that 7 million egg cell precursors are present in the 6-month-old human fetal ovary (compared with 300–1300 spermatogonia in the fetal testis).

Many primary ovocytes become surrounded by a single layer of flat epithelial cells (*granulosa cells*), and the composite structures make up the *primordial follicles*. Cells between the oogonia and primordial follicles later form the *ovarian stroma* (Fig. 19-1C). The fetal gonad is recognizable as an ovary by the beginning of the 3rd month.

Development of the ovary continues through the 3rd month. No new oogonia are formed thereafter. Significant numbers of oogonia and of ovocytes which have failed to become surrounded by granulosa cells die off, while starting at the 6th month some primordial follicles mature into *primary follicles*.

The population of egg cell precursors falls off to about 2 million during the 6th fetal month, and the loss continues until puberty, at which time about 400,000 can be counted. Something like 400 of the latter complete development into egg cells.

The Mullerian Ducts. Long after the Mullerian ducts would have degenerated in the male, they begin development into Fallopian tubes (oviducts), uterus, and part of vagina, while most of the Wolffian duct components atrophy. No humoral factor seems to be needed for formation of the Mullerian duct derivatives; they develop in XX and XY embryos from which gonads have been removed. Atrophy can be accomplished by administration of fetal testis components up to a certain developmental stage; but once the ducts have accomplished considerable differentiation, they become resistant to testicular influences.

Full development of the female reproductive system seems to require the presence of two X chromosomes, (at least in humans and some other species), although partial development proceeds when only one X is present. According to some authors, a humoral substance (*corticin*, *cortexin*) produced by the ovary promotes ovarian and duct development. The name is related to the fact that the *cortical* part of the ovary develops to a greater extent than the *medullary* part (by contrast with the situation in the testis).²

External Genitalia. The genital tubercle develops into a clitoris and the cloacal folds into labia at a later time than genitalia development occurs in the male. The human fetus becomes externally recognizable as female by the end of the 3rd prenatal month. Administration of androgens (e.g., testosterone) can promote development of the tubercle along masculine lines up to the time when feminization is evident. But once female structures have been fully formed, androgenization results mostly in enlargement of the clitoris.

There is no evidence that estrogens are needed for female development of the genital tubercle. Very high concentrations are normally present during the gestation period, but some development has been accomplished *in vitro* without estrogens.

If ovaries of XX females are removed at an early stage, Mullerian ducts and external genitalia develop along female lines. If testes of XY males are removed, reproductive maturation is delayed until the time it normally would occur in a female. The Mullerian ducts and genital tubercle develop along female lines, but the Wolffian ducts do not undergo normal atrophy.

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Comparisons of Male vs Female Developmental Patterns

It is apparent from the preceding that:
 (1) The presence of a Y chromosome directs early rapid growth of the indifferent gonad, association of gonocytes with epithelial elements, and development mostly of the medullary portion into a recognizable testis. (2) The embryonic testis releases regulators which promote early development of the Wolffian ducts, masculinization of the genital tubercle, and atrophy of Mullerian structures. (3) Influences of the Y chromosome and of the immature testis are effective during critical periods of development which are very limited in time. By contrast, (4) female development is delayed and (5) will occur in the presence of a Y chromosome if humoral regulators from the embryonic and fetal testis are excluded. (6) Epithelial components of both testis and ovary seem to inhibit gonocyte proliferation; but (7) whereas spermatogonia do not proceed beyond the stage of formation, oogonia surrounded by granulosa cells may enter into the prophase of the first meiotic division.

DISORDERS OF SEX DIFFERENTIATION^{1, 6, 8, 11}

Chromosomal Aberrations

Problems Arising during Gametogenesis. Chromosome pairs do not always separate in a normal manner; *nondisjunction* can lead to migration of two sex chromosomes to a single gamete, while the "sister" cell receives no sex chromatin. All products of such divisions (XX ova, XY sperm, and O gametes) can engage in fertilization.

Chromosomal lag is a condition in which separation does take place, but one chromosome fails to migrate in a normal manner and may be extruded from the cell.

The sex chromosome makeup of zygotes which can result from fertilization of known abnormal types of gametes is shown in Figure 19-2, in which absence of a chromosome is represented by 0.

A third kind of difficulty arises when only a portion of a sex chromosome is deleted. The chromosome "type" may be normal (XX or XY), but some essential genetic information may be missing. Dele-

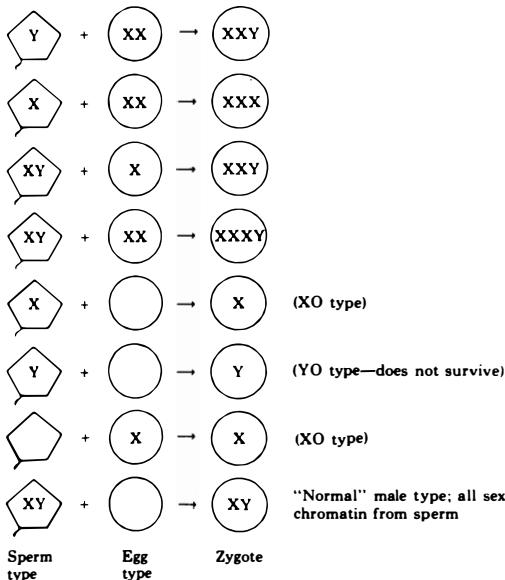


FIG. 19-2. Zygote types resulting from fertilization of gametes with abnormal sex chromosome makeup.

tion of a portion of one or more chromosomes can also occur in combination with an abnormal chromosome number.

Problems Arising during or soon after Fertilization. On rare occasions more than one sperm fertilizes an egg cell. If all of the genetic material is retained and passed on during cell divisions, the embryo usually does not survive past early developmental stages. (Polyploidy is, however, compatible with survival in vertebrates with less highly developed brains, e.g., salamanders.) A portion of the excess chromatin may be retained, so that the zygote has an abnormal makeup.

More commonly, some of the chromatin is passed on to certain cells and different chromatin to others. *Chimaerism* (possession of more than one genetic cell line) can result in this and other ways. Sometimes a polar body (Chapter 21) fails to separate from the egg cell before fertilization. It is also possible for two different morulae (Chapter 21) to fuse into one individual.

Additional problems can arise during early mitotic division of the zygote, resulting in chromosomal aberrations similar to those seen when gamete formation is defective; if some cells are affected during an early cleavage stage, while others are not, the individual that develops may become a *genetic mosaic*. Some of his cells will have one chromosomal pattern, while others will have a different pattern.

Some Generalizations concerning Effects of Chromosomal Aberrations on Reproductive Development in Humans. Any deviation from the normal pattern is likely to result in defective function of systems other than those directly related to reproduction; mental retardation is probably the most common finding.

The X chromosome affects many nonreproductive functions; at least one X is essential for survival. While development follows the female pattern when a single X sex chromosome is present (XO type zygote), two X chromosomes seem to be required for full development of the human female system and fertility; more than two X chromosomes usually lead to some characteristic abnormalities (see below).

The Y chromosome is the determinant of male-type development. Its effects will emerge to at least a limited extent even

when large numbers of X chromosomes are present (e.g., XXXXY pattern). Persons lacking a Y chromosome but exhibiting some masculine characteristics are believed to have had a Y chromosome present at some earlier stage of development or to have come under the prenatal influence of male-type hormones produced by the adrenal gland. Y chromosomes affect a few somatic characteristics, e.g., body length.

Some Diagnostic Methods.* Procedures have been developed for determination of the karyotype (chromosomal pattern) by examination of readily obtainable cells, e.g., scrapings from the inside of the mouth, or leucocytes.

The short arm of the Y chromosome exhibits intense fluorescence when treated with quinacrine hydrochloride. Persons highly skilled in use of the techniques can determine the number of Y chromosomes present from fluorescent points visible on the nucleus.

A densely stainable chromatin mass at the periphery of the nucleus of some cells (Barr body) is seen when more than one X chromosome is present. The total number of such bodies is equal to one less than the number of X chromosomes; normal males with a single X show none, normal females one, and XXX females two. The presence of more than one X chromosome can also be demonstrated by "drumstick" appendages of nuclei of polymorphonuclear leucocytes.

In clinical usage, chromatin is classified as + (positive) if Barr bodies are present. Therefore all normal females are said to be chromatin +, and all normal males are chromatin -. But females of the XO types are negative while males of the XXY type are positive.

Turner's Syndrome. Patients with an XO pattern are usually phenotypic but incompletely developed females. They have recognizable Fallopian tubes, uterus, and female-type external genitalia; but the gonads are usually rudimentary ("streak-like"), the individuals are not fertile, and secondary sex characteristics are poorly developed.

Typical nonreproductive findings include short stature, webbing of the neck, deformities of the forearm (cubitus valgus), shield-shaped chest, mental retarda-

tion, facial deformities, and coarctation of the aorta.

Many variants of the syndrome have been described, often associated with genetic mosaicism. Persons with XX/XO patterns may exhibit the whole spectrum of features ranging from apparently normal to full emergence of the syndrome. Poorly developed phenotypic males with defective Y chromosomes or with XO/XY mosaicism have been said to have "male Turner's syndrome."

The condition is believed to arise predominantly during an early cleavage stage; no correlation has been found between appearance of the syndrome and age of the mother. The reported incidence is about 1 in 2700 live births but is higher in aborted fetuses.

Klinefelter's Syndrome. Patients with XXY chromosomal pattern are phenotypically male but "chromatin positive" since they have more than one X chromosome. Typical findings include undeveloped or degenerated seminiferous tubules and associated sterility, mental retardation, tall stature, poorly developed secondary sex characteristics, and often psychological disturbances and what has been described as "delinquent behavior." The condition is believed to arise most often from nondisjunction of chromosomes during formation of the ovum; it is associated with advanced age of the mother at the time of conception.

The XXX Chromosome Pattern. Individuals with apparently normal function have been found to present this pattern, and it has been suggested that function of the third sex chromosome can be suppressed. The most common abnormal finding is mental retardation. Reproductive function can be normal, but the designation "super female" is inappropriate, and some individuals are infertile. Mental retardation is likely to be severe if four X chromosomes are present.

XYY Pattern. This chromosomal pattern is associated with unusually tall stature. A few years ago it was reported that the incidence of the disorder is especially high among inhabitants of penal institutions, and that the chromosomal aberration plays a role in development of antisocial behavior. Subsequent investigation revealed the presence of the pattern in apparently normal men. Some have discounted any relationship between XYY patterns and be-

havior while others have suggested that effectiveness of the Y chromosome is somehow suppressed in psychologically normal men.

True Hermaphroditism. In one form of true hermaphroditism, an ovary and female duct system are present on one side of the body and a testis and male duct system on the other. In others, an ovotestis may be present on both sides. The condition has been attributed to genetic mosaicism or chimaerism. XY/XX, XXY/XX, and other patterns have been found.

Disorders of Sexual Differentiation Attributable to Hormonal Disturbances

Adrenogenital Syndrome. Although gonads develop in close proximity to the adrenal glands, the latter probably exert very little influence on sex differentiation under normal conditions. But congenital disorders (Chapter 8) can lead to adrenocortical secretion of sufficient androgenic steroid hormones to masculinize an XX embryo or fetus. If this happens early, the external genitalia become completely masculinized. Wolffian ducts exhibit only partial development (possibly because there is no medullarin) while ovaries and Müllerian ducts persist. The resulting condition is *pseudohermaphroditism*. Babies born with this defect are usually classified as males.

If adrenal hypersecretion commences at a later stage of development, external genitalia can become masculinized although the Wolffian duct has regressed and the uterus and Fallopian tubes have started formation. At a still later stage, adrenal hypersecretion will promote enlargement of the clitoris but not regression of already formed female structures; the enlargement may be reversible with hormone treatment.

Precocious maturation of male fetuses and infants has also been reported to result from secretion of adrenal androgens.

Progesterone was commonly administered in the past for the purpose of quieting the uterus during pregnancy. It was not at first appreciated that this hormone can be converted to androgens and that female fetuses can thereby be masculinized.

"Feminizing Testis." A different form of pseudohermaphroditism arises from a congenital defect in which male sex hormones can be synthesized and secreted,

but target organs do not respond to the hormones. In XY embryos, the testes differentiate, the Wolffian ducts show some development and the Mullerian ducts atrophy, but the external genitalia become feminized. Individuals with this disorder develop female sex characteristics at puberty and are believed to be perfectly normal females until it is noted that menstruation is not established. Reproductive structures do not respond to exogenous administration of testosterone.

In some individuals with an incomplete form of the syndrome, a defect in conversion of testosterone to dihydrotestosterone has been demonstrated, and there may be some response to high doses of dihydrotestosterone. In others, there is evidence for defective translocation and/or nuclear binding of the cytoplasmic androgen receptor.¹¹ Development of the Wolffian ducts is of theoretical interest; it has been attributed by some to medullarain and by others to some special properties of these embryonic structures which make them susceptible to testosterone influences.

Congenital Defects Affecting the Leydig Cells. Inability to secrete testosterone in adequate amounts can elicit prenatal conditions similar to those described above. But individuals with this disorder are responsive to exogenous administration of male sex hormones.

Sex Reversal With Hormones^{2, 8}

In many fish species, females have XX sex chromosomes and males have the XY set. Administration of female sex hormones at a *critical* stage of development to XY fishes can lead to formation of a *phenotypic female* complete with ovaries, oviducts, and the feminine pattern of body contours and coloring. Such animals will produce egg cells containing a Y chromosome and will mate with normal males. Similarly, an XX embryonic fish can be made to develop a male phenotype if testosterone is administered at a critical stage.

Studies of this kind are useful for distinguishing between characteristics dependent upon the presence of a Y chromosome, and those attributable to influences of hormones normally secreted by animals with Y chromosomes. It has also been possible to produce fishes with interesting

chromosomal patterns by mating sex-reversed animals.

Certain differences between fish and mammal differentiation are obvious. It will be recalled that mammalian embryos and fetuses seem to be unaffected by estrogens, while male fishes can be transformed into phenotypic females by these hormones. It has been proposed that emergence of insensitivity in mammalian embryos was necessitated by their development in an estrogen-rich environment.

While mammalian embryos deprived of hormones tend to develop along female lines (and this can be shown in organ culture), a different situation is seen in birds. The latter tend to develop along masculine lines except when female hormones direct otherwise. Mullerian duct structures persist in both ZZ and WZ castrated birds, but the genital tubercle develops the male form. Early administration of estrogens can convert what would have been a testis into an ovotestis and can inhibit formation of the penis.

EARLY INFLUENCES OF SEX HORMONES ON THE HYPOTHALAMUS^{1, 8}

Reproductive functions in the female mammal depend upon cyclic or intermittent patterns of secretion of adenohypophyseal hormones (Chapter 21), and the adenohypophysis is in turn dependent upon cyclic secretion of gonadotrophic hormone release factors by the hypothalamus. By contrast, reproductive processes of adult males are sustained by relatively continuous or tonic secretion of hypothalamic and adenohypophyseal hormones.

Although the pattern for hypothalamic activity is not expressed until the time of puberty, the "setting" of the hypothalamus is determined much earlier. In humans, monkeys, and guinea pigs (which are relatively mature at birth), hypothalamic conditioning seems to occur late in fetal life; in animals born in a less well developed stage (rats, mice, etc.), hypothalamic activity is set within the first few days after birth.

Studies on the hypothalamic setting have been carried out largely in rats because of the convenience for the investigator. Experimental evidence supports the concept that small quantities of testosterone secreted by the immature testis act directly on the hypothalamus during a "critical" stage of development and per-

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manently set it for the male pattern. Presumably, no testosterone is present at comparable times in females.

When male rats are castrated within the first day or two after birth, the hypothalamus develops along "female" lines. After puberty it functions cyclically and will support activity of a transplanted ovary. If female rats are given a single injection of testosterone within 2 days after birth, the hypothalamus becomes set for the "male" pattern. Females then undergo anatomical changes characteristic of the sex, but most fail to exhibit ovulatory cycles. Male hypothalamic patterns can also be induced a few days later (with less consistency) if very much larger quantities of testosterone are injected. The ovaries of noncycling females can function normally if transplanted to untreated females; pituitary glands will exhibit cyclic responses if placed in contact with the hypothalamus of a normal female.

Testosterone seems to affect neurons within the preoptic region of the hypothalamus which synapse with those that actually secrete the gonadotrophin-releasing factors. The preoptic neurons are known to avidly bind steroid hormones, and lesions in that location disrupt ovarian cycles when cells secreting release factors are left intact.

There are some unresolved questions. An estrogen injection shortly after birth can also promote masculinization of the hypothalamus. Since both types of steroid hormone inhibit gonadotrophin secretion, one hypothesis states that it is not action of the steroid directly, but the suppression of gonadotrophin secretion at a critical stage, which affects the hypothalamus.

Guinea pigs are born after a gestation period of 67–68 days (compared with 21–22 days for the rat) and the hypothalamus is "set" during fetal life. Androgens injected into pregnant mothers can travel to the fetuses and affect the hypothalamus. This has important theoretical implications for maternal influences over developing fetuses. However, there are sharp limitations on what can be carried over from one species to another. It is known that primate fetuses are resistant to androgen injections into the mother and that masculinized human XX fetuses can grow into normal adults having regular menstrual cycles and fertility.

ACTIONS OF REPRODUCTIVE HORMONES AT PUBERTY AND IN THE ADULT

Following completion of rapid early development, infantile reproductive structures pass through a stage of dormancy before further maturation takes place. There are striking differences between influences of reproductive hormones starting at the onset of puberty and those seen during developmental stages.

(1) Female sex hormones are clearly needed for reproductive maturation and function in female; in their absence, structures remain infantile.

(2) There does not seem to be a critical time during which the sex hormones must be available. Premature development and function can be achieved by early administration of the hormones. And if puberty is delayed (e.g., by withdrawal of the hormones or administration of antagonists), structures retain the ability to respond to hormones at a later stage.

(3) Hormones are needed throughout life; if they are withdrawn at any time, reproductive structures regress. With few exceptions, actions of sex hormones are reversible. The exceptions involve special anatomical changes such as growth of the vocal cords under the influence of androgens.

(4) Within broad limits, there is a quantitative relationship between hormone level and response. For example, the size of seminal vesicles and prostate glands is directly related (over a wide range) to the amount of androgen present.

(5) Females require small amounts of "male" hormones for normal function and can respond to excess. For example, some testosterone seems to be needed for sexual interest in females but too much can promote disfiguring growth of facial hair in humans. Similarly, males require small amounts of estrogens, while the male breast can respond to an excess.

(6) Responses of nonreproductive structures to sex hormones are more obvious in the adult than in the embryo or fetus.

HORMONAL INFLUENCES ON BEHAVIOR⁷

Criteria

Attempts have been made to set up quantitative criteria for measurements of hormonal influences on behavior. For example, "masculine behavior" has been described in terms of *aggressiveness* (the number of times one animal attacks an-

other under specified conditions), *territorial defense* (what an animal is willing to do to keep out intruders), and activities associated with *copulation* (how many times the animal attempts to *mount* within a given time period, the number of *intromissions* and *ejaculations*).

"Feminine behavior" has been described in terms of *passivity*, *receptiveness* towards the male, or the readiness with which the characteristic posture (lordosis) can be elicited. Similarly, "maternal behavior" has been related to the *number of attempts to build a nest*, the *type of nest* built, and reactions to the presence of infant members of the species (*gathering* of the young, *placement* in the nest, *licking* or attempting to *nurse*).

Difficulties arise in study of hormonal influences over behavior because most aspects are quantitative rather than qualitative, and there is a wide range which must be considered "normal." Some fully fertile males are more aggressive than others. Some fertile females highly receptive to males exhibit aggressiveness, mounting, and other "male" behavior; and "aggressive" males may engage in behavior that has been classified as "maternal."

A second kind of problem arises from metabolic transformations of one kind of hormone to another. Progestagens are readily converted to androgens, and androgens to estrogens.

"Strength of Drive"

Hormones seem to act primarily through alterations of thresholds to stimuli; some may do this through direct actions on membrane properties of neurons. They tend to elicit, intensify, or diminish behavior patterns that could proceed in their absence.

Male animals in which behavior patterns are well developed may respond more frequently or more aggressively to other animals when they are injected with substantial (but not toxic) doses of testosterone, and interest in such activity can be diminished by administration of testosterone antagonists. There are very definite limits to hormonal influences. One pertinent observation is that "experienced" male rats (with a life-span of 2½-3 years) may continue to engage in copulatory behavior for as long as 5 months after castration when no hormones are administered.

Female rats exhibit a characteristic posture (lordosis) when approached by males during periods of sexual receptivity. If they are given male-type hormones, the female behavior may be intensified.

"Conditioning Influences" of Hormones

When immature male animals are given injections of testosterone, they may, later in life, exhibit greater sensitivity to hormone administration of the same kind, and reduced susceptibility to injections of hormones of the female type. Similarly, injections of estrogens into immature females may enhance sensitivity to such hormones later in life. The observations suggest that hormones can exert during growth periods, more permanent changes in the nervous system than can the same hormones given after maturity has been attained. Like the preceding phenomena, the effects of the hormones seem to be quantitative rather than qualitative, in that they do not change the *direction* of responses.

"Organizational" Influences of Hormones

During very early *critical* stages of development, hormones seem to influence the *quality* of behavior. It has been reported that administration of androgens to pregnant mothers, and to the young during the perinatal period, can enhance the tendency of both male and female animals to exhibit "masculine" behavior after puberty. Related studies have raised questions about whether female patterns in XX and XY animals emerge because of the presence of high levels of estrogens or because of the absence of androgens.

While application of rodent studies to situations in mammals with more highly developed brains is limited, there have been suggestions that tendencies towards homosexual activity in humans could be conditioned by exposure to prenatal hormone imbalances arising in the mother or fetus, or possibly because of the presence of a fraternal twin of the opposite sex.

GENDER ROLE

The term usually encompasses a broad spectrum of learned behavior associated

with "maleness" and "femaleness", including activities not related to reproduction.

According to some observers, the human infant is born in a completely neutral condition with respect to gender role, but the concept of belonging to a male or female group is built up almost from the moment of birth by such things as the color of the crib blanket, the design of infant clothing, the kinds of toys presented, attitudes of parents and siblings, and later by social conditioning.

Observations on individuals with discrepancies between genetic and phenotypic sex have been used to support the idea. Persons with XY chromosome patterns and congenital inability to respond to testosterone appear externally as normal females, and readily adapt to the expected gender role. They are classified legally as females, and they often enter into happy marriages with normal males. Discovery of the discrepancy between chromosome makeup and phenotype may never be made; or it may be diagnosed only when there is concern over infertility and failure to establish menstrual cycles. The presence of testes, Wolffian duct structures, and high testosterone titers does not seem to affect acceptance of the gender role.

Female infants subjected to prenatal masculinization but recognized as females may also readily adopt the female gender role, and make satisfactory adjustments to life as women.

There are numerous case histories of individuals born with ambiguous genitalia who were assigned a gender role at the time of birth. Even with persistence of the genital abnormalities, many of those designated as girls successfully adopted female roles, while those judged to be boys accepted the male gender role. The group includes persons in which the assigned role agreed with, and those in which the assigned role disagreed with subsequent findings of the nature of the chromosomal sex. The group also includes true hermaphrodites, in which both testes and ovaries were discovered in adulthood.

Other observers believe that the ability to adopt a particular gender role depends on hormonal conditions present during early developmental stages. They attach different interpretations to the same findings, and cite other kinds of observations.

It has been suggested for example, that the XY person unable to respond to testosterone cannot be considered to be subjected to the influence of testosterone, and that the levels of estrogens secreted (which are obviously adequate for stimulation of structures such as the breast) are sufficient to promote acceptance of the female role. A problem which requires resolution before this interpretation can be evaluated is the one of possible differences in responses of neurons vs reproductive structures to testosterone. It is known that some of the "target organs" (e.g., prostate glands) responding to testosterone in normal males have enzymes which convert testosterone to dihydrotestosterone (DHT) and that such organs respond to administration of DHT. In the same individuals, testosterone itself seems to be the hormone which acts directly on certain brain structures. It has not been established whether those individuals with feminizing testis who lack ability to form DHT exhibit normal neuronal responses to testosterone.

Other observations invoked in support of the concept that hormones influence the ability to adopt an assigned gender role include repeated descriptions of "personality" characteristics of prenatally androgenized but otherwise normal girls. "Tomboy behavior," special interest in sports, and "career orientation" with diminished interest in playing with dolls have been cited as evidence of tendencies towards masculinization, and it has been claimed that prolonged secretion of estrogens starting at the time of puberty is needed to overcome some of the early influences of the masculinizing hormones. It has also been pointed out that some prenatally androgenized females do not make good social adjustments. Evidence of a high incidence of psychological problems in children with ambiguous gonads, and decisions to undergo "sex reversal" later in life by such persons have also been cited.

One reaction to some of the preceding is that there is nothing "innate" about the interest of female children in passive activity and doll play, and that the "personality" characteristics of the androgenized girl are expressions of rebellion against stylized social conditioning on the part of youngsters who tend as a group to be stronger, more intelligent, and more physically ad-

vanced than others of their age. The incidence of psychological problems in children with ambiguous gonads could certainly arise in part from recognition that a part of the body associated with strong emotions is different from that of other children (or from recognition that surgery has been performed on such a part). Differences in growth rates or maturation resulting from hormone imbalances can also put youngsters "out of step" with their contemporaries, and attitudes expressed by parents, siblings, and playmates can add to the difficulties.

One question which has aroused a great deal of controversy concerns the etiology of homosexual interests and behavior. It has been pointed out that individual differences are not of an "all or none" nature; most "heterosexuals" seem to have had some kind of interest at some time in their lives in homosexual activity, while most "homosexuals" have varying degrees of interest in heterosexual activity. In males classified by themselves or others as homosexuals, testosterone levels in the blood have been shown to vary from well within the "normal" range to considerably below normal levels. Attempts have been made to affect the behavior by administration of androgens. Usually, the result has been to strengthen and reinforce the kind of behavior already present.

Psychologists and psychiatrists have described in great detail the special social conditions during infancy and childhood said to "predispose" to homosexual interest. Some endocrinologists have pointed to the effects of perinatal hormonal influ-

ences. Other points of view include concepts of complex interactions between hereditary, hormonal, and environmental factors. But many believe that the underlying factors cannot be explained by any of the preceding.

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20. The Male Reproductive System

THE TESTIS^{2, 5, 11, 15}

The testis profoundly influences almost every aspect of the physiology of the individual in whom it resides; and it does so from early embryonic life until the time of death.

The functions can be grouped into categories, but they are interrelated. Sper-

matozoa are produced in the seminiferous tubules, while testosterone is secreted mostly by the interstitial tissue. But sperm cell precursors affect development of the embryonic testis; and epithelial components of the tubules produce binding proteins for sex hormones. The tubules also produce some hormones of their own (and regulators of pituitary gonadotrophin func-

tion), while responding to hormones produced by interstitial cells. Testosterone promotes maturation and transport of gametes and maintenance of the physiological and psychological apparatus needed for realization of their function. It also exerts widespread influences on nonreproductive structures, and the results of such influences secondarily affect the testis.

The Seminiferous Tubules

The seminiferous tubules develop from the sex cords of the fetal testis (p. 231). The structures become long, hollow, and tortuous; their total length in the adult human male has been estimated at 250 meters. They contain *spermatogonia* or sperm cell precursors closest to the periphery and in contact with a *basement membrane*. Germ cells of increasing maturity are seen closer to the centers of the tubules: *primary and secondary spermatocytes*, *spermatids* in advancing states of development, and *young spermatozoa* with their tails extending into the lumen. Closely associated with the germinal cells and apparently essential for their development, the *sustentacular* or *Sertoli* ("nurse") cells mature from epithelial elements of the sex cords. They take up nutrients which pass from the *tunica vasculosa* through the basement membrane. The tunica is a layer of loose connective tissue richly supplied with blood from the spermatic arteries and with lymph capillaries. It is enclosed in a thick, fibrous capsule (the *tunica albuginea*) which contains collagen and contractile elements. In most species (including man) the capsule projects between the seminiferous tubules as the *mediastinum* which gives rise to branches or septula that divide the testis into lobules.

The fluid within the tubule lumina is secreted by the Sertoli cells. Spermatozoa are sent into the cavity after traversing the coiled portions and the straight *tubuli recti*. They enter a network of epithelium-lined spaces (the *rete testis*) and then the efferent ductules leading into the epididymis.

The Interstitial Tissue

The loose connective tissue between the tubules contains (in addition to blood and lymph capillaries, mast cells, and fibro-

blasts) clumps of specialized *interstitial* or *Leydig cells* which synthesize and secrete male sex hormones. Most observers believe that the Leydig cells arise from proliferation and maturation of fetal interstitial cells which undergo a period of dormancy until reactivated at puberty (p. 232).

Testosterone, the major secretory product of the mature testis, acts locally to maintain spermatogenesis. High concentrations in the vicinity of developing germ cells have been attributed to direct uptake through lymphatic vessels; but recent work points to some steroid synthesis by tubular cells.

The hormone also enters the systemic circulation via the spermatic veins. It supports maturation and function of the *accessory reproductive structures* which include (in man and most others) the *epididymis*, *vas deferens*, *seminal vesicles*, *ampullae*, *prostate gland*, *ejaculatory duct*, *bulbourethral* or *Cowper's glands*, the *scrotum*, and the *penis*, all of which are involved in maturation and/or transport of the spermatozoa.

Androgens are needed for emergence of the *secondary sex characteristics* which confirm "maleness" and enhance attractiveness of the male to the female. In humans they promote growth of the mustache and beard, the axillary and pubic hair, the larynx and the vocal cords. They affect distribution of the scalp hair (and play a role in development of baldness), stimulate the sex and sebaceous glands, and coarsen the texture of the skin.

Testosterone affects distribution of body fat, and secondarily influences the amount acquired since it stimulates the appetite. It is partially responsible for the adolescent "growth spurt" and bone elongation, but it accelerates calcification of the epiphyses as well as synthesis of bone matrix. Children with precocious puberty tend to be tall for their age, but suffer shortening of stature because of premature skeletal maturation. Under its influence, the pelvis develops along "male" lines.

Some (but not all) influences on the central nervous system lead to development of "male" type behavior and sexual interest.

Secondary sex characteristics of nonhuman vertebrates responsive to testosterone

include the lion's mane, the antlers of some of the ungulates, the cock's comb, and the coloring of some of the birds and fishes.

Testosterone is an "anabolic" hormone. It promotes retention of nitrogen and electrolytes, protein synthesis, and growth and strengthening of skeletal muscles. The kidneys enlarge, enzyme systems of the kidney and liver mature, more red blood cells are manufactured, and metabolic rate is elevated. But the thymus gland involutes.

Certain muscles, e.g., the levator ani and masseters of the jaw of some species, are especially sensitive to testosterone and may continue to enlarge when food intake is inadequate to sustain generalized growth. The diaphragm muscles are relatively insensitive, while those of the arms, shoulders, and legs are intermediate.

Location of the Testes^{11, 12}

The testes of most mammals descend through the inguinal canals to reside in the scrotal sacs. In humans the descent usually occurs about 2 months before birth; in immature neonates (e.g., rats and mice) it occurs postnatally. The temperature of the scrotum is usually a few degrees lower than that of the abdominal cavity. This is maintained in part by a special arrangement of blood vessels, the *pampiniform plexus* through which blood is cooled on the way to the testis and rewarmed on the journey back to the abdomen. (The plexus may perform other functions as well, e.g., concentration of steroid hormones for use by the seminiferous tubules, and moderation of pulsatile flow to provide a continuous supply of arterial blood.)

In most species spermatogenesis requires the lower temperature. In the condition known as *cryptorchism*, the testes do not descend because of either obstruction of the inguinal canal or testosterone deficiency. Sperm maturation is impaired. (The condition can be mimicked by application of heat to the scrotum of experimental animals.) Artificially warmed or undescended testes maintain almost normal testosterone production at abdominal temperature.

Some mammals have special adaptations. The rat has very large testes for its body size; they can be temporarily withdrawn into the abdominal cavity for short periods (e.g., when the animal is fighting) with no apparent ill

effect. A few of the seasonal breeding species withdraw the testes during inactive phases. Still others (e.g., whales) produce fertile sperm within the abdominal cavity and do not have scrotal sacs.

SPERMATOGENESIS^{1, 2, 5, 7}

The term "spermatogenesis" is usually defined as the process of transformation of spermatogonia to sperm cells, and it can be divided into three stages: *spermatocytogenesis* (formation of the primary spermatocytes), *meiosis* (reduction division of the primary spermatocytes to produce the spermatids), and *spermiogenesis* (maturation of the spermatids into spermatozoa or sperm cells). Some authors prefer to consider spermatogenesis as encompassing only the first two, and spermiogenesis as a separate process.

Spermatocytogenesis

When embryonic gonocytes become committed to future production of sperm cells, they are known as *spermatogonia*. They remain in this state until puberty.

A few observers believe that embryonic and fetal cells die off and are replaced at puberty by a new germ line, but the concept is not widely accepted.

Several morphologically distinguishable types of spermatogonia have been described. There are unsettled questions about the functions of specific types, and species differences seem to have added to the confusion. But it is generally agreed that some persist as long-lived *stem cells* which renew the population from time to time, while others proliferate by mitosis. Some products of mitosis seem to die off as others go on through successive stages of maturation and eventually form type B spermatogonia committed to formation of *primary spermatocytes*.

The names immature, primitive, stem cell, A_a, and A_b have been given by various authors to stem-type spermatogonia. One concept states that a certain group remains dormant most of the time and can be called upon to renew the population when something unusual has depleted the numbers of other A-type spermatogonia, for example after exposure to radiation, infection, or severe malnutrition. Another group may proliferate periodically so that a stem cell

HORMONES AND REPRODUCTION

population is maintained. The names A₁, A₂, A₃, and A₄ have been given to successive stages of maturation, but some authors believe one or another of such types may represent proliferating stem cells. The term A_{pr} has also been applied to proliferating stem cells.

Maturation proceeds in waves or cycles. At any given time, cells in different stages of the cycle coexist within the same seminiferous tubule, with the least mature closest to the periphery.

While daughter cells of the A series

separate completely after mitosis, B-type spermatogonia retain a cytoplasmic bridge and give rise to two connected primary spermatocytes. The bridges are retained throughout meiosis, and eventually sets of eight interconnected spermatids are formed.

Meiosis

The primary spermatocytes may persist as such for a time, or they may begin meiotic division soon after they are formed.

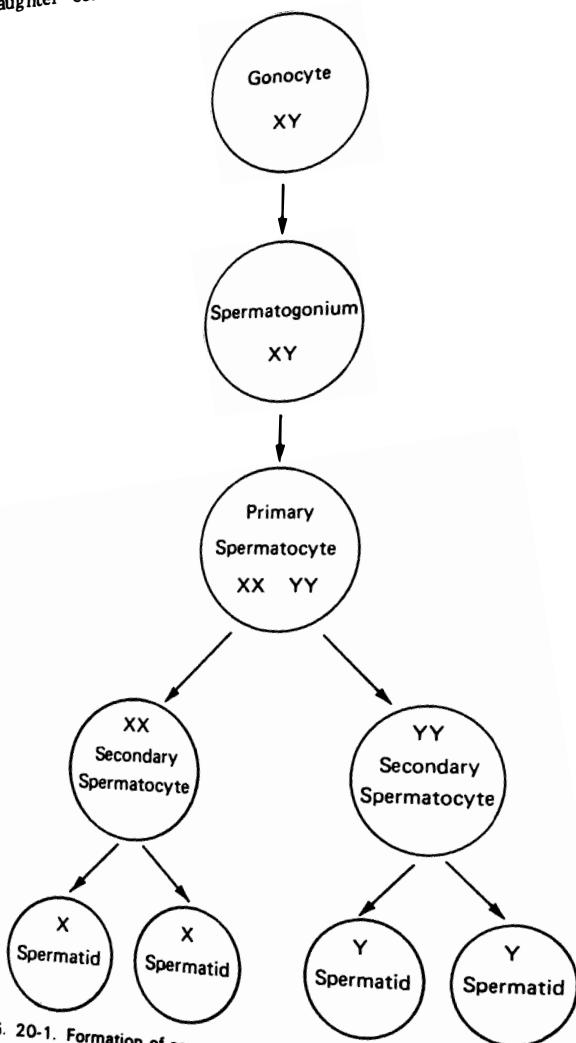
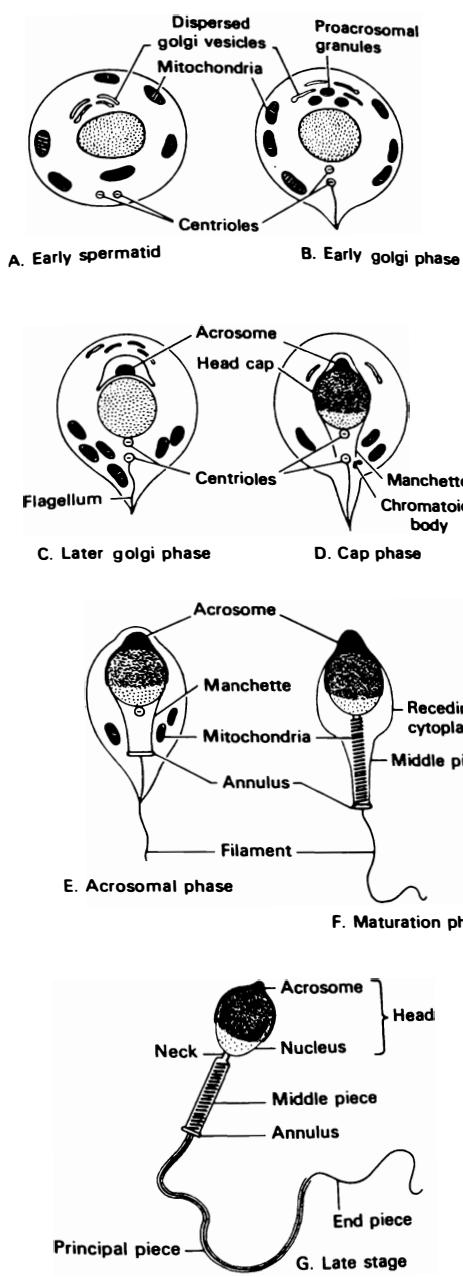


FIG. 20-1. Formation of spermatids (only the sex chromosomes are shown).

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Each gives rise to two **secondary spermatocytes** which differ from each other in genetic makeup. Secondary spermatocytes divide again, and two haploid spermatids are formed from each (Fig. 20-1).

Spermiogenesis^{2, 3}

When first formed, the spermatid is a small, rounded cell with a spherical nucleus located near the center, a prominent Golgi region, and numerous mitochondria (Fig. 20-2A). Two centrioles are visible on the side of the nucleus away from the Golgi.

Spermiogenesis is the process whereby the spermatid becomes gradually transformed into a spermatozoan. Since the spermatid starts out with a limited amount of cytoplasm and much of this is lost, intimate association with the Sertoli cell is essential.

The "Golgi Phase." Small, carbohydrate-rich membrane-enclosed **proacrosomal granules** appear in the juxtanuclear Golgi region. They soon coalesce to form a single **acrosomal granule** surrounded by the **acrosomal vesicle**. The latter adheres closely to the proximal pole of the nucleus and gradually spreads over the nuclear surface.

One of the centrioles gives rise to a membrane-enclosed **flagellum** or **axial filament**, and mitochondria begin migration toward the filament (Fig. 20-2, B and C).

The "Cap Phase." The acrosomal vesicle spreads over the proximal two-thirds of the nucleus, forming the **head cap** which has a ring-like structure at its distal end. What remains of the Golgi matter moves away from the acrosomal vesicle.

Microtubules become visible and associate with the ring-like structure forming a **caudal sheath** or **manchette** (which will soon grow posteriorly). Mitochondria and a dark-staining cytoplasmic structure, the **chromatoid body**, move toward the manchette, move toward the manchette (Fig. 20-2D).

The Acrosomal Phase. The acrosomal granule spreads over the upper part of the nucleus, as the latter begins to flatten, condense, and move toward the cell periphery. The chromatoid body organizes into a ring-like **annulus** marking the posterior border of the manchette which now extends from the lower pole of the nucleus backward over the proximal part of the growing axial filament. (The annulus has been implicated in synthesis of contractile proteins.) Mitochondria form a collar around the manchette. Cytoplasmic volume decreases (Fig. 20-2E).

The Maturation Phase. The cell continues to elongate as the cytoplasm is further reduced and the nucleus becomes flattened and condensed. The acrosome spreads over much of the head cap. Mitochondria wind diagonally around the portion formerly occupied by the manchette, and the latter is replaced by the *middle piece* which encircles the upper portion of the growing filament. The distal centriole disappears (Fig. 20-2F). In some animal types, the acrosome undergoes additional specialization and assumes a species-characteristic shape.

When ready to leave the testis, the spermatozoan consists of a *head* containing all of the condensed nuclear material, an acrosomal cap which partially covers the nucleus, and a *tail*.

The tail is similar in all mammalian species studied. The widest portion containing the mitochondria is the *middle piece*; it is connected to the head by a very short, narrow *neck*. The annulus marks the terminal end of the middle piece. Most of the tail length makes up the *principal piece* (about $45\ \mu$ long in the human sperm, compared with about $5-7\ \mu$ for the middle piece). A short thread-like portion forms the *end piece*.

Time Required for Spermatogenesis.⁵ The time required for formation of spermatozoa from type A spermatogonia is genetically determined. Hormones influence the number of cells which reach maturity, but do not affect the timing.

Periods ranging from 48 to 53 days have been reported for different strains of rats, 34.5 for the mouse, and 49 for both the bull and the ram. The figure most often cited for man is 64 days.

The entire process of spermiogenesis takes place while the developing cell is completely embedded within the cytoplasm of the Sertoli cell; only the axial filament projects outward, towards the tubular lumen.

SPERMIACTION

Spermiation (extrusion of the spermatozoa into the tubular lumen) is an active function of the Sertoli cell which is regulated by pituitary luteinizing hormone (LH). The Sertoli cells take up fluid; cytoplasmic swelling, cell elongation, and opening of the cisternae and channels of the endoplasmic reticulum have been attributed to reduction of ATPase activity

(and presumably, blocking of the sodium pump). Unfolding of the apical cytoplasm and movement of the spermatozoa may require active contraction of the Sertoli cell. Slender cytoplasmic projections are seen around the emerging spermatozoa; the threads finally break, and a small bit (the *cytoplasmic droplet*) remains in contact with the sperm as it emerges, while the *residual body* is ingested by the Sertoli cell.

It has been proposed that ingestion of the residual body leads to formation of a "Sertoli cell hormone" which initiates the next spermatogenic cycle.

Since the pregnancy hormone, chorionic gonadotrophin, acts on Sertoli cells in a manner which appears identical to that of LH, spermiation in amphibians is utilized in one form of pregnancy test (Chapter 22).

The spermatozoa travel via the fluid-filled lumen of the seminiferous tubule through the rete testis into the efferent ducts leading to the epididymis. They are apparently swept along by fluid currents that result from absorption of fluids by the cells of the proximal part of the epididymis.

SPERM MATURATION WITHIN THE EPIDIDYMIS.⁶

Although spermatozoa entering the epididymis have well developed tails, they are not yet capable of either independent motility or fertilization. Further maturation takes place during the long, tortuous journey through the head (*caput*), body (*corpus*), and tail (*cauda*) of the epididymis.

Changes which can be detected in sperm leaving the epididymis include backward movement of a cytoplasmic droplet present at the time of entry, reduction in size and modification of the acrosome (more obvious in some species than in others), changes in tail organelles (which in some cases seem to make the sperm less flexible and less likely to swim in circles), and changes in properties of the plasma membrane. Modification of the nucleoprotein content of the head has been detected, and there are biochemical changes which have been associated with ability to become motile when exposed (on ejaculation) to higher oxygen tensions and to fructose present in seminal fluid.

It is not known whether epididymal cells simply provide an environment favorable for sperm maturation, or whether they contribute materials essential for the process. Sperm have been removed directly from the testis and incubated. Some mature in an apparently normal manner; but only small numbers survive and some are abnormal in appearance or unable to engage in fertilization.

Androgenic hormones are needed to maintain structure and function of the epididymis and are believed to act on the sperm cells as well. There are indications that an optimal level maintains functions while excessive concentrations are inhibitory to sperm maturation.

Testosterone is secreted mostly by the interstitial cells (although small amounts are formed by Sertoli cells). The Sertoli cells synthesize an *androgen-binding protein* (ABP) which binds the hormone with high affinity, aids in its concentration, and may also play a role in hormone transport. (It differs chemically from plasma proteins which bind the hormone.) Epididymal and interstitial cells contain enzymes which convert testosterone to metabolites that bind to ABP and may exert special actions. *Dihydrotestosterone* (DHT) has been directly implicated in maintenance of epididymal epithelial functions, while androstenediol may be important for the development of fertilizing ability of sperm cells.

Epididymal fluid contains high concentrations of glycerylphosphorylcholine. It was at first suggested that this substance is used as an energy source, but more recent work indicates that it accumulates because it cannot be metabolized by sperm (and possibly not by epididymal epithelium), and it has been implicated in either stabilization of the sperm or maintenance of the osmotic pressure of the fluid. Large quantities of sialic acid-rich glycoproteins are also present, and may play some role in reduction of friction between the spermatozoa. The spermatozoa within the epididymal fluid seem to depend heavily upon lipids and possibly amino acids as energy sources. High concentrations of carnitine found in some species aid the transfer of fatty acids to sperm mitochondria. Epididymal cells may also elaborate temporary inhibitors of motility. The fluid is a component of semen.

Time spent within the epididymis depends in part on the frequency of ejaculation; immature sperm can be removed by

repeated stimulation, while mature ones can be stored for a time. It is estimated that the average sojourn ranges between 14 and 23 days in humans; some investigators believe that the sperm deteriorate within days after that, while others maintain that they can survive in good condition for several months. Bats regularly retain sperm for several months.

Sperm that are stored too long lose the ability to participate in formation of normal zygotes (as judged by zygote survival and development in animals); later, ability to fertilize diminishes; with further deterioration, the potential for motility is lost. Finally, the sperm undergo morphologically demonstrable deterioration and die. Epididymal cells phagocytize the deteriorating cells, and may also play a role in selective destruction of abnormal sperm. Lysosomal enzymes within epididymal cells complete the destruction.

Spontaneous peristaltic contractions of smooth muscle components move the sperm through the epididymis. Cilia may also aid in transport of the sperm toward the cauda. The cauda is innervated, and participates actively in ejaculation.

SPERM TRANSPORT IN THE MALE TRACT^{1, 2, 8, 11}

Sperm leave the epididymis during ejaculation (or following spontaneous contractions) and pass via the long, tubular *vas deferens* into the *ampulla* in which they can be stored for short periods (Fig. 20-3).

The *ejaculatory duct* is a tube less than 1 inch long formed by the union of the ampula and the *seminal vesicle* ducts; it passes through the *prostate gland* to terminate in the genito-urinary *urethra*. In man and many other mammals, the seminal vesicles provide the bulk of the seminal fluid. (Contrary to previously held beliefs, it is unlikely that sperm enter the seminal vesicles.) Prostatic fluid reaches the urethra directly via small ducts.

Strong contractions of the ejaculatory duct and urethra propel the sperm and seminal fluid past the *Cowper's* or *bulbourethral* glands (which add a highly viscous secretion) through the penis. But at other times, some sperm from the ampulla enter the urinary bladder.

There are species variations in accessory structures. For example, while prostate glands

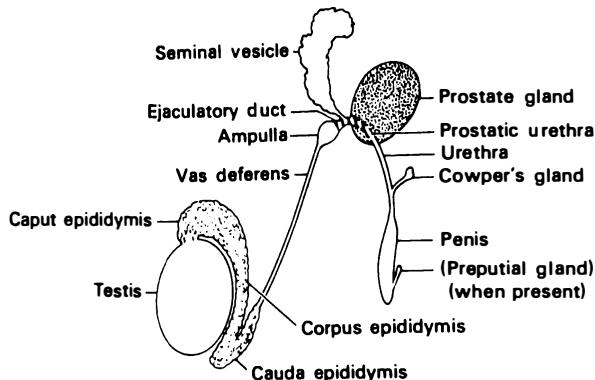


FIG. 20-3. Schematic representation of structures through which spermatozoa travel, and glands contributing to seminal fluid.

have been found in all mammals studied, the dog, ferret, whale, and others lack seminal vesicles. In rodents, *preputial glands* close to the penis are well developed, and may produce pheromones.

SEmen^{2, 5, 11, 15}

Semen is a mixture of seminal fluid and spermatozoa. The fluid provides a medium for movement of the large numbers of cells leaving the epididymis. Fructose is produced by seminal vesicles of man and many others, but it is formed in prostate glands of some species including rats. It provides the major energy source for most mammalian sperm cells, but a mixture of glucose and fructose is used by some species.

Prostatic secretions are rich in *citrates* which may protect against precipitation of calcium and other cations. Both calcium and citrate have been implicated in coagulation and subsequent liquefaction of semen; the latter contains *fibrinogen* and *thromboplastin* which participate in formation of a "plug" that increases vaginal retention of the semen. Citrate has also been implicated in activation of phosphatase and hyaluronidase.

Phosphate and bicarbonate buffers protect the spermatozoa against the acidity of the female tract. *Phospholipids* and *cholesterol* give the semen an opalescent appearance and contribute to the viscosity.

No satisfactory explanation has been offered for the ability of prostate glands to concentrate very large quantities of zinc and to transfer them to seminal fluid. The amount present exceeds by many-fold what is needed for activity of zinc-dependent enzymes. Some zinc may

be utilized by the early conceptus after it has entered the uterus. *Spermine* is present in human and some other semens; its function has not been established, but it has been used in the legal identification of semen.

Seminal fluid is rich in an assortment of *prostaglandins* which may promote motility of the female tract (Chapter 22). Also present are a variety of *amino acids*, *enzymes*, *choline*, and *vitamins*. Factors affecting capacitation (see below) have been identified, and others may inhibit sperm motility.

CAPACITATION²

Spermatozoa of most species are incapable of fertilization at the time of entry into the female tract. The process of maturation or *capacitation* is only partially understood. It may take place in two phases, the first in the uterus and the second in the Fallopian tube (oviduct). The estrogen-dominated uterus provides a favorable environment, while progesterone promotes creation of conditions which retard the reactions. (Tubular capacitation may be less strongly hormone-dependent.)

Seminal fluid seems to contain something which inhibits capacitation. An important function could be served, since capacitated sperm which do not engage in fertilization deteriorate rapidly.

It has been reported that when spermatozoa that have been capacitated are returned to seminal plasma they undergo a process of *deacquisition*, and that they can later be *recapacitated*.

tated if returned to the uterus. A substance with a molecular weight of less than 2,000 and resistant to procedures which destroy most proteins, has been identified in seminal plasma and has been given the name *decapacitation factor (DF)*. It is suspected that DF is a carbohydrate which is associated with a specific protein.

The described phenomena may have a different meaning. According to some investigators, seminal fluid selectively destroys the more delicate capacitated sperm, and recapsicitation is nothing more than activation of a new population of germinal cells.

Attempts to find DF in the seminal plasma of animals such as the rooster and dog, which lack seminal vesicles, have been unsuccessful. It is believed that rooster spermatozoa do not require capacitation; the need in dogs has not been established, but dog spermatozoa are unusual in that they retain ability to fertilize for up to 10 days after ejaculation.

Some authors note similarities between epididymal or freshly ejaculated (uncapacitated) and recapsicitated sperm while others state that they are very different.

HORMONE SYNTHESIS IN THE TESTIS^{2, 5, 16}

Most of the hormone synthesis is accomplished by the Leydig (interstitial) cells via the pathways described below. Steroids found in the seminiferous tubules are believed to arise partly from absorption and concentration of hormones synthesized by the Leydig cells; and concentration is facilitated by the presence of ABP (p. 247). Some limited steroid synthesis also seems to take place within the Sertoli cells, but this may be heavily dependent upon uptake of intermediates furnished by the Leydig cells.

By contrast with adrenocortical cells, the Leydig cells take up very little cholesterol from the blood plasma. The major hormonal substrate seems to be plasma-derived fatty acids which are converted first to acetyl coenzyme A, and then used to synthesize cholesterol.

All of the enzymes present in the zona fasciculata-reticularis of the adrenal cortex (with the exception of 11 β - and 21-hydroxylases) have been identified in the testis, and the same cofactors seem to be utilized. Pathways for synthesis of *pregnenolone* from cholesterol (Chapter 7) seem to be identical. Pregnenolone is the

most important intermediate for hormone synthesis.

It is possible that small amounts of cholesterol are directly converted to *dehydroepiandrosterone (DHEA, DHA)* via 17 α ,20 α -dihydroxycholesterol, bypassing pregnenolone formation (Fig. 20-4); but this is no longer considered to be a quantitatively important pathway. It is likely that most DHEA arises from 17 α -OH-pregnenolone.

Once formed, pregnenolone leaves the mitochondrion and can be converted to *pregnenolone-3 β -yl-sulfate*, to 17 α -OH-pregnenolone, or to *progesterone*. All three are secreted by the testis. In humans and many others, some is converted to DHEA and this, too, is secreted. Some species also secrete DHEA-sulfate. DHEA and its sulfate are "weak" androgens, i.e., target organs respond to very high doses in a manner qualitatively similar to that seen after administration of very much smaller amounts of testosterone.

Major pathways for synthesis of $\Delta 4$ -androstenedione and testosterone are shown in Figure 20-4. Minor pathways seem to involve similar actions of enzymes on sulfated derivatives, and androstanediol 3-sulfate, androstanediol 3,17-disulfate, androstanediol 17-sulfate, and testosterone 17-sulfate have all been identified in the testis and in spermatic vein blood. Developmental changes in testicular secretory products have been studied in some species. The fetal rat produces testosterone; during infancy it tends to put out small amounts of androsterone and 5-androstanediol; later, considerable quantities of androstanedione are released. The testosterone:androstenedione ratio rises sharply as sexual maturity is approached.

Androstanedione is more potent than DHEA but less potent in many ways than testosterone. (Relative potencies of androgens can be described only in terms of the test system used; thus, while androstanedione is only about 20% as effective on a molar basis as testosterone for promoting growth of seminal vesicles, it is almost 40% as effective on the prostate gland and actually more potent than testosterone when assayed on the capon's comb. By contrast, DHEA is almost as potent as androstanedione when tested on the prostate but has about 13% relative potency when tested on the capon comb.)

HORMONES AND REPRODUCTION

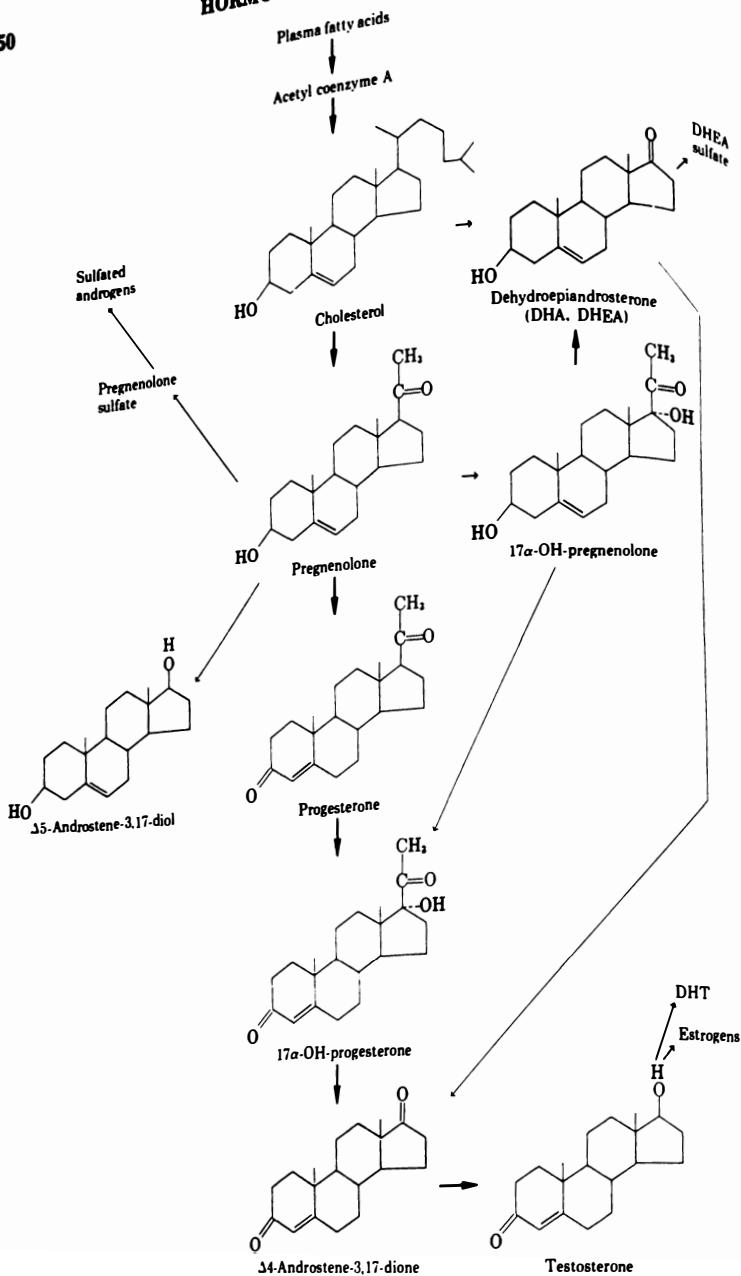


FIG. 20-4. Steroid hormone synthesis in the testis.

Both Leydig and Sertoli cells contain enzymes which convert a small fraction of the testosterone to dihydrotestosterone (DHT, see below) and even smaller amounts to estrogens. Very small quanti-

ties of both are released into the circulation.

One difficulty with determination of exactly what the testis synthesizes directly is that

epididymal activity contributes to products found in the spermatic veins.

Under normal conditions, up to 90% of the secretory product of adult testis may be testosterone. As with the adrenal cortex, congenital defects in steroid synthesis have been found. When the 17α -hydroxylase and 17β -ketosteroid dehydrogenase systems are affected, potent androgens cannot be produced, and this leads to development of pseudohermaphroditism in XY embryos.

METABOLISM OF TESTICULAR ANDROGENS^{8, 16}

Hormone Activation

Testosterone may function directly as a hormone when it acts on neurons, bone, and skeletal muscle. These structures respond directly to testosterone but not to some of its metabolites. It may also act directly on embryonic reproductive duct tissue.

Many testosterone-responsive tissues (including embryonic genital tubercle and

cloaca, as well as prostate glands, seminal vesicles, penis, skin, pituitary gland, liver, and parts of the hypothalamus) have a 5α -reductase which converts testosterone to 5α -dihydrotestosterone (DHT, 17β -hydroxy- 5α -androstan-3-one, Fig. 20-5). The tissues also contain a protein which binds DHT with high affinity; and they respond to direct administration of DHT.

A second metabolite is 3β -androstane-diol.

It has been suggested that testosterone functions as a prohormone in those tissues. There are indications that the two metabolites subserve different functions. For example, it has been reported that DHT preferentially promotes growth and mitosis of prostatic epithelium, while the diol promotes secretory activities. (The diol has also been implicated in sperm maturation in some species). Since testosterone is the precursor of both metabolites, the parent compound exerts both actions. DHT can serve as an intermediate for diol synthesis; but when administered alone, it is avidly taken up by binding protein. Testosterone

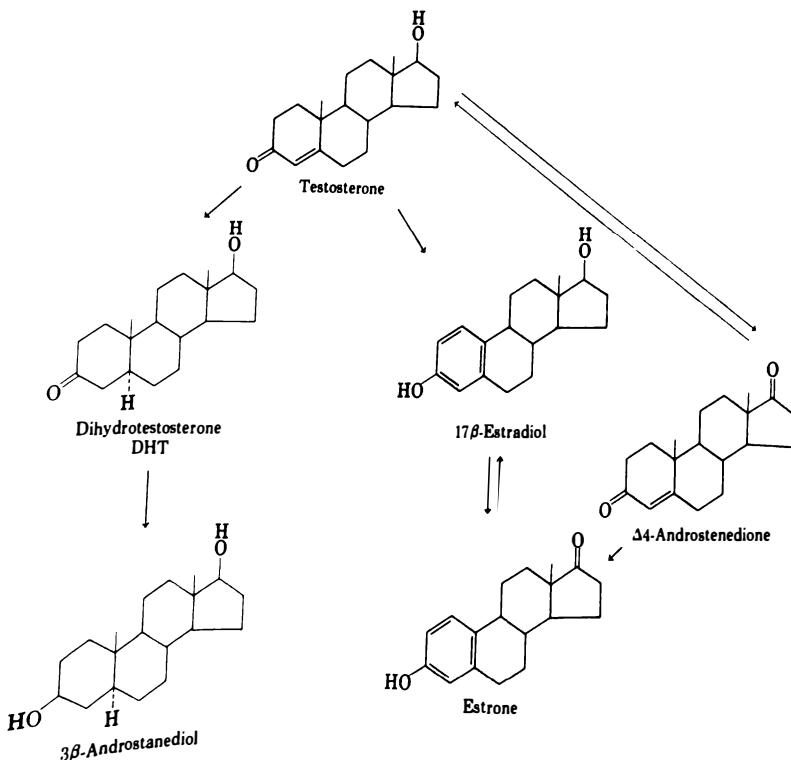


FIG. 20-5. Formation of "active metabolites" of testosterone.

HORMONES AND REPRODUCTION

(and androstenedione) can be converted to estrogens which may participate in "testosterone" actions.

One interesting concept recently introduced is that DHT formation may serve the function of increasing testosterone uptake by lowering its intracellular concentration and thereby enhancing the plasma:tissue gradient. Another (concerning which there is much controversy), is that testosterone may not function at all as a hormone; in tissues that do not respond to DHT, responses have been elicited with a combination of DHT and estradiol, and testosterone may simply provide the starting material for both. There is strong evidence for participation of both estrogens and androgens in regulation of sexual behavior in both sexes.

Formation of the metabolites is under hormonal control; but the control varies with different tissue types. In male accessory reproductive

structures, testosterone increases (while castration decreases) 5-reductase activity. Control seems to be exerted in the opposite direction in pituitary gland.

Hope has been raised that knowledge of testosterone metabolism may shed some light on the poorly understood phenomenon of prostatic hypertrophy which occurs commonly in humans, dogs, and apparently in lions, but not in many of the laboratory species. The incidence of prostatic hypertrophy in human males has been placed at 50% by the age of 50, and at close to 100% in those surviving to age 80. It is claimed by some observers that malignant transformations which are asymptomatic but can be demonstrated at autopsy, occur in a high percentage of males. The changes take place at a time in life when androgen secretion is either stabilized or declining, and when changes in hormone clearance rates can be demonstrated. One suggestion (based on experi-

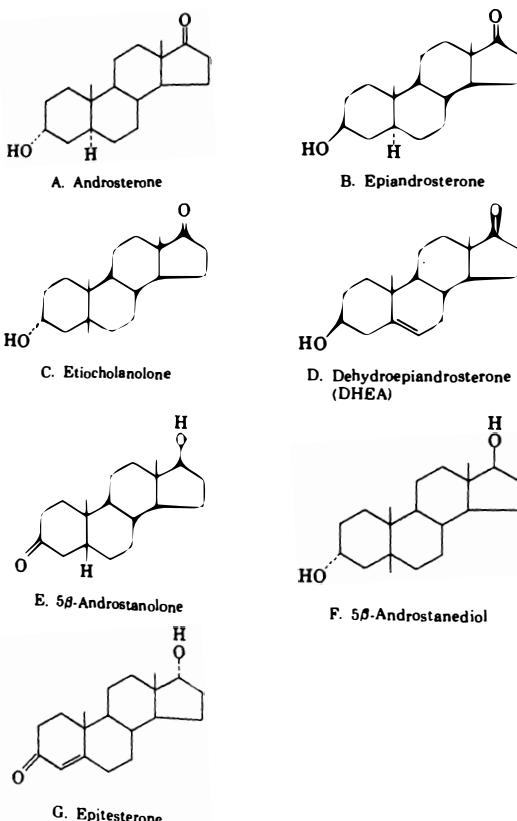


FIG. 20-6. Degradation products of testosterone. A, B, C, and D are 17-ketosteroids; G is a stereoisomer of testosterone, arising through androstenedione as an intermediate.

mental findings) is that there is an exceedingly high rate of conversion of testosterone to DHT in hypertrophied tissue, and that the disorder is related to accumulation of excessive quantities of the metabolite. Another point of view is that some regulatory mechanism which should be inhibiting both DHT formation and DHT effectiveness has gone awry. Animal species in which prostatic hypertrophy does not occur spontaneously, exhibit limited responses to administration of very large doses of DHT; similar observations have been made for other tissues, e.g., penis of species exhibiting prostatic hypertrophy. Definitive answers will have to be reconciled with other kinds of evidence pointing to high responsiveness of proliferating prostatic tissue to insulin.

Sex Hormones in Blood Plasma^{8, 10}

Testosterone and DHT of blood plasma bind strongly to a β -globulin (testosterone binding globulin, TeBG) which also binds estrogens, and has been named *sex steroid-binding globulin* (SSBG). It is clearly different from androgen-binding protein. SSBG is utilized in procedures for estimation of plasma concentrations of androgenic hormones.

Testosterone and DHT bind (but less strongly) to the plasma albumin fraction, and also to transcortin.

In males, a very high percentage of plasma testosterone arises from the testis; small amounts may be contributed by the liver. (In females, most testosterone takes its origin from hepatic transformation of adrenocortical hormone precursors.)

A considerable portion of the circulating DHT seems to arise from testosterone metabolism in "target" tissues and liver. Most of the plasma estrogen in males is of nontesticular origin; some arises within the liver, and some may come from target organs. It has recently been shown that considerable conversion of androgens to estrogens can occur in adipose tissue especially if the latter is abundant. In highly obese males, and in males with certain liver disorders, sufficient titers of estrogens can accumulate to promote marked breast enlargement.

Metabolic Degradation of Testosterone⁹

In the liver, testosterone is subjected primarily to three kinds of transformations: (1) oxidation at the 17 carbon, leading to production of 17-ketosteroids; (2)

reduction of the A ring; and (3) conjugation directly or after the other reactions, with glucuronide or sulfate. Conjugated steroids are more readily excreted in the urine than are the free steroids. But both free and conjugated steroids appear in the bile and urine.

Reduction of the A ring leads to formation of a number of stereoisomers. Some of the more important degradation products found in urine are shown in Figure 20-6. Some of the 17-ketosteroids are "weak androgens" but others are devoid of such potency. (Much of the 17-ketosteroid content of male urine, and almost all in females arises from degradation of adrenocortical hormones.)

Etiocolanolone does not have androgenic activity, but it can cause fever and has been implicated in some pathological conditions.

SYNTHETIC ANDROGENS

Agents Used for Testosterone-like Action

In addition to their obvious value as replacement therapy for hormone deficiency in the male, androgens have proven useful for a variety of purposes. They can suppress postpartum breast engorgement and lactation, are palliatives in treatment of inoperable breast cancer, and have sometimes been useful in treatment of menstrual disorders (especially dysmenorrhea) and frigidity. In some forms of inadequate sperm production, the "rebound" effect on secretion of gonadotrophins which follows temporary suppression by androgens has proven effective (see below).

Because of the efficiency with which the liver disposes of naturally occurring androgens, testosterone is not suitable for oral administration. Addition of an α -methyl group to the 17-carbon decreases the rate of hepatic degradation. *Methyl testosterone* tablets can be administered orally or sublingually. Unfortunately, methyl testosterone exerts inhibitory influences on secretion of bile, and prolonged administration may lead to development of jaundice and other liver disorders. *Fluoxymesterone* has largely replaced methyl testosterone because it is more potent and less likely to induce jaundice.

A variety of testosterone esters have proven useful for parenteral administra-

HORMONES AND REPRODUCTION

tion, because they have a longer duration of action than the native hormone, and some are more readily suspended in aqueous solutions. Those in common use include the *propionate*, *enanthate*, and *cypionate* of testosterone. They are widely used in experimental animals. Structures of these agents are shown in Figure 20-7.

by removal of the methyl group at the C-19 position. Agents in clinical use include 17 α -ethynodiol (norethandrolone, nilevar, Fig. 20-8A), nandrolone phenylpropionate (Fig. 20-8C), and oxymetholone (Fig. 20-8D). Methandrostenolone (Fig. 20-8B) has a C-19 methyl group, but high anabolic potency.

Anabolic Steroids

Under some circumstances it is desirable to take advantage of the protein anabolic actions of the sex steroids but to avoid the "masculinizing" effects. For example, the anabolic actions can be useful in treatment of patients convalescing from prolonged illnesses. Both stimulation of protein synthesis and stimulation of the appetite induced by the preparations are desirable. Similar androgens have been administered to premature infants to promote growth. The hormone analogs have found a place in treatment of elderly patients with bone fractures (Section IV).

Anabolic actions can be retained and androgenic or virilizing effects minimized

ANTIANDROGENS

Cyproterone and its acetate (Fig. 20-9) compete with testosterone and with DHT for binding proteins in target cells and therefore block androgen actions. They have been especially useful for production of experimental androgen deficiency uncomplicated by problems arising from gonadectomy. However, cyproterone acetate exerts progesterone-like actions.

There are also clinical needs for antiandrogens, for example in combating virilism in females with ovarian and adrenocortical disorders. No satisfactory hormone treatment is useful on a long-range basis for cancer of the prostate gland, but in cases where palliation can be achieved by castra-

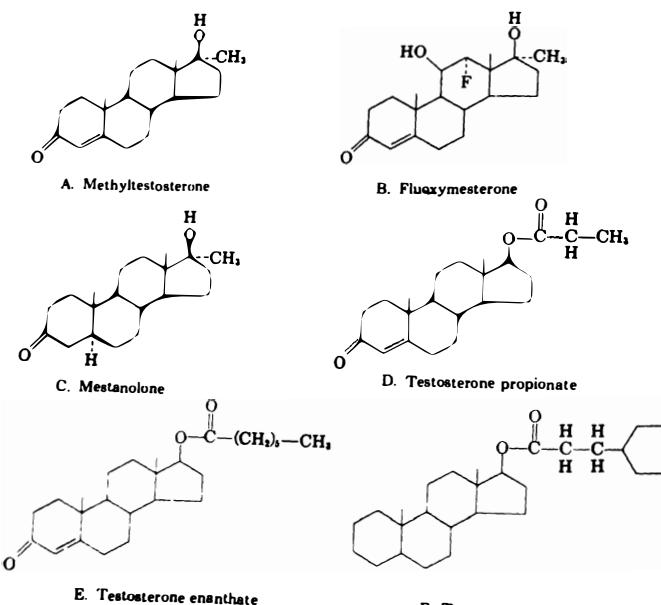


FIG. 20-7. Clinically useful synthetic androgens.

THE MALE REPRODUCTIVE SYSTEM

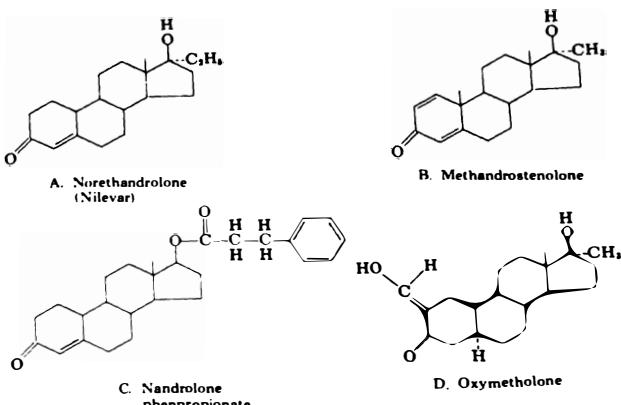


FIG. 20-8. Synthetic anabolic steroids related to testosterone.

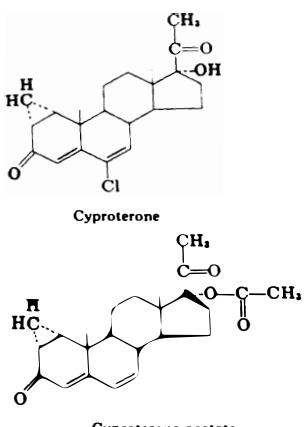


FIG. 20-9. Testosterone antagonists.

tion or estrogen administration, antiandrogens may prove to be the agents of choice.

MECHANISM OF ACTION OF ANDROGENS^{6, 14, 22}

It is not yet known whether common denominators exist between actions of androgens on embryonic differentiation, growth of muscle cells, hypertrophy, hyperplasia and secretory activity of accessory reproductive structures, inhibition of gonadotrophin secretion, involution of the thymus gland, and on sexual behavior. It is

possible that the actions exerted by the hormones find very different expression because of the properties of the target cells and susceptibility of the latter to simultaneous influences of other hormones.

Androgens readily penetrate plasma membranes and can be detected within the cells minutes after exposure to the hormone. Many of the reproductive tissues concentrate the hormones and retain them for long periods of time; and most target organs metabolize them.

An androgen binding protein (ABP) is synthesized in the Sertoli cells of the testis and is transported to the epididymis. It binds testosterone and DHT, but has been implicated in concentration of the hormones rather than in mediation of hormone actions.

As noted above, many tissues contain 5 α -reductase activity which rapidly converts testosterone to DHT. The enzyme has been found in both endoplasmic reticulum and in the nucleus.

A β -protein has been identified in prostate glands which avidly binds DHT but has little affinity for testosterone. It is clearly different from ABP, SSBG, and the α -protein described below. A two-step, temperature-dependent set of reactions has been described in which the DHT- β protein complex (sometimes called complex II) undergoes a change, and the modified complex is transported to the nucleus.

The situation is reminiscent of that described for estrogens (Chapter 21), but there are also differences. Estrogens do not require metabolic transformation before they are bound to cytoplasmic "receptor" protein. Moreover the estrogen-receptor complex becomes associated with chromatin and acidic nuclear proteins, and has been implicated in formation of new messenger RNAs as a result of the association. Complex II seems to bind preferentially to ribonucleoprotein particles within the nucleus. It may function differently. It has not been determined whether DHT itself is the active molecule (while the protein serves to efficiently concentrate and transport the steroid to the nucleus); there have been reports of direct influences of DHT on isolated nuclei. The protein itself may perform functions within the nucleus (and DHT may be needed to put it in the active form, to stabilize the active form, or to translocate it). Another suggestion is that the "cytoplasmic" form of the β -protein acts as a repressor, and that modification of those molecules which enter the cytoplasm leads to removal of the repressor from nuclear sites.

The connecting events have not been clarified, but it is known that within 1 hr after exposure of accessory reproductive structures to androgenic hormones, there is increased RNA synthesis which seems to be predominantly of the nucleolar type, associated with heightened RNA polymerase activity.

Since complex II does not preferentially bind to nucleoli, its influences may be indirect. Some androgen influences can be blocked with actinomycin D while others cannot; it has been proposed that the DHT-protein complex may affect sequestration of preexisting RNA polymerase, and thereby promote RNA synthesis. There are other reasons to believe that the complex in some way promotes formation or availability of new messenger RNAs. (Studies in which RNA extracted from androgen-treated tissues has been shown to stimulate protein synthesis in androgen-sensitive cells not exposed to the hormone have been interpreted in different ways by various observers.)

Some hours after accelerated RNA synthesis is apparent, isolated ribosomes have been shown to more rapidly utilize amino acids, and increased populations of ribosomes are seen within the cells. An associated elaboration of endoplasmic reticulum membranes has been described in seminal vesicles. There is also evidence for an increased rate of phospholipid synthesis.

Still later, mitochondrial protein synthesis is enhanced.

An α -protein has been described in prostate glands which binds several steroids including estradiol (which can arise from testosterone metabolism) and progesterone as well as androgens. (It does not bind glucocorticoids.) The α -protein cannot serve as a substrate for DHT transformation and translocation to the nucleus.

Certain tissues, e.g., prostate glands, exhibit a delayed stimulation of cell proliferation, while others, e.g., skeletal muscle, respond only by increases in cell size, protein, and RNA content. Available data do not provide much information on mechanisms leading to cell replication. One possibility is that certain kinds of cells will undergo mitosis without further hormonal stimulation when they have been suitably "prepared" by cytoplasmic events (while others lack machinery for such responses). Another possibility is that androgens exert more direct influences on cell replication, but only after the cells have been made ready to respond.

Stimulation of protein synthesis in muscles responsive to testosterone is accompanied by increased rate of uptake of some amino acids. Increased uptake is not observed until several hours after hormone administration. Therefore, it probably represents a secondary effect, in response to increased utilization of cytoplasmic amino acids.

Evidence has been presented for synergism of testosterone with hormones known to promote protein synthesis. Both insulin and prolactin have been shown to be important.

Reasons for conflicting data on whether androgens stimulate adenylate cyclase are not immediately apparent. Administration of D-cAMP in combination with theophylline can induce changes in carbohydrate metabolism similar to those seen after androgen administration; there have been reports that effects on RNA synthesis (which can be blocked with actinomycin D) can also be achieved.

Transient reduction of ATP content followed by a secondary rise has been reported and confirmed. The observations are consistent with rapid ATP utilization for synthesis of mac-

romolecules, followed by delayed formation of metabolic machinery for more rapid ATP synthesis. Direct stimulation by androgens of sodium-potassium ATPase has also been proposed. There are conflicting interpretations concerning observations that androgens promote incorporation of ATP phosphate into regulatory proteins (including histones).

Concentrations of spermine and spermidine have been observed to increase when androgen treatment of accessory reproductive tissues is maintained for 2 days or longer. Since no significant elevations were found earlier, it seems unlikely that such molecules can serve as "second messengers."

Regression of reproductive glands following hormonal withdrawal involves something more than simple removal of a stimulant. There is time lag, and it seems probable that new proteins involved in degradation are synthesized. Involution can be retarded with agents that stabilize lysosomes. This raises the possibility that the machinery for degradation is ever present but is inhibited by the hormones.

REGULATION OF TESTOSTERONE SECRETION^{10, 11, 14}

Role of Luteinizing Hormone (LH):

During puberty and afterward, Leydig cells are tonically stimulated by pituitary *interstitial cell-stimulating hormone* (ICSH) which is similar to or identical with luteinizing hormone (LH) described in Chapter 21.

There is indirect evidence for a prenatal role of LH. The pituitary gland becomes active in the fetus, and fetal cells show responsiveness to the hormone.

Anencephalic and experimentally decapitated XY fetuses exhibit atrophic interstitial tissue and disorders of sex differentiation; but of course they are lacking much more than LH.

Fetal cells are probably also stimulated by chorionic gonadotrophins (Chapter 22) present throughout gestation. The fact that human infants exhibit Leydig cell hyperplasia and increased secretory activity just before and for a short time after birth has been taken as evidence that the influence of the chorionic gonadotrophins is important. The chorionic gonadotrophins seem

to utilize the same hormone receptors as LH, and exert qualitatively similar actions. But there are those who believe that the greater stimulation of interstitial cells which can be achieved with chorionic hormones has some explanation other than longer duration of action.

Full appreciation of the roles of gonadotrophins requires understanding of the target cells. Fetal cells of rats seem to acquire maximal LH sensitivity, and to produce the largest amounts of testosterone around the 19th day of a 21-day gestation, and to regress afterward. Interstitial cells of the infant testis respond to gonadotrophins by hypertrophy; but they produce little testosterone until the time of puberty is approached.

It is believed by some that a population of fetal interstitial cells follows a genetically programmed developmental pattern during which transient sensitivity to LH (or chorionic gonadotrophin) is attained. The cells then die off, to be replaced by an entirely new set of Leydig cells which are also genetically programmed and must acquire a certain state of maturity before they can secrete substantial quantities of testosterone.

The observation that human infants show Leydig cell hyperplasia around the time of birth, but do not exhibit substantial regression until 4-6 weeks after loss of chorionic gonadotrophin influence, may have a similar explanation.

Sources of LH

LH-secreting gonadotrophs are described in Section VII. They have been identified by immunological procedures, and by histological changes resulting from disruption of normal hormonal balance. Under physiological conditions they are subjected to negative feedback control which depends upon testosterone, and positive control from the hypothalamus. Castration can lead to enlargement, vacuolization, and transformation into "signet-ring" or "castration" cells.

The question of whether there are distinct *interstitiotrophs*, morphologically and functionally different from gonadotrophs which secrete follicle-stimulating hormone (FSH), is discussed in Section VII.

Actions of LH

Trophic influences of LH on the Leydig cells clearly involve stimulation of new protein synthesis, as enzymatic machinery for steroid hormone synthesis is augmented. Additional influences on steroid hormone synthesis may be accomplished via different mechanisms.

An action of LH on conversion of cholesterol to pregnenolone seems well established. It can be demonstrated even when administration of puromycin blocks LH stimulation of rate of incorporation of amino acids into new proteins. Additional data point to influences of the hormone on conversion of acetate to cholesterol and on enzymes involved in the pathway between pregnenolone and testosterone.

Actions of LH on Leydig cells may be similar to those of ACTH on adrenocortical cells (Chapter 8). LH stimulates testicular adenylate cyclase, and many actions can be mimicked by administration of D-cAMP.

Most influences of LH on the reproductive system result from stimulation of testosterone secretion by the Leydig cells. But LH also affects the Sertoli cells, and may play a direct role in spermatiation. In addition, it affects the secretion of hypothalamic hormones. Large doses promote lipolysis in adipose tissue, but this probably has no physiological significance.

One method for bioassay of LH depends on the ability of the hormone to promote darkening of the feathers of the weaver finch, an influence which can be elicited in castrated animals and is therefore not related to stimulation of testosterone secretion.

Luteinizing Hormone-releasing Factor (LRF)^{4, 21}

Adenohypophyseal cells secreting LH require stimulation by a hypothalamic hormone for maintenance of structure and secretory functions. The hormone reaches the pituitary via the hypothalamo-hypophyseal portal system (Section VII); ordinarily, little gains access to the systemic circulation. When the pituitary gland is removed from hypothalamic influences and transplanted to another site within the same animal, the LH-secreting cells

undergo atrophic changes, and LH secretion falls to very low levels.

Ectopic pituitaries receive some LRF stimulation, because loss of feedback inhibition (see below) leads to increased LRF secretion and some of the hormone enters the peripheral circulation. (The very low levels of LH can be even further reduced if LRF influences are removed.)

Pituitary cells maintained in tissue culture respond to LRF in hypothalamic extracts by increasing synthesis and release of LH into the medium. Suitably purified hypothalamic extracts can selectively promote LH secretion (without influencing secretion of TSH, STH or ACTH) when administered to intact animals. Similar increases in LH secretion can be elicited by stimulation of the *arcuate-ventromedial* region of the hypothalamus (believed to contain LRF-secreting cells) or the *suprachiasmatic* region (believed to contain neurons that synapse with and carry stimulatory impulses to the cells that secrete LRF). Destruction of the *arcuate-ventromedial* area reduces LH secretion and leads ultimately to decline in testosterone output and atrophy of the Leydig cells.

A decapeptide having the structure shown below has been isolated from ovine and porcine hypothalami, and the peptide has also been synthesized.



Because of its small size, it is suspected that it is synthesized by cytoplasmic enzyme systems not directly dependent upon ribosomes.

Both the naturally occurring and synthetic peptides are highly potent stimulators of LH secretion. The names luteinizing hormone release factor (LRF) and luteinizing hormone-releasing hormone (LRH) have been given to the peptide. However, since it also promotes release of follicle-stimulating hormone (FSH), it is also known as *FSH-RH/LH-RH*.

The question of whether the hypothalamus secretes a single release factor for the two gonadotrophins (and therefore what is released is determined by prevailing hormonal conditions), or whether there are perhaps three distinct hypothalamic hormones, one each for FSH and LH and one affecting both (or two hypothalamic hormones, one acting on one gonadotrophin and the other affecting two) has not been answered.

It has been reported that preferential release of LH results from stimulation of the arcuate region, while preferential release of FSH can be achieved by stimulation of the paraventricular hypothalamic nuclei, and that prevailing levels of steroid hormones (especially of estrogens) affect the nature of responses to administered LRF.

Analogs of the decapeptide have been prepared. Some behave as weak LRFs and others as LRF antagonists; but no one molecule is known which promotes selective release of only one of the gonadotrophins.

There are also unsettled questions about whether LRF controls both gonadotrophin release and gonadotrophin synthesis.

LRF promotes prompt gonadotrophin release, and this can be accomplished when puromycin blocks new hormone synthesis. The fact that *depletion* of LH stores has not been demonstrated despite repeated attempts, has been interrupted in several ways. One is that LRF promotes configurational changes in LH molecules and thereby increases *apparent* LH content (as usually measured) while it may be reducing total LH.

There is strong evidence that LRF promotes LH synthesis, and that it can elevate total LH content of the pituitary gland. The possibility that LRF-stimulated release of stored hormone leads secondarily to hormone synthesis has not been ruled out.

Influences of LRF on the pituitary seem to be mediated via activation of adenylyl cyclase. Requirements for calcium and potassium in the medium have been shown. LRF may induce depolarization of the pituitary cell membrane. High (supraphysiological) concentrations of potassium depolarize the LH-cell membrane and promote LH secretion. Some studies indicate an inhibitory influence of prostaglandins on responses of the cells to both potassium and LRF stimulation.

There are controversies concerning whether LH secretion is associated with extrusion of secretory granules. The cells are not readily degranulated under conditions in which LH secretion is markedly stimulated.

LRF secretion is affected by many fac-

tors (Chapter 22). It is reduced in response to certain forms of "stress," and such reduction may play a role in the reduced fertility seen when animals are well fed but crowded together.

Light and temperature are certainly important for regulation of LRF secretion in seasonal breeders, and some influences are mediated via the pineal gland (Chapter 25). But additional seasonal factors may operate; it is well known that laboratory rodents tend to exhibit low fertility in January even when temperature, lighting, and humidity are controlled.

There are several reasons for believing that testosterone secretion is influenced (via the hypothalamus) by psychological factors. In some cases of impotence related to emotional problems, testosterone secretion is low; and it may be elevated following alleviation of the psychological disturbance.

It was reported that beard growth (a presumed indicator of testosterone secretion) can be accelerated in male members of the armed forces who are isolated from women, when such men are furnished with photographs and other stimuli which arouse thoughts of women. (Men not given the stimuli were used as the basis for comparison.)

Dopamine has been implicated as the neurotransmitter promoting LRF release. Stimulatory influences of high (but not of moderate or low) doses of norepinephrine and epinephrine have been reported, while serotonin seems to be inhibitory in females.

Feedback Mechanisms Regulating the LRF-LH-Testosterone System^{2, 17}

Castration leads consistently to elevation of plasma LH concentrations and to morphological changes in LH-secreting cells indicative hyperactivity. The changes can be prevented by administration of testosterone to castrates. Chronic administration of large doses leads to reduction of LH levels in plasma and to morphological changes in LH cells consistent with reduced secretory activity.

Inhibitory influences of testosterone are believed to be exerted largely on the hypothalamus. Implantation of testosterone

into appropriate hypothalamic sites leads to reduced LH secretion and secondarily to lowered testosterone output.

The problem with such studies is that it is virtually impossible to rule out leakage of testosterone (via the portal circulation) to the pituitary gland. However, it has also been shown that pituitary glands transplanted ectopically and responding to LRF in the systemic circulation show reduced activity when testosterone is implanted into the hypothalamus.

Testosterone also seems to directly affect pituitary cells. Unilateral implants of the hormone into the pituitary gland were shown to reduce LH secretion on the side of the implant only; and reduced responsiveness to LRF has been shown *in vitro*.

Inhibitory influences of testosterone have been utilized in human fertility control. It has been observed that some men producing inadequate numbers of apparently normal sperm cells reduce LH secretion in response to testosterone administration. When the steroid is later withdrawn, there is a "rebound" hypersecretion of LH of sufficient magnitude to favorably influence spermatogenesis.

Another application is for reduction of fertility; it is possible to chronically administer sufficient androgen to inhibit LH secretion while maintaining secondary sex characteristics and metabolic functions. The lowered LH leads to *localized* reduction of testosterone within the seminiferous tubules and thereby reduces sperm maturation. Testosterone itself is not useful for such procedures. A problem which arises, at least in some species, is that excessive systemic doses of androgens can *directly* stimulate the testis.

There are indications that high levels of LH may inhibit LRF secretion via a "short feedback loop"; implantation of LH into the median eminence has been reported to reduce LH output. The existence of an "ultrashort feedback loop" for FSH raises the possibility that LRF may inhibit its own secretion.

Testosterone secretion may also be affected by local factors acting within the testis, e.g., accumulation of pregnenolone and progesterone may inhibit enzymes of the steroid pathway. The inhibitory compounds are also secreted by the adrenal cortex and could be involved in "stress"

inhibition of gonadal function. Established and proposed influences on testosterone secretion are summarized in Figure 20-10.

HORMONAL REGULATION OF SPERMATOGENESIS^{11, 18, 19}

The problems associated with investigation of hormonal control of spermatogenesis are formidable. The hormone most obviously involved is testosterone; since it is produced in the same organ in which spermatozoa are formed, there is no simple way to remove it to study deficiency states. Testosterone antagonists (e.g., cyproterone acetate) are effective only when they can gain access to hormone receptor sites; moreover, they can exert actions unrelated to testosterone antagonism.

Gonadotrophin deficiencies cannot be easily induced by removal of the pituitary gland since multiple defects are thereby created which cannot easily be overcome through selective hormone administration. Antibodies to gonadotrophins are useful, but they can affect only the hormone with which they manage to combine, and cross-reactions can be troublesome.

Available gonadotrophin preparations are contaminated with unknown substances as well as with traces of other hormones. There is the problem of getting them to the desired sites in the right quantities. And bioassays for potency are not completely satisfactory. Moreover, many different cell types are involved in spermatogenesis. It is extremely difficult to make quantitative measurements of all of the types.

Role of Testosterone

Germinal epithelium does not develop when there is inadequate testosterone. If the hormone is withdrawn after spermatogenesis has become established, the seminiferous tubules undergo atrophy, and spermatogenesis comes to a halt.

When spermatogenesis is established in the laboratory rat, and the animal is subsequently hypophysectomized, the processes can be maintained at approximately 80% of normal levels by injections of testosterone alone. However, if testosterone injections are delayed after hypophysec-

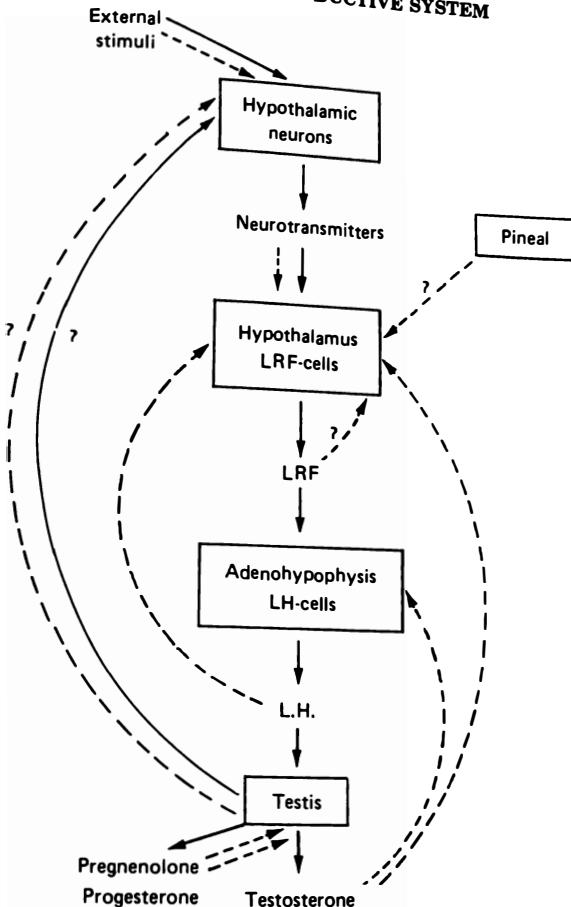


FIG. 20-10. Factors influencing secretion of testosterone, LH, and LRF. Broken arrows, inhibition; solid arrows, stimulation.

tomy, so that the testis undergoes atrophy, then spermatogenesis cannot be reestablished with testosterone alone. Unlike rats, primates do not maintain spermatogenesis when testosterone treatment is instituted immediately after hypophysectomy.

Gonocytes form in the embryo, replicate, and migrate to the gonad before testosterone-secreting cells develop; and they can continue replication without the hormone. But there is evidence that testosterone promotes maturation of the gonocytes into Type A spermatogonia. If this is so, it can

be appreciated that removal of the hormone leads to depletion of precursors for all later stages of spermatogenesis.

Type A spermatogonia seem to be capable of replication, progression to type B spermatogonia and to primary spermatocytes, and initiation of prophase of the first division without steroids, but stimulatory influences of STH on these processes have been described. Completion of meiosis beyond prophase seems to be again under the control of testosterone, and the steroid may be needed for certain stages of spermiogenesis.

Follicle-Stimulating Hormone (FSH)^{1, 10}

The chemistry of FSH is presented in Chapter 22, and cells which secrete it are described in Section VII.

Early development of the testis takes place before the pituitary gland secretes hormones. Small amounts of FSH may be secreted late in fetal life, and low levels can be detected in the plasma of young, rapidly growing animals; it is likely that the hormone contributes to prepubertal testicular development.

It is generally agreed that FSH is needed for growth and maturation of the testis in preparation for spermatogenesis, and for repair and restoration if posthypophysectomy regression has taken place. It also seems to be needed for the late stages of spermogenesis.

FSH binds principally to Sertoli cells (and to a lesser extent to spermatogonia and spermatocytes); it does not seem to be specifically taken up by interstitial tissue.

A specific receptor which seems to be a protein has been identified in Sertoli cell membranes. Hormone binding is soon followed by activation of Sertoli cell adenylyl cyclase, and hormone actions seem to be mediated via cAMP. Specific cAMP-dependent protein kinases have been identified in the testis. They can be activated by FSH treatment even in the presence of inhibitors of protein synthesis.

When no inhibitor is present, FSH promotes a delayed increase in protein synthesis which has been attributed to influences on RNA synthesis. The action can be blocked with actinomycin D and does not seem to involve stimulation of either new ribosome formation or amino acid transport through the plasma membrane.

Ability of FSH to augment protein synthesis is dependent upon age and other conditions associated with target cells. In the rat, maximal protein synthesis response is seen only during infancy (ages 16–22 days), or after hypophysectomy has led to atrophy of the seminiferous tubules. A specific phosphodiesterase isozyme has been identified in mature testis, and has been directly implicated in reducing effectiveness of FSH for promoting protein synthesis. FSH also exerts delayed influences on lipid synthesis.

Many FSH actions cannot be elicited in

the absence of testosterone. A major function of FSH may be induction of the ABP needed to achieve localized high concentrations of testosterone. FSH has also been directly implicated in promoting Sertoli cell synthesis of regulators of gonadotrophin secretion which seem to include estrogens (see below), and in exerting influences on testosterone secretion by the interstitial cells.

Spermatogenesis seems to be enhanced by FSH. This has been attributed to reduction of the rate of degradation of type A spermatogonia. The effect may be indirect, via the Sertoli cells which enlarge and increase their secretory activity under the influence of the hormone.

Pituitary hormones, and especially STH and prolactin, may synergize with growth promoting actions of FSH. These hormones may also synergize with testosterone.

Luteinizing Hormone¹²

The most important action of LH seems to be that of maintaining testosterone secretion by the Leydig cells. In some species, it does not seem to be required for spermatogenesis if sufficient testosterone is administered.

LH has been implicated in maintaining localized concentrations of the steroid in the Sertoli cells, possibly through influences on ABP formation. In humans and primates there are indications that it plays additional roles in maintenance of Sertoli cells and in regulation of testicular blood flow.

It is not known whether influences on spermatiation are secondary to elevation of testosterone concentrations.

Growth Hormone and Prolactin

These hormones may play an ancillary role in maintenance of accessory reproductive structures in addition to the influence on the testis mentioned above. It is possible to achieve full spermatogenesis and sperm maturation in hypophysectomized animals with a combination of FSH, LH, and testosterone; but this does not rule out functions of the pituitary protein hormones in the intact animal. Evidence for influences on the prostate gland is strong.

REGULATION OF FSH SECRETION^{11, 12, 13, 14}

Castration leads to increased secretion of both FSH and LH. Administration of testosterone in moderate dosage effectively returns plasma and pituitary LH levels to presurgery levels but has little influence on FSH hypersecretion. Chronic administration of supraphysiological doses of testosterone can, however, inhibit FSH secretion in castrates.

When animals are hemicastrated, compensatory hypertrophy of the remaining testicle seems to be directly dependent upon the rise in FSH secretion, and the latter falls off as hypertrophy nears completion.

There seems to be a quantitative relationship between the degree of suppression of spermatogenesis with pharmacological agents, radiation, or testicular ischemia, and the magnitude of elevation of plasma FSH concentrations. A decline in levels of the pituitary hormone is associated with withdrawal of the suppression and recovery of spermatogenesis. Cryptorchism also leads to increased FSH secretion.

During prepubertal maturation in several species, FSH levels tend to fall as spermatogenesis gets under way. In humans, oligospermia and azoospermia are associated with high plasma FSH, whether or not the testosterone and LH concentrations are within the normal range.

All of the preceding are consistent with production of something by functioning seminiferous tubules which exerts negative feedback control over FSH secretion. The hypothetical substance involved was given the name *inhibin*; according to some observers it is a peptide produced by Sertoli cells. When repeated attempts to isolate inhibin proved unsuccessful, alternate hypotheses were formulated.

One concept is that *FSH* is "used up" by seminiferous tubules engaged in spermatogenesis, and that the high FSH levels in blood and urine following damage to or removal of the tubules result from failure to metabolize the hormone. The concept is not widely accepted because it fails to explain histological changes in the pituitary gland which indicate accelerated FSH synthesis and release. Moreover, no evidence has been presented that the tubules

actually do metabolize significant quantities of FSH. The concept has also been rejected by some on the basis that endocrine glands in general do not seem to effect trophic hormone regulation in this way.

A concept more widely accepted is that Sertoli cells actively engaged in spermatogenesis (and, in association with testosterone-producing interstitial cells), produce and release sufficient quantities of *estrogens* to inhibit FSH secretion. Injections of estrogens can suppress FSH secretion in castrate males; and decreased estrogen levels has been described in the plasma of some patients with inadequate sperm production.

It has been proposed that estrogen secretion by the Sertoli cells can be stimulated by FSH. Another concept is that the estrogen secretion is triggered by Sertoli cell ingestion of sperm cell residual bodies (p. 246); but this is not universally accepted. Yet another variation of the concept places estrogen formation at the level of spermatozoa emerging from the Sertoli cells.

Objections to the estrogen concept include statements that there is insufficient estrogen released by Sertoli cells to effectively suppress FSH secretion in normal males, and that the amounts that must be given to castrate males are supraphysiological. It has also been pointed out that most of the estrogen in males arises from nontesticular sources, and the latter may not be affected by seminiferous tubule damage.

Recently an antigenadotrophin isolated from rete testis fluid has been described. The inhibin concept is still believed by some observers to offer the most reasonable explanation. (See also p. 289.)

PUBERTY¹⁰

Puberty is usually defined as the developmental stage in which individuals first become capable of reproduction. In the male this is equated with the earliest appearance of mature spermatozoa. It is preceded by a juvenile period in which reproductive structures appear dormant, and is followed by a later stage in which a spurt of somatic growth leading to attainment of almost adult body size is accompanied by maturational changes in the primary and accessory reproductive structures, sec-

ondary sex characteristics, and the central nervous system.

During infancy, responsiveness of the reproductive system to stimulation is minimal. But this is followed by a period in which reproductive structures and the pituitary gland are capable of precocious maturation. The most widely accepted concept for the normal delay of puberty is that the hypothalamus is very sensitive to negative feedback influences exerted by the very low levels of steroid hormones secreted during childhood, and

that a change in hypothalamic sensitivity leads to the onset of puberty.

The timing of puberty is genetically controlled, but is subject to a wide range of influences including nutritional status and stimuli to the central nervous system. Precocious or delayed puberty can be induced by appropriate manipulations of the hypothalamus.

More is known about the onset of puberty in females than in males, and the subject is discussed in greater detail in Chapter 22.

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21. The Female Reproductive System

SOME COMPARISONS WITH THE MALE SYSTEM

Despite obvious differences, there are many similarities between female and male reproductive systems. Ovaries and testes develop from "indifferent gonads" which are morphologically indistinguishable in very young male and female embryos (Chapter 19). Both are comprised of coelomic epithelium and mesenchymal derivatives, and they contain gonocytes or germ cells which migrate in from the yolk sac. They undergo a period of rapid differentiation which involves interaction between germinal and nongerminal components. This is followed first by a relatively quiescent phase, and later by gonadotrophin-stimulated prepubertal development in preparation for production of mature gametes. Electron microscopy has revealed numerous similarities between ovogenesis and spermatogenesis.

Epithelial components of the gonad become intimately associated with the gonocytes. The epithelial cells inhibit early maturation of the germinal cells, and later provide nutrients for their development. Mesenchymal components of the gonad give rise to interstitial cells which secrete steroid hormones needed for gamete maturation, growth and function of accessory reproductive structures, emergence of secondary sex characteristics, and modulation of sex behavior.

The steroids are synthesized along similar biochemical pathways. The mature testis puts out mostly testosterone but also releases small amounts of progestagens and estrogens, and additional estrogen is formed peripherally. The ovary secretes mostly estrogens and progestagens, but also small amounts of androgens; additional androgen is formed peripherally. A combination of estrogens and androgens affects behavior in both sexes.

Parallels have been drawn between spermatiation (in which incompletely matured spermatozoa are passively propelled into a fluid-filled cavity to start their journey through accessory reproductive structures) and ovulation in which cells that will become ova are extruded to enter the fluid-filled oviducts.

Gonadotrophins which are identical or nearly so in the two sexes are produced by morphologically similar gonadotrophs of the pituitary gland. They influence gamete development, steroidogenesis, and gamete passage through the accessory structures. Gonadotrophin secretion is regulated by gonadal steroids and by hypothalamic hormones, and is influenced by stimuli which affect hypothalamic activity.

THE OVARIES^{1, 2, 8, 12, 14}

Prepubertal Oogenesis and Development of Follicles

Proliferation of Germ Cells. As noted in Chapter 19, differentiation of the ovary occurs later than emergence of a definitive testis. Delayed association of gonocytes with epithelial cells permits a longer period of mitotic proliferation. It has been estimated that the 1,000-2,000 germ cells which migrate to the human embryonic ovary give rise to about 600,000 cells by the end of the second prenatal month. When they become committed to the female pattern of development, they are known as oogonia.

Some germinal cells continue rapid proliferation and attain greatest numbers during weeks 8-20; others mature into primary oocytes (*primary ovocytes*), a process which is evident by the 15th week and reaches a peak during weeks 20-28. Still others degenerate and disappear.

Maximal germinal cell population (estimated at 6-7 million) is achieved by the end of the 5th month; of these about 5 million are primary oocytes. From the middle of the fetal period to the time of menopause, the number declines. By birth all gonocytes and oogonia have disappeared from the human ovary, and the number of primary oocytes is between 700,000 and 2 million. The number surviving at the time of puberty has been estimated by various authors as between 40,000 and 400,000.

In addition to up to 3-fold *individual variation*, differences in estimates are partly attributable to use of different techniques for measurement and to the fact that some authors

HORMONES AND REPRODUCTION

count all germinal cells while others disregard those that are obviously degenerating.

A total of about 8,000 surviving oocytes eventually undergoes partial maturation, but only 400-500 ever become potential egg cells (ova). At menopause the ovary may be completely depleted of viable germinal elements (but descriptions of menopausal ovaries by various authors differ). Degeneration may be a selective process in which large numbers of abnormal cells are discarded.

Comparable changes take place in the ovaries of other mammals, but the time-tables are different, the numbers of gonocytes formed bears some relationship to the size of the ovaries, and the numbers of ova eventually reaching maturity usually show a relationship to litter size.

The rat is born after a gestation period of only 21-23 days, and maximal ovocyte population is achieved about 3 days after birth. A potentially fertile but unmated female may experience 150 estrous cycles in which sufficient oocytes mature for production of litters of up to 20 pups each. The hamster, which is born after 16 days, develops most of its oocytes after birth. The rabbit has a 32-day gestation period, but ovaries exhibit developmental changes after birth comparable to those occurring prenatally in many of the smaller animals.

In some of the prosimians (bushbabies, lorises), oogonia persist postnatally and provide stem cells for new oocytes. In certain other mammals (e.g., the domestic cat) oogonia have been recognized in ovaries of older animals but they are not believed to remain functional.

Formation of Primordial Follicles. Cytoplasmic bridges between primary spermatocytes have been described in Chapter 20. Similar bridges are formed between primary oocytes, and connected cells tend to undergo synchronous development. They soon become associated with *prechondrocytic cells* which arise from epithelial components of the ovary. The primary oocytes enter into the prophase of the first meiotic division. When they reach the late pachytene or diplotene stage, they separate.

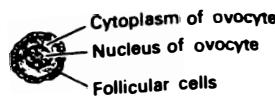
Individual germinal cells then become surrounded by epithelial cells which are now known as *follicular* or *granulosa cells*. The ovocyte and its surrounding cells comprise the *primordial follicle* which is said to be *unilaminar* since there is a single layer of epithelium (Fig. 21-1A). The granulosa

cells will later rest on an acellular basement membrane (lamina propria) containing collagenous fibrils.

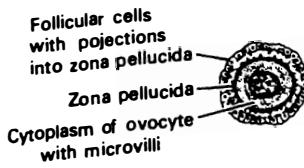
Most of the follicles lie near the periphery (cortex) of the ovary, separated from each other by stromal cell derivatives of the embryonic mesenchyme. The distribution probably results from selective destruction of germinal cells in the more central (medullary) regions. Occasional primordial follicles containing two or more oocytes have been described and the incidence is said to be higher in hamsters than in most others.¹

Large numbers of follicles and their oocytes degenerate (undergo atresia) and disappear during late fetal life and early childhood, and the process continues until maturity and beyond.

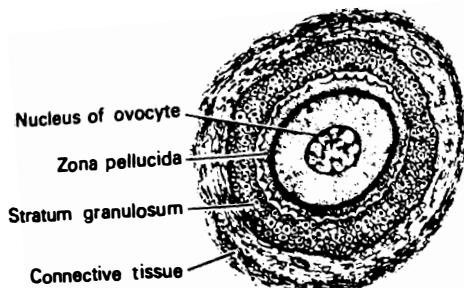
Meiosis.^{1, 2, 3} The stage of meiosis at which development becomes arrested seems to vary with the species; but it does



A. Primordial follicle



B. Early unilaminar primary follicle



C. Multilayered primary follicle
FIG. 21-1. Development of primary follicles.

not proceed as far as diakinesis in any known case. The term *dictyate* is applied to the "resting stage" characteristic of certain rodents; on the basis of morphological differences and the greater susceptibility of rodent primordial follicles to radiation injury, it is believed that meiosis is arrested at a slightly earlier (diplotene) stage in primates.

Starting at puberty, certain oocytes resume cell division while others remain "dormant." In humans (in which ovarian cycles have been known to continue into the 6th decade), cells which mature toward the end of the fertile period do so after 40 or 50 years of "arrested development."

There is something special about the "resting stage" which favors cell survival; cells which proceed beyond that point have a very limited life span. But it is suspected that degenerative changes occur if the "dormant" period is excessively prolonged. Certain kinds of chromosomal aberrations of the zygote occur with high frequency when women are more than 42 years old at the time of conception, and the incidence rises if fertilization occurs still later. (An alternate possibility is that reduction of available oocytes with advancing age favors development of greater proportions of abnormal cells.) Parallel changes have not been found in aging males in which spermatocytes undergo full development soon after formation. Follicular cells probably play a key role in maintenance of the "dormant" condition. Oocytes separated in culture from granulosa cells rapidly resume meiosis.

Formation of Primary Follicles. Although the ovocyte is said to remain "arrested" because meiosis is not completed, it grows rapidly and gradually becomes one of the largest of mammalian cells (exceeded in size only by some of the neurons); the process is associated with formation of large quantities of macromolecules including substantial amounts of RNA. Changes in ultrastructure have been described in the literature.²

It has been proposed that changes in the ovocyte promote development of the surrounding follicular cells. Small numbers of primordial follicles resume growth soon after birth in the human.

A clear area made up of proteins and polysaccharides forms between the ovocyte and the inner surface of the granulosa; it is

believed that contributions are made by both the ovocyte and the follicular cells to what will soon be the *zona pellucida*. Microvilli from the ovocyte project into the zone, and these interdigitate with processes of the follicular cells which extend toward the plasma membrane (oolemma) of the ovocyte. Nutrients reach the ovocyte, and wastes are removed through this region.

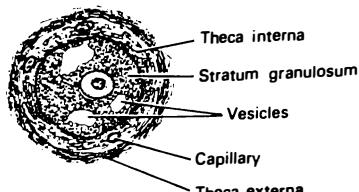
At this stage, the follicle and its ovocyte are often called the *unilaminar primary follicle* (Fig. 21-1B). Others prefer the term *growing follicle* to designate all stages between early enlargement of the granulosa cells and the vesicular stage described below, since once the follicle enters this stage it seems to be committed to either continued growth or to destruction.

The follicular cells undergo further growth and proliferate by mitosis to form first two and later three or four concentric layers around the ovocyte. The multilayered ring of follicular cells is then called the *stratum granulosum* (granulosa membrane). The outermost granulosa cells are in contact with a basement membrane and the latter becomes associated with surrounding connective tissue cells of the ovarian stroma which may contribute to the granulosa (Fig. 21-1C). Development to this stage may not require pituitary hormones.

It has been described in hypophysectomized animals. However, there is evidence that some gonadotrophin is available to affect development, and advancement of increased numbers of follicles to this stage has been reported following administration of gonadotrophins to hypophysectomized animals.

Few follicles advance beyond this point before the onset of puberty. It is likely that pituitary gonadotrophins and steroid hormones are required. But variations in sensitivity to the hormones (at certain stages of maturation of the animal as a whole, in some follicles of an ovary as compared with others, and in one species compared with another) make it virtually impossible to define quantitative relationships. Gonadotrophins can affect the ovaries late in gestation and during childhood, and a few follicles advance before the others. But no orderly maturation process emerges until cyclic secretion of the needed hormones is established.

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A. Early vesicular or secondary follicle.

ration of selected follicles. Small amounts of luteinizing hormone (*LH*) are also required and the term *folliculotropin* designates the hormone combination acting on the follicles.

Granulosa cells grow and proliferate, and new cells are added from the ovarian epithelium. Connective tissue cells of the surrounding stroma encircle the follicle to form a new layer, the *theca folliculi*, which enlarges and is invaded by blood and lymph vessels. The theca remains separated from the granulosa cells by the basement membrane through which nutrients enter; no capillaries penetrate the granulosa before ovulation.

The layer first formed becomes the *theca interna* as the growing follicle becomes surrounded by fibroblasts that organize into the *theca externa*. The latter develops collagen fibrils, an amorphous ground substance and fibromuscular elements, and contains blood vessels that pass through to the theca interna.

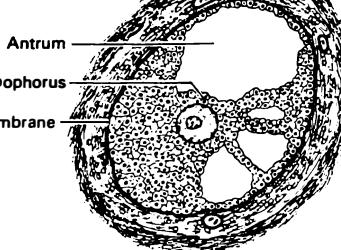
Elasticity and distensibility of the follicle have been attributed to properties of the mucopolysaccharide ground substance. Tensile strength is conferred by alignment of the collagenous fibrils and the interconnections between fibroblasts with each other and with the fibrils. At its most peripheral surface, the theca externa is in contact with a layer of connective tissue, the *tunica albuginea*.

Granulosa cells take up precursors from the capillaries and secrete a fluid which accumulates between the cells; the process seems to require *LH*. The granulosa cells synthesize proteins, but no protein specific for follicular fluid has been identified, and there is an unsettled question about whether follicular proteins are derived completely from the blood plasma.

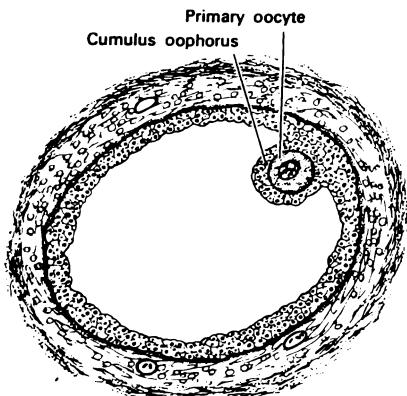
The structure at this stage may be called a *secondary follicle* (Fig. 21-2A). It is also called a *vesicular follicle* by some authors, while others reserve the term for the next stage, in which the follicle continues to enlarge, elongates, and forms a single large vesicle or *antrum* through coalescence of the smaller vesicles. When fully developed, the structure is called a *Graafian follicle* (Fig. 21-2C).

While the antrum is a prominent feature in most mammals, a few (e.g., some of the hedgehogs) do not form antra.

A direct correlation has been noted between



B. Late secondary follicle.



C. Mature Graafian follicle

FIG. 21-2. Preovulatory maturation of follicles.

Postpubertal Oogenesis and Follicular Maturation

Formation of Secondary Follicles. As puberty approaches, the adenohypophysis begins intermittent (cyclic) secretion of sufficient quantities of *follicle-stimulating hormone* (*FSH*) to promote further matu-

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the size of the Graafian follicle and the size of the animal; but ovocytes of large and small animals are similar in size.

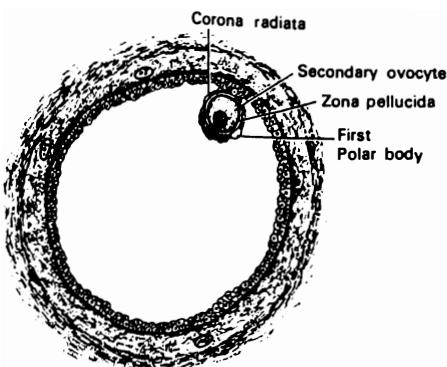
Graafian follicle fluid contains considerable quantities of steroid hormones (mostly estrogens). The hormones can be directly synthesized by the follicular cells, but some may also be taken up from the surrounding stroma. The fluid also contains a variety of proteins including hyaluronidase and proteolytic enzymes. Thromboplastin activity is present, and fluid taken from the follicles clots slowly *in vitro*.

Estrogens seem to be essential for maturation of the follicle and its ovocyte, and they play a role in development of *competence* (sensitivity to gonadotrophins). This seems to involve estrogen-mediated formation of estrogen receptors and also estrogen facilitation of FSH-mediated induction of receptors for FSH and LH.²¹ The steroids also influence gonadotrophin secretion (see below).

The granulosa cells most closely associated with the ovocyte and providing its connection with the follicle form the *cumulus oophorus*: they maintain higher metabolic activity than other follicular cells as the time of ovulation is approached. The single layer in direct contact with the ovocyte is the *corona radiata*. The number of cells adhering to the ovocyte at the time of ovulation is variable, and some authors do not distinguish between the two terms.

Follicles vary in their sensitivity to pituitary hormones; only limited numbers respond to the amounts present during each ovarian cycle, but it is known that others can be made to respond if the gonadotrophin level is artificially elevated. In humans, about 20 advance simultaneously to the secondary stage; two of these characteristically mature beyond this point, but usually only one attains the *tertiary stage* and is extruded. Follicles which mature incompletely are called *cohorts*; they probably perform hormonal functions contributing to development of the single most sensitive follicle.

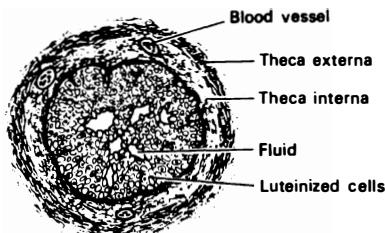
The incidences of birth of nonidentical twins, triplets, and larger numbers of non-identical siblings provide some indication of the frequency with which multiple ova mature simultaneously in the human. But not all ova are fertilized, and not all that are fertilized complete gestation.



A. Follicle at time of ovulation



B. Follicle after ovulation



C. Corpus luteum

FIG. 21-3. Ovulation and formation of the corpus luteum.

The number undergoing simultaneous maturation is under genetic control, but can be influenced by a variety of factors. In recent years, inadvertent overdosages of "fertility drugs" (e.g., pergonal, a gonadotrophin obtained from menopausal urine) have led to heightened incidence of multiple births and have underscored the desirability of limiting the number of fetuses to

that which can be effectively accommodated by the uterus. On the other hand, perfection of methods for increasing twinning in cattle may lead to augmentation of future meat and milk supplies.

Many small mammals regularly produce multiple fertilizable ova during each reproductive cycle. But their uteri are proportionately large and dual-chambered, and the young are retained for short gestation periods. Rats carry their young for 21–23 days, but a 260-g rat typically gives birth to neonates weighing about 4 g each. Does can have up to 20 pups per litter but the average number is closer to 6–10 (depending upon the strain). Guinea pigs with a 63- to 70-day gestation period usually produce two, three, or four young per litter but often have only one.

While production of large litters can contribute to perpetuation of the species, there are advantages to longer gestation periods. Guinea pig young are so mature at birth they require minimal maternal care. In primates, the long gestation period makes possible a higher development of the central nervous system.

Some variations in sizes and numbers of follicles maturing during each ovarian cycle are not so readily related to specific advantages. The plains viscacha (a small animal related to the chinchilla) for some reason produces 300–700 ova per cycle but carries only two young through gestation.

In species with very short ovarian cycles, follicles seem to start development during one cycle and to complete the stages to ovulation at a later time.

Tertiary or "Preovulatory" Follicles. Maturation of the follicle beyond the secondary stage involves additional fluid accumulation associated with marked swelling of the follicle and thinning of its walls.

The role of LH has not been completely defined. It is known that the oncotic pressure of the fluid rises. This has been attributed by some to LH-stimulated release of histamine from mast cells in the ovarian stroma close to the follicle and from basophilic leucocytes. Histamine may increase membrane permeability and permit entry of proteins. Depolymerization of mucopolysaccharides probably contributes further to the rise in oncotic pressure; changes in staining properties of the fluid and the existence of the needed enzymes support this concept.

The theca is stretched and thinned to a fraction of its former width. Proteolytic enzymes of the follicular fluid and others released from vesicles cast off by fibroblasts of the tunica albuginea have been implicated in thecal cell destruction. But some of the stretching and distensibility seems to be related to realignment of the collagenous fibrils and to continued depolymerization of the ground substance.

The basement membrane between the granulosa and the theca disappears, many granulosa cells disintegrate, and those of the cumulus oophorus become attenuated. The ovocyte is displaced to the part of the follicle closest to the external surface of the ovary.

A blister-like bulge or *stigma* forms just above the ovocyte, at a point where the follicular wall receives the least mechanical support from surrounding tissue. Necrosis of cells in the region of the stigma has been attributed to anoxia, since pressure from below reduces oxygen availability to cells far removed from the nearest capillary. There is also disruption of the blood supply to some of the cells of the theca at this time.

Stimulatory influences of LH on activity of proteolytic enzymes could be mediated by either a rise in progesterone or a fall in estrogen concentration of the follicular fluid. Actinomycin D abolishes LH stimulation. It could be interfering with LH actions on either progesterone secretion or induction of some regulatory protein involved in tissue destruction.

While preovulatory changes are proceeding in the follicle, the primary ovocyte resumes the processes of cell division. It soon gives rise to a single large cell, the *secondary ovocyte*, which contains one half of the genetic information of the parent cell but most of the cytoplasm. The other half of the genetic material goes into the *first polar body*, a minute cell which contains small amounts of cytoplasm morphologically similar to that of the original ovocyte. The first polar body usually remains in contact with the secondary ovocyte for a time within the zona pellucida (Fig. 21-3A). Most often it disintegrates and disappears soon afterward.

Resumption of meiosis has been attributed in part to reduction of previously existing inhibitory influences exerted by the granulosa cells.

As a result of the uneven division of the primary ovocyte, (1) excess nuclear material is discarded, and (2) the bulk of the cytoplasm is reserved for use by the surviving cell.

The secondary ovocyte then starts the second meiotic division, but development is arrested at the metaphase stage and is not usually completed until after fertilization in most species. At that time, the secondary ovocyte gives rise to one egg cell or *ovum* containing most of the cytoplasm and half of the genetic material, and to a very small *second polar body* which usually deteriorates. Sometimes the first polar body survives long enough to undergo cell division, yielding very small third and fourth polar bodies (Fig. 21-4).

Ovulation and Formation of the Corpus Luteum^{1, 2}

Under the influence of LH, continued weakening of the follicular wall culminates

in rupture of the follicle and extrusion of the ovocyte and the surrounding corona radiata cells (Fig. 21-3B).

At one time it was believed that pressure built up within the follicle played a role in the rupture, but it is now known that intrafollicular pressure does not rise appreciably. Ovulation is attributed to thinning of the wall to the breaking point, but recent work has also implicated prostaglandin stimulation of a contractile process.³

Fluid oozes out of the follicle, and the emptied structure is transformed into a yellow body or *corpus luteum* (Fig. 21-3C). In most species both granulosa and theca cells enter into its formation, but in some it is comprised predominantly of granulosa derivatives.

While morphological changes associated with luteinization are recognized, little is known of underlying mechanisms. LH has been implicated; but follicles from which the ovocyte has been removed can undergo spontaneous lutein-

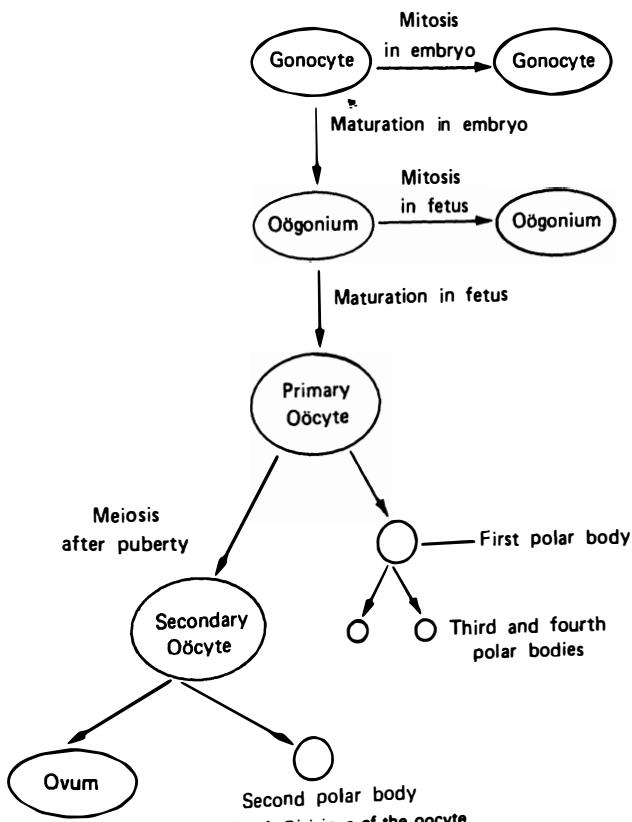


FIG. 21-4. Divisions of the oocyte.

ization, and changes can be induced by administration of progesterone without LH. LH influences seem to be mediated via cAMP. Prostaglandins and ovarian testosterone have also been proposed to play some role.²¹ The oocyte may inhibit luteinization.

Ovarian stromal cells can develop ability to secrete progesterone and have sometimes been called *paralutein glands*. Some authors have described changes in stromal cells as "luteinization," but others object to use of the term for nonfollicular cells.

If fertilization occurs, the corpus luteum persists for a time (Chapter 22); otherwise, it remains viable for a genetically determined period and then deteriorates, to be replaced by a *corpus albicans* (white body) comprised largely of scar tissue.

Accessory corpora lutea may form from partially matured follicles; but most of the latter degenerate and persist as *atretic follicles*. The number within the ovary increases with advancing age. Atresia does not seem to be hormone-dependent.

The secondary ovocyte and its surrounding cells are cast into the abdominal cavity in the immediate vicinity of the enlarged, fimbriated (fringed) opening of the oviduct (Fallopian tube) to start the journey toward the uterus. The structures through which the human ovocyte travels are shown diagrammatically in Figure 21-5. Fertilization normally takes place in the oviduct, with cell division completed after fertilization. Usually the fertilized ovum is the only product of the cell division which survives. Unfertilized egg cells disintegrate.

HORMONE SYNTHESIS AND SECRETION BY THE OVARY^{1, 2, 5}

Estrogens

Chemistry. Naturally occurring estrogens are typically 18-carbon steroids which have an *aromatic A ring*, an OH group attached to carbon 3 and an alcoholic or ketone group attached to carbon 17.

Estradiol- 17β is the major estrogen secreted by the ovary of humans and most other mammals. Substantial quantities of *estrone* (which is by several criteria less potent) are secreted by many species, along with smaller amounts of related steroids which have not been fully characterized. Estradiol and estrone are biologically interconvertible, but prevailing conditions usu-

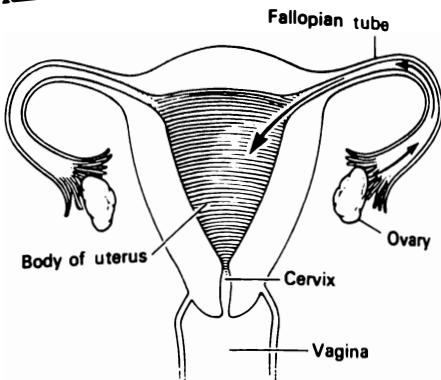


FIG. 21-5. Female reproductive organs.

ally favor formation of estrone. Additional estrogens identified in follicular fluids or ovarian tissues of laboratory animals include 6α -hydroxyestradiol- 17β , estradiol- 17α and 16α -estriol (Fig. 21-6).

Cellular Origins of Ovarian Estrogens. The major site for formation of estrogens during the follicular phase seems to be the vascular *theca interna*. Enzymes catalyzing steroid synthesis have been identified in the granulosa cells; but the latter are not believed to contribute substantially to estrogen production because they do not receive a direct capillary blood supply and because activities of enzymes catalyzing cholesterol cleavage and 17α -hydroxylation are low. (They may convert pregnenolone to progesterone, aromatize the A-rings of androgens, and exert stimulatory influences on theca cells.) The estrogens are picked up by vessels of the theca interna and sent to the ovarian veins. (Some steroid may also enter the lymphatic vessels).

At the time of ovulation, considerable quantities of estrogens are discharged from the follicular fluid into the abdominal cavity. Some hormone probably also enters the oviducts. A portion finds its way into the peritoneal vessels. Concentrations of estrogens in the systemic blood rise during the follicular phase and reach a peak at the period known as proestrus (p. 294).

In those species (humans, rats, sheep, etc.) in which theca interna cells enter into formation of the corpus luteum, estrogen is also secreted during the luteal phase. In others (e.g., the cow) the corpus luteum is formed by granulosa cells; it secretes progesterone but little or no estrogen.

Cells of the ovarian stroma secrete androstenedione which is a "weak androgen

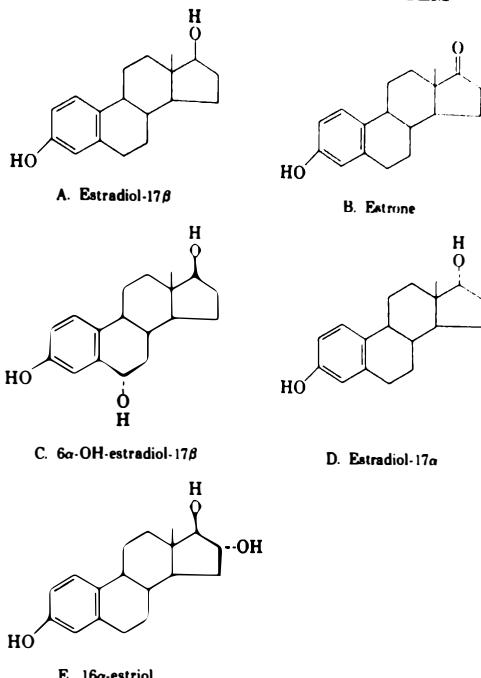


FIG. 21-6. Ovarian estrogens.

(p. 249)." Small amounts of this steroid and the more potent testosterone are normal constituents of adult female blood plasma; but most arises from hepatic metabolism of adrenocortical steroids. Much of the androstenedione formed is converted to estrogens in the liver. (Therefore the androstenedione functions as an estrogenic prohormone; this assumes special importance in post-menopausal human females in which follicular estrogen production ceases).

Stromal tissue also synthesizes small amounts of DHEA (Chapter 20), and may release this along with some pregnenolone, 17 α -hydroxyprogesterone and traces of testosterone, estradiol, and estrone.

Non-ovarian Sources of Estrogens. The adrenal cortex directly releases small amounts. Larger quantities may be formed in adipose tissue, from adrenocortical and ovarian stroma steroid precursors.

Biosynthetic Pathways in the Ovary. Biosynthesis of 3,17-androstenedione and of DHEA from acetate or cholesterol seems to be accomplished via pathways identical to those described for the testis. Major pathways for conversion of these precursors

to estrone and estradiol are summarized in Figure 21-7.

Alternate pathways involving sulfated steroids have been described in the testis and adrenal cortex. The quantitative significance of these in the ovary awaits evaluation.

Intermediates in estrogen synthesis seem to include 19-hydroxytestosterone (Fig. 21-8A) which is known to serve as a precursor. There is some evidence that it is oxidized to 19-oxotestosterone (21-8B), but additional steroids which may arise from 19-hydroxytestosterone are shown in Figure 21-8, C and E. At some point in the biosynthesis of estrogens, carbon 19 is removed. It has not been established whether 19-nortestosterone (Fig. 21-8F) serves as an intermediate or whether a second double bond is formed in the A ring simultaneously with removal of carbon 19. (Low yields of labeled estradiol have been obtained after administration of labeled nortestosterone.) Additional steroids within the pathway may include some with as yet unidentified substitutions at carbon 1.

Regulation of Estrogen Synthesis. It is clear that gonadotrophins are essential for promotion of estrogen synthesis by the ovary; but it is difficult to define their role. Impurities in gonadotrophin preparations

HORMONES AND REPRODUCTION

(including both unknown contaminants and traces of other hormones), inadequate definition of overlapping biological properties of FSH and LH, uncertainties about physiological dosage, recognized species variations, and incomplete knowledge of endogenous hormone levels, all contribute to problems of interpretation.

While gonadotrophin administration can lead reproducibly to increased estrogen secretion, most investigators believe that the gonadotrophins function mainly to prepare cells for autonomous hormone synthesis. FSH especially has been implicated in promoting growth and mitosis of cells, and synthesis of new cell proteins. LH seems to augment blood supply, but it may also enhance hydrolysis of cholesteryl esters and activate enzymes catalyzing cleavage of cholesterol.

Estrogens in Blood Plasma.¹² *Estrone sulfate* is the major estrogen of the plasma.

It is formed in the liver from estrogens and related steroids of ovarian and adrenocortical origin. Concentrations in mature non-pregnant women are in the range of 80–90 ng/100 ml. (Adult males have about half this amount.) Concentrations of free estradiol-17 β are between 5 and 21 ng/100 ml, depending on the phase of the ovarian cycle, while while values for total estradiol are between 14 and 60 ng/100 ml.

Estrogens bind with high affinity to *sex steroid-binding globulin (SSBG)* (p. 253). Hepatic synthesis of the protein is augmented when plasma levels of natural or synthetic estrogens are high, e.g., during pregnancy. Estrogens also bind to plasma albumins which have lower binding affinity for the steroids but large total binding capacity.

Metabolism of Estrogens. The liver is the major site for degradation. In addition to conjugation with sulfate and to a lesser

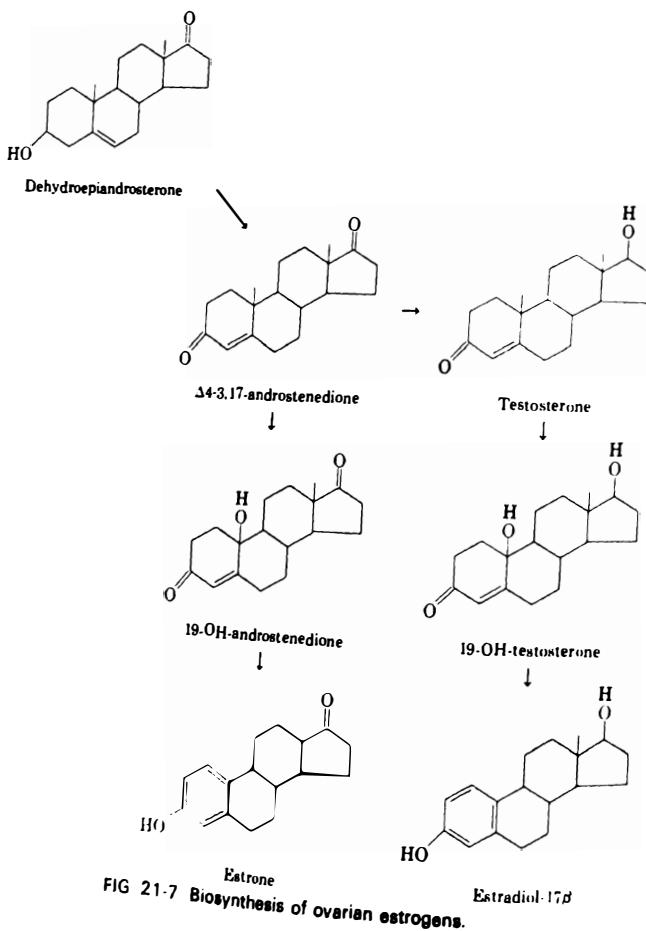


FIG 21-7 Biosynthesis of ovarian estrogens.

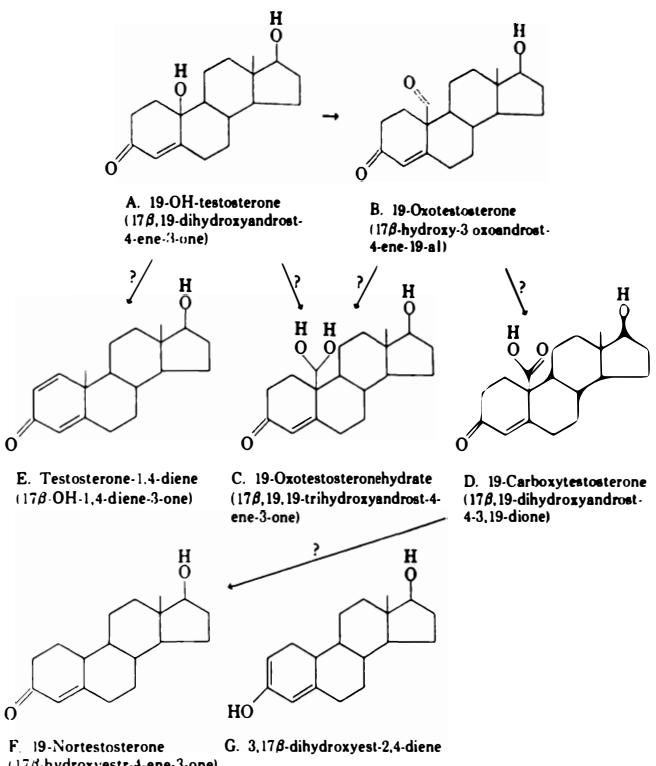


FIG. 21-8. Some proposed intermediates in the biosynthesis of estrogens.

extent with glucuronate, estrogens are subjected to hydroxylation, oxidation, and methylation. The products are generally either less active than the original hormone or totally devoid of estrogenic potency. Most are excreted by the kidney.

Estriol is produced in large quantities by the placenta. In nonpregnant females it arises mainly from hepatic metabolism.

Some of the known degradation products are shown in Figure 21-9. All have been identified in the urine. Both free and conjugated estrogens are secreted into the bile, from which they may be reabsorbed or excreted with the feces.

Ovarian Progestagens

Progesterone is the major progestagen (progesterogen, progestin) secreted by the ovary of many species, and it is, by many criteria, the most potent of the naturally occurring steroids of this group. But significant quantities of 20α -hydroxyprogester-

one, 20β -hydroxyprogesterone, and 17α -hydroxyprogesterone (Fig. 21-10) are synthesized and released. In some species the amounts of 20 -hydroxyprogesterone are substantial when LH levels increase, and their formation may be reduced by prolactin. In others, amounts relative to progesterone increase as the corpus luteum ages. The hydroxy forms may have specific functions; a facilitatory influence on LH release has been proposed for the rabbit.

Most of the progesterone is synthesized by cells of the corpus luteum (and this is true of both species in which the latter is derived largely from the stratum granulosum and those in which theca interna cells are incorporated). Some is synthesized by the ovarian stroma; the contribution may be considerable in certain species, but it does not seem to exhibit cyclic fluctuations. Small quantities of progesterone are also produced by developing follicles, and the hormone has been identified in follicular fluid shortly before the time of ovulation.

HORMONES AND REPRODUCTION

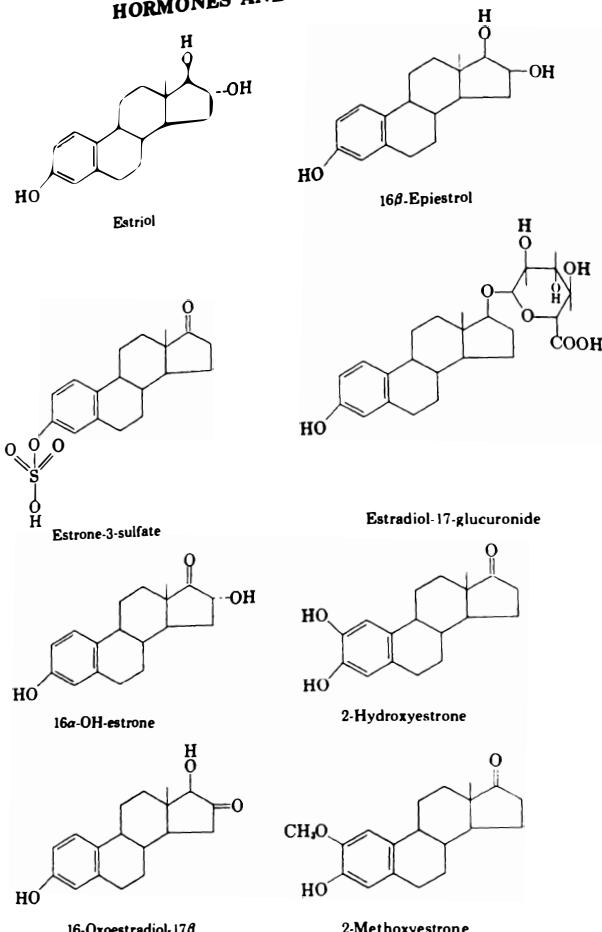


FIG. 21-9. Degradation products of estrogens.

Progesterone is needed in larger amounts than are estrogens. Plasma concentrations of free hormone of 50–90 ng/100 ml and of 1,100 ng/100 ml, respectively, have been found during follicular and luteal phases of the menstrual cycle in normal women.

Progesterone binds strongly to corticosteroid-binding globulin (CBG, Section II) and to plasma albumin, but not to SSBG. It is taken up by peripheral tissues, especially those with high lipid content, and is transformed by them into numerous metabolites; but most degradation takes place in the liver. Some progesterone and its metabolites are secreted into the bile and later reabsorbed (enterohepatic circulation).

Pregnane 3 α ,20 α -diol is a major degradation product. Other metabolites include pregnane 3,20-dione and pregnanalone (Fig. 21-11).

Progestagens can be metabolized to androgens; this has presented problems when large doses were administered to pregnant women (Chapter 19).

The naturally occurring progestagens have 21 carbons, and the potent molecules have one double bond in the A ring and oxygen groups at carbons 3 and 20. Clinical use is limited by the rapid degradation, the need for frequent parenteral administration of large amounts, and by transformation to androgens.

Ovarian Androgens

Under normal conditions, most of the androstenedione produced by the ovarian stroma is converted by hepatic enzymes to estrogens. However, about 150 ng/100 ml of androstenedione of both adrenal and ovarian origin is found in the plasma of normal women. (This is higher than the androstenedione concentration found in adult men.) Women also have about 350–400 pg/ml of testosterone, compared with 5,000–10,000 for men.

Testosterone in normal females arises almost exclusively from hepatic transformation of ovarian and adrenocortical steroids. Very minute amounts may be directly secreted by normal ovaries. Ovarian tumors and ovaries deficient in enzymes needed for estrogen synthesis may, however, release very large quantities, and this causes "virilization." An interesting observation is that normal ovaries grafted to peripheral sites where the temperature is lower than that of the abdominal cavity, tend to secrete large amounts of androgens.

The very small quantities of androgens normally present affect secondary sex characteristics and especially growth of axillary hair. They also stimulate libido in females as well as in males.

FUNCTIONS OF ESTROGENS^{1,2}

Influences on reproductive structures are described below. But estrogens affect virtually every cell type. References have been made in earlier sections to roles in regulation of carbohydrate, water, sodium, calcium, and lipid metabolism, food intake, and secretion of pituitary hormones. The estrogens affect (among other things) the texture and secretory functions of the skin, fat distribution, body temperature and metabolic rate, enzyme activities and protein synthesis in the liver, blood clotting, erythropoiesis, involution of the thymus gland, intraocular pressure, and sensitivities to other hormones. Metabolic actions have been utilized for fattening and tenderizing cattle and poultry for the food market,

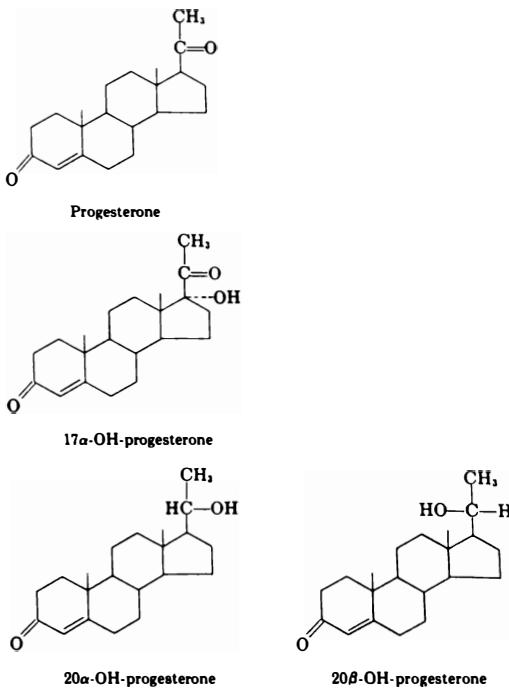


FIG. 21-10. Ovarian progestagens.

HORMONES AND REPRODUCTION

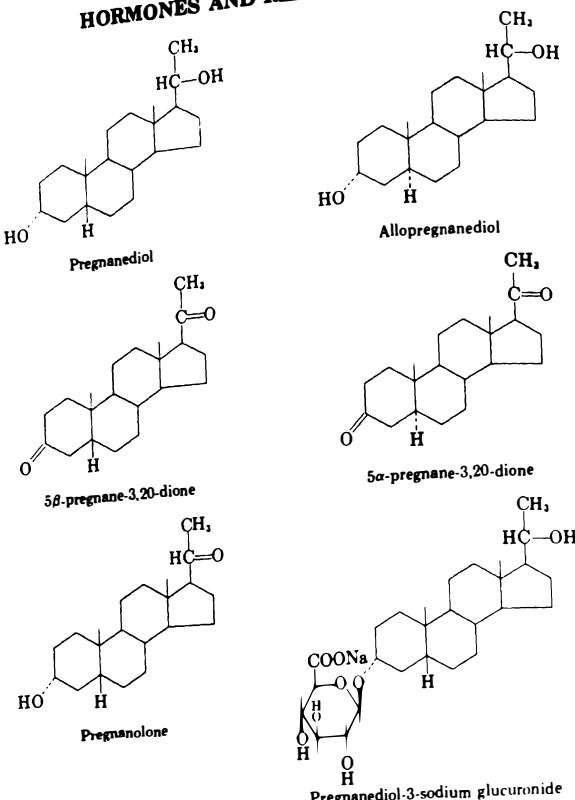


FIG. 21-11. Some degradation products of progesterone.

and hormonal influences are utilized in several ways for control of conception.

Because of powerful influences on mitosis of several kinds of cells, there is interest in a possible role in etiology of malignant growth. Under specified (but highly artificial) conditions, repeated application of estrogens can induce neoplastic changes in the skin. Recent statistical studies strongly implicate some of the estrogenic compounds in the etiology or aggravation of endometrial, vaginal and mammary gland cancers in certain groups of women.^{18, 19} Removal of sources of estrogens (via ovariectomy) is an accepted procedure in management of some forms of cancer. But estrogen administration may be beneficial in selected cases.

Influences of estrogens on reproductive behavior are described in the section on ovarian cycles (see also Chapters 19 and 22). Influences on hypothalamic and pituitary hormones are noted in several places throughout the chapter.

Actions on the Ovary

Mention was made above of an essential role of estrogens in the maturation of follicles and ovocytes; influences on the corpus luteum are described later in the chapter. Some estrogen actions seem to be exerted directly; they can be demonstrated after highly localized topical application, and when given systemically to hypophysectomized animals. Others involve regulation of gonadotrophin secretion both directly and indirectly, and still others depend upon interactions with the gonadotrophins.

Actions on the Uterus

Estrogens initiate cyclic preparation of the uterine lining (endometrium) for implantation by promoting hyperemia, more rapid uptake of water, electrolytes, and small organic molecules, and stimulation of synthesis of RNA and protein. In some species, influences on electrolyte metabo-

lism and on contraction of the uterine cervix lead to "ballooning" of the uterus. The distention in turn enhances some effects of the steroids. (Similar enhancement can be achieved by artificial distention.)

Both epithelial and stromal components of the endometrium proliferate rapidly by mitosis, uterine glands elongate, and the tissues are "primed" for subsequent actions of progesterone. Close to the time of ovulation, the uterine environment is made favorable for sperm capacitation. Later, if conception occurs, estrogens are needed in most species to provide conditions for implantation of the blastocyst (Chapter 22).

Estrogens influence the secretion of gonadotrophins which promote ovulation and formation of the corpus luteum; and they affect the life span of the corpus luteum. Therefore they indirectly affect progesterone influences on the uterus in several ways. In addition, they induce synthesis of progesterone receptors.

Estrogens also promote growth and strengthening of the muscle components (myometrium); they induce formation of contractile proteins and of collagen, and increase excitability through influences on the cell membranes. Responses to oxytocin and to other muscle stimulants are augmented. In some of the larger mammals, uterine contractions play a role in transport of sperm prior to conception. During pregnancy, estrogen influences on the myometrium are important for growth and strengthening of the muscle to accommodate the developing fetuses. Later, they play a role in parturition and in postpartum uterine involution.

Influences on the Uterine Cervix

In addition to promoting transient contraction of the cervix before ovulation, and preparing for the relaxing influences of progesterone, estrogens exert profound influences on activity of the cervical glands. Shortly before ovulation, they promote secretion of copious quantities of a watery fluid whose composition is favorable for sperm survival. The hydrogen ion and sialic acid content are decreased, electrolyte content is increased, and there are indications that the secretion contains substances which are protective to the sperm. Such changes may assume special impor-

tance in those species in which sperm is temporarily stored within the cervix.

Glycoprotein filaments are oriented so that spaces between them are sufficiently large for passage of sperm into the uterus. Alterations in composition of the cervical mucus seem to enhance migration and orientation of healthy sperm, but to retard passage of less motile or abnormal sperm.

Spinnbarkeit (fibrosity) is a term which describes ability to form mucus threads when cervical fluid is stretched between two glass slides (or a slide and cover slip). Estrogens increase the length of the thread which can be drawn without breakage. Estrogen-dominated cervical fluid also exhibits characteristic crystallization patterns (*fernning*) when permitted to dry on a glass slide.

Cyclic variations in properties of cervical secretions can be used in some species for obtaining information on the timing of ovarian cycle events, and attempts have been made to apply the observations to procedures for fertility control.

Shortly before the onset of parturition, relaxin promotes softening and dilation of the birth canal; estrogens are needed for responsiveness to relaxin.

Influences on the Vagina

Estrogens promote growth and maturation of the vagina. In immature rats they hasten the vaginal opening associated with onset of puberty. In guinea pigs the vaginal membrane opens and closes with ovarian cycles, and this too is affected by estrogens.

Effects on mitosis of the vaginal epithelium and later on keratinization of newly formed cells is described below. (The processes of mitosis and cornification may depend upon different mechanisms; they can be differentially affected by pharmacological agents.) The exquisite sensitivity of the vagina to local application of estrogens forms the basis for bioassay procedures.

Estrogens increase the acidity of vaginal secretions; this accounts for reduced susceptibility of fertile women to invasion by microorganisms. (Acidification of the secretions is sometimes induced in children and in postmenopausal women as part of the treatment for vaginal infection.) Influences of progesterone on vaginal mucus secretion require estrogen "priming."

Influences on the Oviducts

The effects are most obvious in species with long ovarian cycles. Both secretory and ciliated cells increase in size and number, and exhibit changes in ultrastructure. The composition of oviducal fluid favors sperm capacitation and fertilization around the time of ovulation. Fluid formed later in the cycle is inhibitory. Influences on muscular and ciliary activity provide for optimal timing of movement of the ovum and conceptus toward the uterus so that the blastocyst arrives when the uterus is maximally receptive.

Influences on the Mammary Glands

The role of estrogens in development and function of the mammary gland is described in Chapter 22.

SYNTHETIC ESTROGENS

Naturally occurring estrogens have limited usefulness as therapeutic agents because they are rapidly degraded and excreted after oral administration. Several highly potent synthetic compounds have been developed which resist degradation and are effective orally. Some resemble the natural hormones; others are not steroids but are apparently able to effectively combine with estrogen receptors. The nonsteroidal compounds have two phenolic groups at opposite ends of the molecule; there is evidence that they must have molecular diameters similar to those of the hormones to exert estrogen-type activity.

Diethylstilbestrol (DES) is a highly potent nonsteroid agent which has been widely used for clinical and experimental purposes. Recent indications that daughters of women receiving this agent during pregnancy tend to develop an unusual form of vaginal cancer have discouraged its use in women with potential for future pregnancies. It has not been established whether pathological findings are related to potent estrogen-like action or to pharmacological actions. Structures of DES and some other synthetic estrogens are shown in Figure 21-12.

MECHANISM OF ACTION OF ESTROGENS^{1, 2, 4, 10, 11, 20}

Much of the information on mechanism of action has been obtained from studies of estradiol-17 β influences on uteri and uterine preparations of laboratory rats.

Binding of Hormones to Specific Cytoplasmic Receptors

Specific macromolecular cytoplasmic estradiol (E_2) receptors have been identified in uterine endometrium and myometrium, vagina, oviduct, mammary gland, pituitary gland, and hypothalamus. Unlike the situation described for androgens (Chapter 20), estradiol seems to function directly without prior metabolic transformation.

The macromolecules (*estrophiles*) bind with high affinity to synthetic agents which exert estrogen-like actions (DES, hexestrol, 17 α -ethynodiol) and to some of the "antiestrogens," e.g., chloro-

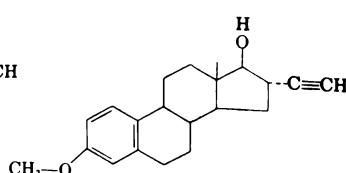
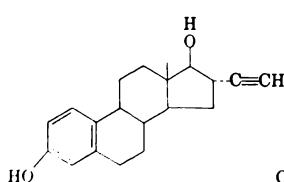
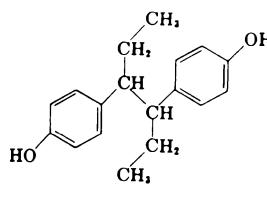
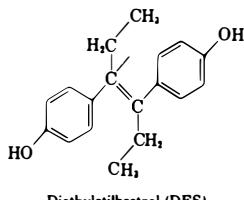
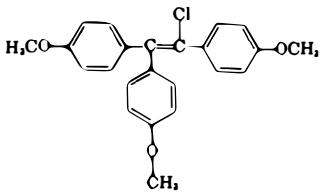
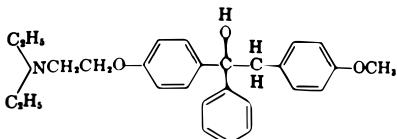


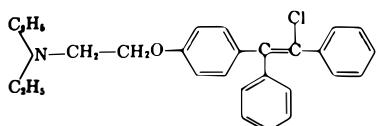
FIG. 21-12. Orally effective agents with estrogen-like activity.



Chlorotrianisene (TACE)



Ethamoxytriphetol (MER-25)



Clomiphene

FIG. 21-13. Some antiestrogens.

trianisene or TACE, MER-25, clomiphene (Fig. 21-13), and nafoxidine. In common with other competitive inhibitors, the antiestrogens may exert some weak estrogen-type actions.

A few cell types take up limited amounts of estrone. But biological conversion to E₂ seems to be essential for actions on others. Estradiol competes to a limited extent for E₂ receptors; it exerts weak estrogen-like actions but reduces effectiveness of E₂ when presented with the more potent hormone. (The receptors do not bind progesterone, testosterone, or glucocorticoids.)

Observations of the kind presented support the concept that free hydroxyl or phenolic groups at both ends of the molecule are involved in physiological binding.

Estrogens play a role in induction of E₂ receptors. This provides an explanation from the long-recognized positive influence of "estrogen priming" on responses to a second dose of E₂ administered a day later. In addition to the readily saturated high affinity, low capacity specific E₂ receptors of "target" organs, binding molecules with lower affinity but high capacity have been found widely distributed in mammalian cells.

The estrophiles have properties of proteins. They can be destroyed with proteolytic enzymes but not with RNases or DNases, and they are heat labile. Binding to high affinity receptors is impaired with sulphydryl-blocking agents which do not affect binding to low affinity receptors.

Under specified conditions, a complex of tritium-labeled E₂ with the receptor sediments as a discrete 8S band. When salt concentrations are varied, a 4S band is found. An intermediate (6S) has also been described. The E₂-receptor complex can be demonstrated in the cytoplasm within minutes after exposure to the hormone; maximal binding requires 15–20 min under the usual conditions.

Inhibitors of RNA or protein synthesis do not interfere with the binding, but they block actions of the hormone; since the processes of binding and action are separable, some authors object to designation of the binding molecules as hormone receptors.

Specific Nuclear Receptors

Within minutes after appearance of the cytoplasmic hormone-receptor complex, there is a transfer of the steroid hormone to the nucleus. A complex with a sedimentation coefficient of approximately 5S has been isolated from the nuclei and been shown to be clearly different from the one found in the cytoplasm. The nuclear substance cannot be obtained by direct exposure of isolated nuclei to E₂; but it will appear if cytoplasmic components are present.

All available evidence is consistent with the concept that the steroid hormone in some manner induces changes in the cytoplasmic macromolecule with which it combines, and that the modification is essential for translocation of the complex to the nucleus (or for its retention at that site). The cell content of cytoplasmic receptor becomes depleted as the complex builds up in the nucleus.

Both the steroid and the protein-like component bind to specific chromatin sites. It has been proposed that the hormone complex has two binding sites, one for the DNA and the other for an acidic nuclear protein.

The nuclear binding can be demonstrated both *in vivo* and *in vitro*. Treatment with DNase releases bound E₂, but RNase has little influence. Nuclear histones do not seem to be

directly involved; under some conditions, binding is enhanced by removal of the histones.

Consequences of Binding to Nuclear Components

A 40% increase in the rate of synthesis of rapidly labeled RNA has been described within 2 min after binding of the complex to uterine nuclei. The synthetic rate was observed to reach a peak of 400-500% above control values within 20 min, and to decline afterward over the next 24 hours (but not to control levels). Since the effect is seen very early and is not blocked by cycloheximide, it may represent a primary even in E_2 stimulation of target tissue.

A biphasic response has been described in which an actinomycin D-sensitive initial phase reaches a peak in 20-30 min and falls abruptly while a second phase starts at 2 hr and remains high for at least 24 hr. According to at least one report, incorporation of uridine into nuclear RNA rises early, but incorporation of adenine is not increased until after several hours. Enhancement of chromatin template activity has been described as early as 10 min after administration of the hormone.

Soon afterward, it is possible to demonstrate the existence of one or more specific proteins in both cytoplasm and nucleus. According to one hypothesis, a very early event is synthesis of an acidic protein (IP) in the cytoplasm which is rapidly transferred to the nucleus. IP may interact with histones to remove their inhibitory influence on transcription of specific RNA sequences.

Reproducible changes following E_2 administration have been reported, but an exact sequence of events has not yet been worked out. The findings are consistent with E_2 promotion of some small changes in RNA and protein metabolism which lead secondarily to larger and broader changes in RNA and protein metabolism. Influences of E_2 on adenylate cyclase have been described, and it is possible that cAMP participates in amplification of early E_2 -induced events.

Increased nucleolar RNA polymerase activity has been described within 1 hr after E_2 administration. Early changes have been attributed to activation of existing enzyme; they seem to require the formation of small amounts of a specific protein

with a short half-life, since E_2 stimulation of the nucleolar polymerase can be blocked with cycloheximide. There are conflicting reports about the timing (but not the occurrence) of increased activity of a second RNA polymerase. It seems certain that at some point there is accelerated formation of new species of RNA and of new proteins.

RNA extracted from estrogen-treated tissues has been reported to induce estrogen-like effects in target tissues not exposed to the steroid. This supports the concept of appearance of new mRNAs. Increased rate of transcription of unique gene sequences seems to be involved in E_2 action (rather than changes in the nature of the DNA).

Under the influence of E_2 , new populations of ribosomes appear. The hormone may also affect the rate at which the ribosomes form new protein.

Generalized stimulation of protein synthesis and cell growth is a still later event. Prolonged actions of the hormone lead to cell proliferation. There is no evidence for direct influences of the steroid on DNA replication. It seems likely that influences on protein and RNA synthesis lead to changes in cells which prepare them for entry into processes of cell division.

Some of the nuclear E_2 seems to return to cytoplasm to pick up additional cytoplasmic macromolecules. The process may continue until the supply of cytoplasmic receptor is depleted or the hormone is metabolized or extruded. A question under active investigation is that of what limits steroid hormone action on target cells.

Other Proposed Mechanisms⁴

According to some authors, undue emphasis has been placed on binding of estrogen-macromolecular complexes to nuclear components as the single primary site for hormone action. Some estrogen-induced effects are seen within seconds after hormone administration, and a few cannot be blocked by inhibitors of RNA and protein synthesis.

Estrogens invoke a very early hyperemia in the uterus, and there is a rise in permeability to water, salts, and small organic molecules measurable within seconds after

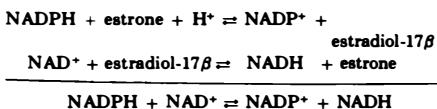
exposure to the hormone. Estrogens are known to be capable of releasing histamine and other amines from binding sites; and many estrogen actions can be mimicked by administration of histamine.

A doubling of uterine cAMP has been described within 15 sec after hormone administration. The cAMP may contribute to primary membrane effects, to activation of glycogen phosphorlyase, and to amplification of hormone influences on the nucleus. Catecholamine release could have direct functions and also contribute to activation of adenylate cyclase.

Estrogens *labilize lysosomal membranes*, and some actions are antagonized by glucocorticoids (which exert stabilizing influences on lysosomes under some conditions). It has been proposed that release of lysosomal enzymes contributes substantially to over-all estrogen actions on cell metabolism through *selective degradation* of macromolecules and release of regulatory substances.

Some earlier concepts of mechanism of action of estrogens have been discarded in the light of recent developments. It has been observed, for example, that steroid hormones including estrogens can affect aggregation and activity of hepatic glutamic dehydrogenase¹⁴; but the high concentrations required, and the failure to find differences in activity of the enzyme in female as compared with male liver make this seem an unlikely site of physiological action.

It has also been proposed that estrogens participate directly in transhydrogenase reactions, perhaps as follows:¹⁵



Among other reasons for discarding such reactions as part of estrogenic functions are the observations that many synthetic agents which mimic the hormone cannot be reversibly oxidized and reduced in this way.

FUNCTIONS OF PROGESTERONE^{1,2}

Most of the known actions of progesterone require estrogen "priming." Excep-

tions include influences on water and electrolyte metabolism involving interaction with aldosterone receptors, some effects on protein metabolism, and possibly actions on the hypothalamus affecting body temperature and appetite.

Species variations in response to or need for progestones are more obvious than is the case for the estrogens. At least some target organs metabolize progesterone and actively bind certain metabolites. There are species as well as tissue differences in the nature of the products formed and the manner in which they are utilized. There are also differences in the relative proportions of the various progestagens synthesized by different animal types, and in the amounts secreted during ovarian cycles.

Progesterone secretion is minimal during the phase of the ovarian cycle in which follicles are ripening in most of the mammals. But fairly large amounts are put out by some ruminants. Progesterone has been identified in follicular fluid and has been implicated directly in mechanisms of ovulation. The hormone and its metabolites may also participate in promotion of LH release around that time. Relatively large amounts of progesterone are secreted by the corpus luteum of primates and other mammals with a luteal phase that lasts many days; but little is put out by species with very short cycles. Characteristically, progesterone secretion falls off markedly toward the end of the longer cycles. Considerable amounts may be secreted by rats, mice, and others during pseudopregnancy.

Actions on the Uterus

Progesterone acts on the estrogen-primed endometrium to complete preparation for implantation. Estrogen stimulation of mitosis is terminated. (This is a physiological action; progesterone does not build up in sufficient quantities until proliferation has been accomplished. But premature progesterone administration impairs the process.)

Endometrial glands enlarge, become coiled, and assume secretory functions. In species with menstrual cycles, the spiral arteries grow and become tortuous. The stroma becomes highly vascular and edematous, and glycogen accumulates.

The volume of luminal fluid diminishes

because of progesterone influences on water and electrolyte metabolism, and also because the previously constricted cervix relaxes to permit drainage. The progesterone-dominated uterus provides an unfavorable medium for sperm capacitation. Since this condition does not emerge until after the normal time of fertilization, it may protect against participation of overripe ova in zygote formation or against a second conception at an unfavorable time (superfecundation).

When progesterone preparation has been completed, the endometrium will respond to appropriate stimulation by participation in the "decidual reaction" (Chapter 22). If conception does not occur, the surface is sloughed off (and this is accompanied by necrosis of blood vessels and obvious bleeding in menstrual cycles). *Physiological* sloughing requires the sequence of estrogen, then progesterone, and finally progesterone withdrawal.

If estrogen alone is given to ovariectomized animals, the lining builds up but does not undergo specialization; it is retained for long periods. Withdrawal of estrogen is followed by some shedding. If estrogen is given first and then progesterone, but progesterone administration is prolonged, sloughing is delayed but eventually a "breakthrough" is seen. Progesterone alone does not promote extensive endometrial proliferation.

In at least some species, progesterone hyperpolarizes cell membranes of the myometrium, promoting relaxation and preventing coordinated contraction. It also reduces responses to oxytocin and to other muscle stimulants. The actions seem to be important for preventing premature expulsion of the conceptus. Parturition is preceded by a rapid decline in progesterone levels.

Progesterone does not seem to exert these actions on uterine muscle of guinea pigs, and effects on primates are inconsistent. The hormone and its synthetic analogs have been administered to patients with a history of repeated abortion, in an attempt to prolong the gestation. Such agents are usually ineffective except in the rare cases in which endogenous progesterone is inadequate. As noted (Chapter 19), conversion of administered progestagens to androgens

has led to masculinization of female fetuses.

Influences on the Uterine Cervix

Relaxation of the musculature was described above. Actions on estrogen-primed glands lead to production of a highly viscous cervical secretion, rich in sialic acid and hydrogen ion, which is hostile to sperm. Orientation of fibrillar components impedes sperm passage, and spinnbarkeit (p. 279) is lost.

Influences on the Vagina

Progesterone antagonizes estrogen influences on proliferation of the superficial layers of the vaginal epithelium, but synergizes with estrogen in action on intermediate layers. Late in the ovarian cycle, progesterone enhances sloughing of the peripheral cells, and promotes secretion of vaginal mucus. In some species, postcoitus formation of a mucus plug aids in retention of sperm.

Influences on the Oviduct

The progesterone-dominated oviduct provides an unfavorable medium for sperm capacitation and fertilization. Regulatory influences on contractile properties of oviducal muscle contribute to early retention and later passage of the conceptus toward the uterus so that it arrives at a time favorable for blastocyst survival and implantation.

Influences on the Mammary Gland

In many species, progesterone acts on estrogen-primed mammary glands to promote development of the alveolobular system for milk secretion; but a fall in progesterone levels at parturition seems to be needed for initiation of lactation (Chapter 22).

Progesterone and Behavior

In most "spontaneous ovulators" (p. 292), progesterone precipitates onset of "behavioral estrus" when neurons have been primed with estrogen. In "induced ovulators" coitus takes place before the rise

in progesterone secretion, and in such animals, estrogen alone may be sufficient for onset of sexual receptivity; but progesterone has been implicated in the "paradoxical sleep" characteristically seen after coitus. Drowsiness of early pregnancy has also been attributed to progesterone.

In ruminants, high levels of progesterone have been found early in the ovarian cycles; in these, sexual receptivity seems to be triggered by a transient fall in progesterone.

Progesterone participates in several ways in the onset and maintenance of maternal behavior in mammals, mostly through interaction with other hormones. It has been implicated in "broodiness" in some of the birds and in behavioral responses of poikilothermic vertebrates.

A stimulatory influence on *appetite* has been described and implicated in the *weight gain* of pregnancy associated with lipid storage. It has also been proposed that the hormone participates in estrogen-induced development of the female phenotype and the associated *distribution* of depot fat.

Some women taking large doses of synthetic progestagens for contraception have complained of *psychological depression*; in selected cases, the condition has been alleviated by switching to a contraceptive with reduced progestagen content. Attempts have been made to relate the effects to influences on tryptophan metabolism and formation of serotonin (5-hydroxytryptamine). But hormonal influences on emotions and behavior are notoriously difficult to evaluate in humans. Depression could arise from nonpharmacological factors (e.g., a desire for conception which the woman does not accept at the conscious level, or a recognized wish combined with resentment at having to take the contraceptives); it has even been proposed that responses to alterations of progestagen dosage have nonhormonal explanations.

Body Temperature

Both endogenous secretion of progesterone starting around the time of ovulation, and administration of small doses of the hormone, are known to induce a transient

elevation of body temperature of between 0.4 and 1.0°F. Unlike most influences of progesterone, no prior treatment with estrogens is required.

It has been demonstrated that radioactively labeled progesterone binds to hypothalamic cells, and a direct influence of the hormone on cells involved in body temperature regulation has been proposed. However, specificity of the binding has not been established, and agents such as salicylates and aminopyrine (believed to act directly on the hypothalamic cells to reduce pyrogen-induced but not physiological elevations of body temperature) do not counteract the progesterone influence.

While no direct effects of progesterone on thyroid hormone secretion rate, plasma concentration of protein-bound iodine, or basal metabolic rate have been demonstrated, thermogenesis can be blocked by hypophysectomy or treatment with propylthiouracil (which impairs synthesis of thyroid hormones but may exert other action). A "permissive" action of thyroid hormones has been proposed. Increased height of thyroid epithelial cells has been described after chronic progesterone treatment in rats. Poorly understood influences of progesterone on protein metabolism may contribute to the thermogenesis. No function has been ascribed to the changes in body temperature, but attempts have been made to utilize the effects for timing of events of the ovarian cycle (Chapter 22).

Other Actions of Progesterone

Incompletely defined actions on carbohydrate metabolism, insulin secretion, and insulin resistance can enhance danger of use of oral contraceptives by women with overt or latent diabetes mellitus, and may contribute to alterations in carbohydrate metabolism sometimes seen in otherwise normal pregnancies. (However, estrogen influences are probably of greater importance.)

Progesterone competition for aldosterone receptors has been mentioned (Section III). This can lead to natriuresis and reduction of edema under certain conditions. However, large amounts can mimic aldosterone actions, and chronically elevated levels of

lower magnitude may induce compensatory rises in aldosterone output.

MECHANISM OF ACTION OF PROGESTERONE^{9, 11, 20}

Most of the work in this area has been carried out on preparations of rat uterus or on chick oviduct. The chick system is convenient to use because a specific protein (avidin) is produced in large quantities under the influence of the hormone. Mouse vagina and uteri of guinea pigs, rabbits, and humans have also been studied.

Estrogen "priming" seems to accomplish two purposes: (1) proliferation of a population of cells responsive to progesterone, and (2) induction of cytoplasmic progesterone receptors. The number of progesterone-binding sites in rat uterus has been reported to rise to a maximum during proestrus (p. 294), to be reduced after ovariectomy, and to be restored by estrogen treatment.

5α -Pregnane-3,20-dione has high progestagen activity in the chick and binds strongly to the receptor. The same metabolite has little activity on rat uterus and does not effectively compete with progesterone for the hormone receptor.

There is good evidence that the complex formed by progesterone and the cytoplasmic macromolecule is translocated to the uterus. Two subunits have been described, one which binds directly to chromatin and a second which binds to an acidic nuclear protein. Available data are consistent with the concept that the binding leads to increased synthesis of DNA-dependent RNA, and that formation of new messenger RNA species leads in turn to synthesis of specific cytoplasmic proteins.

Progesterone does not bind directly to nuclear components when isolated nuclei are exposed to the steroid; but a macromolecular-progesterone complex can be found if the steroid is permitted to bind first to cytoplasmic components, and appearance of the nuclear receptor has been directly related to decline in content of the cytoplasmic macromolecule. The complex isolated from nuclei binds to nuclei of other progesterone "target organs" but not to nuclei of lung, heart, spleen, or intestine.

Specificity of binding seems to be related to the nature of the acidic nucleoproteins (rather than to differences in DNA). Most actions of progesterone are blocked by administration of actinomycin D or inhibitors of protein synthesis; and there is clear evidence for formation of new RNA species. Actions on cell and lysosomal membranes comparable to those described for estrogen have not been found for progesterone.

SYNTHETIC PROGESTAGENS

Progesterone must be injected repeatedly in high dosage to induce sustained effects. It has been largely replaced for therapeutic and contraceptive purposes by synthetic agents. Some are shown in Figure 21-14.

FOLLICLE-STIMULATING HORMONE (FSH)^{2, 6, 17}

Chemistry

FSH is a species-specific glycoprotein secreted by the pituitary gland. It is made up of α - and β -subunits held together by noncovalent linkage. (As noted in Section V and later in this chapter, thyroid-stimulating hormone and luteinizing hormone are also glycoproteins made up of subunits.)

The molecule as a whole is similar in size for all species examined, and has a weight of about 30,000. Differences in amino acid composition account in part for differences in biological potencies of FSH preparations from diverse animal types; but methods for purification of the hormone have not as yet been standardized and all available preparations contain unknown components.

Because of the low pituitary content of FSH and lability of the hormone during extraction, it has not yet been possible to completely work out the structure, but amino acid compositions have been determined (and shown to be quite different from those of the LHSs). The carbohydrate content has been variously estimated at between 7 and 26%. It is not known how much of the range is related to true biological heterogeneity (and how much to conditions associated with the extraction procedures). There are indications that

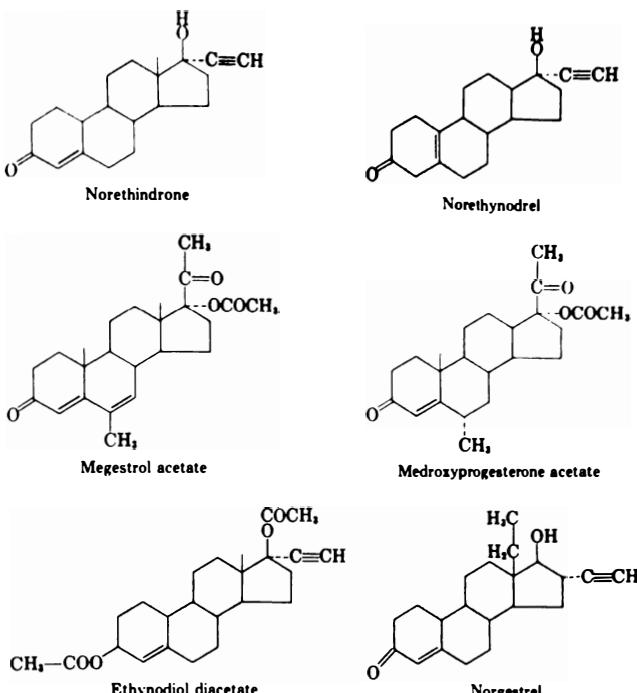


FIG. 21-14. Some clinically useful synthetic progestagens.

multiple forms of the hormone exist within the same species, and that castration and administration of sex steroids can affect the composition. Known carbohydrates are sialic acid, mannose, galactose, fucose, glucosamine, and galactosamine.

Neuraminidase treatment removes the sialic acid and renders the hormone inactive by certain bioassay tests. Such treatment is sometimes utilized to remove FSH contamination of LH preparations. Mild treatment with periodate or borohydride modifies the carbohydrate and also influences biological potency. There are unsettled questions about whether changes in carbohydrate content directly affect interaction with receptors or whether they render the molecule extremely susceptible to rapid degradation.

The hormone is relatively resistant to proteolytic enzymes and to urea denaturation; these procedures have been utilized to remove biological effects of LH contamination. But there are still many uncertainties concerning how much overlap in properties

of FSH and LH will be found when totally pure preparations become available.

The α -subunit is almost identical with that of TSH and LH; it does not seem to have hormone potency, but has been implicated in binding of the hormone to receptors and in protection against metabolic degradation. Hormone potency seems to reside in the β -subunit. According to some authors, low levels of hormone activity can be obtained with β -subunits alone; but according to others, this is explained on the basis of "contamination" by α -units. Combination of an FSH β -subunit with an α -subunit of the other glycoprotein hormones restores FSH activity. The α -subunits from different species also show similarities and may account for some of the observed cross-reactivities. A potent FSH preparation can be obtained by combination of an α -unit of one species with a β of another.

There is speculation on the possibility that adenohypophyseal cells produce an excess of α -subunits, and that formation of the β -unit only is closely regulated. Under

some conditions it has been possible to demonstrate the presence of excess α -units in the blood plasma.

Sources of FSH

The term *gonadotroph* designates cells of the adenohypophysis which secrete either FSH, LH, or both hormones. Methods for establishment of identity of the cells are described in Section VII, and the problems of determining whether separate cell types (folliculotrophs which secrete FSH and interstitialtrophs which secrete LH) co-exist are discussed.

Regulation of FSH Secretion^{1, 2, 3}

Hypothalamic Hormones. It is established that hypothalamic hormones exert stimulatory influences on synthesis and release of FSH. Problems of determining whether one, two, or more hypothalamic principles are involved in control of FSH and LH were mentioned in Chapter 20 and are discussed further in Section VII.

During estrous and menstrual cycles, release of LH is associated with simultaneous release of FSH. But there are also conditions under which differential hormone release can be accomplished, and concentrations of sex steroids in the blood plasma seem to play a role in determining what is secreted by pituitary gonadotrophs. There are reports that stimulation of specific sites within the hypothalamus can lead preferentially to release of FSH alone or LH alone. According to some observers this provides evidence for the existence of separate release factors; but according to others, alterations in the microenvironment may account for differences in responses of pituitary cells to hypothalamic stimulation.

Role of Estrogens. Low levels of estrogens can exert a *positive feedback control* leading to increased FSH secretion. During early phases of the ovarian cycles (see below), such influence may provide for gradual increases in amounts of FSH secreted which in turn lead to rising estrogen levels, until a sufficient steroid concentration is attained to trigger release of LH. Positive feedback may also play a role in maintaining hormone levels in immature females, and in mechanisms leading to onset of puberty. Positive control seems to be exerted at the level of the hypothala-

mus, so that the FSH-secreting cells are indirectly stimulated by hypothalamic hormones.

Higher estrogen concentrations exert a *negative feedback control* over FSH secretion. Inhibition of FSH secretion occurs during normal ovarian cycles and can also be demonstrated after exogenous administration of estrogens.

When one ovary is removed, the remaining one undergoes compensatory hypertrophy. This is known to be related, in part, to removal by surgery of the inhibitory influence of estrogens on FSH release; FSH secretion is elevated during the time the remaining ovary undergoes rapid growth. During pregnancy in many species (including humans), FSH synthesis and secretion are very low, and this has been attributed to negative feedback influences exerted by the high plasma estrogen concentrations.

Studies involving implantation of estrogens into specific sites, and others in which estrogens have been directly applied to pituitary cells *in vitro*, are consistent with influences of steroid on both the hypothalamus and the pituitary gland.

On the basis of observations that relatively low doses of estrogens can suppress *hypersecretion* of FSH in ovariectomized animals whereas only large doses reduce FSH secretion of the intact animal, it has been proposed that estrogens function to maintain gonadotrophs in a "normal" condition; but that in the presence of "conditioning" levels of steroid hormones, pituitary cells are insensitive to relatively wide fluctuations of plasma estrogen concentrations.¹ The implication is that estrogen suppression is *pharmacological* except during pregnancy or after ovariectomy.

Other Hormonal Influences. High concentrations of progesterone can inhibit FSH secretion, but it is not known whether progesterone plays a *physiological* role in regulation of secretion of this gonadotropin during nonfertile cycles. Sustained high progesterone levels during long luteal phases, pregnancy, and pseudopregnancy may reduce the incidence of follicle maturation by suppression of FSH; but a direct inhibitory influence of progesterone on the ovary has also been proposed. There are indications that FSH may act on both the hypothalamus and the pituitary gland to influence FSH secretion.

It has been proposed that FSH exhibits functional pleomorphism.¹⁷ Administration of testosterone propionate to rats leads to production of *andro-FSH* with high bioassay: immunoassay potency, increased sialic acid content, prolonged circulatory survival time, and increased apparent molecular weight, whereas administration of estradiol benzoate promotes formation of *gyno-FSH* showing alterations opposite in direction. Related differences in properties of FSHs synthesized by intact, untreated males as compared with normal females have also been found, while untreated castrates of either sex produce an intermediate-type *neuter-FSH*. The finding of such FSH heterogeneity may provide new insights into regulation of FSH secretion in males as well as in females.

Functions of FSH in the Female^{1, 2, 3}

It is certain that FSH is required for prepubertal maturation of the ovary, for growth and development of immature follicles, and for accomplishment of compensatory hypertrophy following unilateral ovariectomy. It prepares follicular cells for secretion of estrogens, but formation of follicular fluid and steroid synthesis seem to require the additional presence of at least small amounts of LH.

It is extremely difficult to sort out "pure" FSH actions because of all FSH functions seem to be carried out in the presence of estrogens, and FSH release from the pituitary is accompanied by LH release. (Administration of LH antibodies may not completely remove effects of very small amounts of that hormone.)

The corpus luteum of many species secretes estrogens. FSH may play a role in maintaining estrogen secretion at this time and also during early pregnancy. In the hamster, and probably in other species, FSH has been implicated in maintaining the corpus luteum.

Little attention has been directed to the possibility that FSH performs additional nonreproductive functions, and there is no evidence for the existence of FSH receptors outside the ovary. In view of the profound influences exerted on follicular cells, and growing evidence that most hormones act on a wide variety of tissues, the possibility is worth considering.

LUTEINIZING HORMONE

Chemistry and Origins^{1, 2}

LH (ICSH) is a glycoprotein hormone made up of α - and β -subunits. The β -unit contains less carbohydrate than that of FSH. It appears to be relatively rich in mannose. Sialic acid is present in very small amounts in LHS obtained from some species and appears to be totally absent in others; it is not surprising, therefore, that neuraminidase treatment has little effect on biological activity.

More is known about amino acid composition and arrangement than is the case for FSH; but LH is more sensitive to proteolytic degradation, and it is not yet clear whether the hormone exists in multiple closely related forms (or whether some of the reported differences arise during extraction and analysis). The pleomorphism described above for FSH does not seem to apply to LH.

Although clear differences in makeup of bovine, ovine, porcine, murine, equine, and primate hormones have been found, most mammals respond to LHS derived from other species; and differences in potency by bioassay are smaller than those seen for FSH.

Molecular weights seem to range between 26,000 and 34,000 for most mammals, but a weight of 44,500 has been reported for the horse.

Biological properties of LH overlap substantially with those of chorionic gonadotrophins. Since the chorionic hormones are readily obtained from pregnancy urine, they have been used in many "LH" studies. There are experimental conditions under which it becomes necessary to make distinctions, although the two hormones are known to compete with each other for receptor sites.

Regulation of LH Secretion^{1, 2, 3}

Hypothalamic Control. LH is needed in small amounts during most of the ovarian cycle; but there is also a requirement for a rapid "surge" of LH release just prior to ovulation. LH surges may regulate atresia of some follicles while promoting ovulation in others.

Neurons of the *ventromedial arcuate* region of the hypothalamus seem to be directly involved in synthesis and release of a hypothalamic hormone (LRF, p. 258) which maintains structure and function of the LH-secreting cells of the adenohypoph-

ysis. Tonic control exerted by this part of the hypothalamus provides for secretion of small amounts of LH during most of the cycle. If this region is "deafferented," i.e., severed of synaptic connections, the LH-secreting cells sustain certain aspects of reproduction, but no preovulatory surges occur and follicles do not ovulate.

Neurons located more anteriorly, and especially within the preoptic area, synapse with the LRF-secreting neurons. They are receptive to appropriate stimuli; when activated, they promote the LH surge. Dopamine has been implicated as the major transmitter, but there is also some evidence for participation of norepinephrine; and influences of epinephrine and of acetylcholine have been described. Serotonin may be involved in inhibitory control.

In "spontaneous ovulators" (p. 292) the preoptic neurons are believed to be stimulated by rising estrogen concentrations which occur toward the end of the period in which follicles mature and prepare for ovulation; and the neurons respond to exogenous administration of estrogens. In "induced ovulators," the surge is believed to be triggered by neurogenic stimuli arising during coitus; similar effects can be induced by artificial stimulation of the uterine cervix.

LH surges can be blocked by (1) administration of certain central nervous system depressants, e.g., nembutal, (2) severing of connections between the preoptic and ventromedial-arcuate regions, (3) interference with LRF synthesis or release, (4) reduction of sensitivity of pituitary cells to LRF, and (5) promoting a sustained "leak" of LH from the pituitary gland so that not enough can build up for the surge.

Preoptic cells receive inputs from other parts of the brain and are affected by environmental stimuli. If rats or mice are maintained under conditions of continuous environmental lighting, they tend to go into a condition of "continuous estrus" (CE, persistent estrus, PE) in which follicles prepare for ovulation but no LH surge occurs to promote the ovulation. Such animals will exhibit the LH surge if they are activated by mating or by application of stimuli to the preoptic area. It is believed that the continuous light affects sensitivity of the preoptic cells to estrogen concentrations.

Female rats given a single potent dose of androgen on the first day or two after birth usually do not ovulate after the onset of puberty. Many observers attribute this to developmental influences on the preoptic cells which render them insensitive to the usual physiological stimuli. (But others have suggested that there are defects in the ventromedial-arcuate region associated with subnormal synthesis of LRF.)

Some cyclical changes in reproductive function seen in hibernating animals and seasonal breeders have been attributed to alterations of LRF secretion mediated via hypothalamic neurons; the pineal gland has been implicated in certain of the conditions (Chapter 25).

Recent findings implicate neurons outside the ventromedial arcuate region in synthesis and phasic release of LRF.²²

Steroid Hormones. As noted above, rising levels of estrogen are believed to trigger LH release through action on hypothalamic neurons. High doses of estrogens provide inhibitory influences, and there is evidence that these are exerted on both the hypothalamus and on the pituitary gland. They probably contribute to physiological reduction of LH secretion toward the end of each ovarian cycle.

Small amounts of progesterone and of 20 α -hydroxyprogesterone have been implicated in facilitation of LH release around the time of ovulation. But high doses are inhibitory. In some species, the high levels of progesterone which build up during the luteal phase of the ovarian cycle may participate in reduction of LH secretion at that time. LH secretion is very low during pregnancy in some mammals, and this has been attributed to combined inhibitory effects of both estrogens and progestagens.

Other Influences. LH may directly regulate its own secretion via short feedback loops acting on the hypothalamus. "Ultrashort" negative feedback of LH on LH secretion and of LRF on LRF secretion has also been proposed.

MECHANISM OF ACTION OF FSH AND LH²³

Specific hormone receptors have been identified in cell membranes of target organs, and purified receptors are now being utilized in extremely sensitive gonadotro-

phin assays. FSH binds to granulosa cells and has been found in follicular fluid; LH binds to theca and interstitial cells; but there is also indirect evidence for FSH influences on thecal cells and for actions of LH at least on immature granulosa cells.

Relatively little is known of the mechanisms of action. Both of the gonadotrophins stimulate adenylate cyclase; and exogenous cAMP mimics hormone actions. Most of the hormone actions can be blocked with actinomycin D and with puromycin. It is therefore suspected that they depend largely upon synthesis of new RNA. But the gonadotrophins can also promote glucose uptake, glycolysis, and lactic acid formation *in vivo* and *in vitro*, and these actions are not blocked by the inhibitors.

PROLACTIN

Prolactin (PRL; lactotrophic hormone, luteotropic hormone, LTH; mammotrophin, MTH) is a species-specific protein hormone secreted by acidophil cells of the adenohypophysis (Section VII). Influences of the hormone on carbohydrate, water, and electrolyte metabolism, on body composition, and on male accessory reproductive structures have been described in earlier chapters. A role in implantation and in lactation is considered in Chapter 22.

Specific PRL receptors have been recently identified in a large number of cell types not formerly classified as "target organs" including those of the liver, kidney, and adrenal glands. There is growing evidence for a much broader role than was previously suspected. PRL has been recently implicated in mechanisms for onset of puberty in male and female mammals.

Recent attention has been directed to the cyclic variations in PRL secretion which accompany the secretion of gonadotrophins during ovarian cycles, and to the marked increases in PRL secretion with advancing age. The full significance of the findings has not yet unfolded. Influences this chapter. LH plays a role in induction of PRL receptors.

ESTROUS AND MENSTRUAL CYCLES^{1, 2, 14}

Female reproductive cycles have been the most widely studied of all rhythmic biological functions. The menstrual cycles

of primates have much in common with the estrous cycles of other mammals; and patterns of a different but related nature are seen among other vertebrate classes.

The cycles can be conveniently divided into two parts: (1) a *follicular or preovulatory phase* in which follicles and ovocytes mature in preparation for ovulation, accessory reproductive structures prepare for copulation and fertilization, and the uterine lining initiates changes needed for reception of the conceptus; and (2) a *post-ovulatory or luteal phase* during which the corpus luteum develops from the evacuated follicle and secretes the progesterone needed for completion of the uterine lining.

Differences between Estrous and Menstrual Cycles

There are two obvious differences; they are quantitative rather than qualitative.

Uterine Differences. In females with menstrual cycles, there is extensive preparation of the uterine lining for implantation of the conceptus (Chapter 22). If fertilization does not occur, small blood vessels are ruptured as the lining is sloughed off at the end of the cycle, and there is a *menstrual flow* (which is far more obvious in humans than in most other primates). Some non-primates (e.g., the dog) have a bloody discharge during estrus which is unrelated to sloughing of the uterine lining.

Behavioral Differences. In animals with typical estrous cycles, females become receptive to the males close to the time when ova are ready for fertilization; and they exhibit characteristic postural responses to vulval stimulation. Greater physical activity is readily measured by use of "running wheels" which animals can enter voluntarily.

There are limited behavioral changes associated with menstrual cycles in some nonhuman primates, but, as noted in Section I, at least part of this can be attributed to the fact that males are more attracted to (and therefore likely to stimulate) females approaching the fertile time of the cycle. Among women, individuals may experience predictable variations in sexual interest associated with ovarian cycles; but the timing of such interest varies from one individual to the next, and in the same person at different periods. Hormonal in-

fluences are easily overridden by psychic factors. Perhaps the very vague associations between reproductive cycles and human behavior, plus the fact that animals with definite estrous cycles fail to exhibit "raging hormonal storms which impair judgment" should be pointed out to certain (male) vocal but untrained commentators on human performance.

Genetic and Environmental Influences on Ovarian Cycles

Among the aspects controlled by genetic factors are *lengths of the cycle as a whole*, *relative lengths of the component parts* (and of "resting periods" between cycles), the *degree to which there is dependence upon environmental stimuli*, the *kinds of stimuli* to which the animal is susceptible, and certain aspects of hormonal control.

Species differences are obvious. But even within the same strain of rats housed in common cages and fed an identical diet, some will exhibit a regular 4-day cycle while others require 5 days. Selective breeding can enhance the probability that the offspring will have a predictable cycle length; but environmental manipulation affects expression of the genetic tendency.

Animals with very short cycles (e.g., rats, mice, hamsters) have a much abbreviated luteal phase, with corpora lutea which disintegrate soon after formation if fertilization does not occur. By contrast, the domestic dog has a sustained luteal phase, and long intervals elapse between cycles (most have 2 per year). Primates have a luteal phase almost equal in length to the follicular period, and a new cycle commences soon after completion of the previous one.

There are seasonal breeders which exhibit a single cycle per year and are therefore said to be *monoestrous*. Polyestrous seasonal breeders may experience several cycles in rapid succession, followed by a long quiescent period. Some animals reproduce only at specific times even when uniform environmental conditions are artificially maintained; others display obvious seasonal patterns in the wild but may cycle throughout the year if kept in a laboratory or zoo. Most "year-round" breeders show greater fertility during certain months even when environmental temperature, lighting, and humidity are kept constant.

In addition to temperature and humidity, some animals respond to the amount of rainfall, changes in the intensity or wavelength of environmental illumination, the photoperiod, the abruptness with which light-dark changes occur, food availability, and the presence of other animals.

While there is a tendency for very small animals to have short cycles, no consistent relationships are found between size of the animal, length of the cycle, duration of the gestation period, or number of young per litter. Thus, while the rat ovulates every 4-5 days, the only slightly larger mink has a quite different cycle of 8-9 days, and the guinea pig requires 16-17 days. A 90-day rhythm has been described in foxes, while sheep complete the process in 16-17 days. Women average about 28 days (but healthy fertile individuals have been known to menstruate as frequently as every 24 or as seldom as every 35 days and 30-day intervals may be the most common); the chimpanzee cycle averages 35 days, while the periodicity of the elephant has been estimated at 21 days.

The 4-day cycle of the hamster is associated with a 16-day gestation period, but 4-day mice carry their young up to 23 days, and the gestation period of the sheep (with 16-day cycles) averages twice that of the guinea pig. Data on cycle lengths, duration of gestation periods, and average number of young per litter are shown in Table 21-1.

"Spontaneous" vs "Induced" Ovulators

Most mammals (including humans, monkeys, hamsters, and mice) ovulate at the end of the follicular phase and proceed according to a predictable timetable to develop corpora lutea, without need for external stimulation. They are classified as *spontaneous ovulators*.

Some mammals well known for their fertility (cats, rabbits, ferrets) go through the first or follicular phase and remain in a state of sexual receptivity for relatively long periods with follicles ready for ovulation. Mating (or artificial stimulation, e.g., rubbing the cervix with a glass rod) rapidly induces ovulation and onset of the luteal phase. Animals of this type are *induced or reflex ovulators*.

It was once believed that animals could be rigidly classified into one group or the

TABLE 21-1
Cycle Length, Number of Young, and Duration of Gestation for some Mammals

Animal	Cycle Length	Number of Young usually Produced at One Time	Length of Gestation Period
Mouse	4 or 5	3-8	19-23
Rat	4 or 5	3-17	21-23
Hamster	4	2-6	16
Guinea pig	16-17	1-6	63-70
Fox	90	1-8	52
Dog	60	2-6	61
Sheep	16	1-2	144-180
Cow	21	1-2	277-290
Elephant	21	1	607-641
Chimpanzee	35	1-2	227-240
Rhesus monkey	28	1	159-174
Human	28	1	271-289

other; but it now seems more appropriate to consider the differences quantitative rather than qualitative. Ovulation can be induced in "spontaneous" species at times when it would otherwise not occur. A variety of stimuli have proven effective, including nonspecific "stress" and coitus. It has been observed that the frequency with which conception occurs in the human after (stress-related) rape is much higher than would be expected on the basis of known timing of the menstrual cycles. And most of the failures of the "rhythm" method of contraception (Chapter 22) are probably attributable to induction of ovulation at unusual times.

Moreover, "spontaneous" animals can be converted to "induced ovulators" by manipulation of environmental factors. For example, rats maintained under continuous environmental illumination tend to go into a state of "constant estrus" (CE, persistent estrus, PE); but ovulation can be brought about by appropriate stimulation, such as coitus.

HORMONAL CONTROL OF OVARIAN CYCLES^{1,2}

It is possible to piece together a unified picture which is largely but not completely

supported by experimental evidence. All of the species variations have not yet been defined, and it is possible that changes in concentration of a hormone in one group may be replaced in another by changes in sensitivity to that hormone.

During the follicular phase, both FSH and LH concentrations in the plasma rise; the change in FSH may be greater. FSH stimulates maturation of the follicles, but small amounts of LH seem to synergize with FSH in promoting antrum formation and estrogen secretion.

- Estrogens are needed directly for maturation of the follicles, and they exert a positive feedback influence on FSH secretion which contributes to buildup of the latter. There is no real evidence for positive feedback of estrogens on LH secretion at this time. Estrogens released into the systemic circulation exert influences described above on the uterus, cervix, vagina, oviducts and hypothalamus, and they prepare the animal for mating.

In "spontaneous ovulators," estrogens rise at a critical rate (or to a sufficient level) to "trigger" suitably prepared cells of the preoptic region of the hypothalamus, and this leads to the LH "surge." Progesterone and its metabolites may facilitate additional LH secretion after some has been released.

In "induced ovulators," the preoptic cells await neuronal stimulation from the cervix which occurs during mating. Similar neuronal inputs may play a role in spontaneous ovulators. (In some species, more follicles ovulate if the animals are permitted to mate.)

LH promotes ovulation and formation of the corpus luteum and contributes to progesterone secretion. The LH surge is accompanied by release of smaller amounts of FSH. It is not known if FSH contributes to the ovulatory events; anti-FSH sera given at this time do not prevent ovulation, but ovulation can be triggered by administration of very large amounts of FSH which contain minimal LH contamination. FSH may facilitate estrogen secretion by the corpus luteum.

Prolactin secretion during the cycle follows elevation of plasma estrogen concentrations. It has been implicated in maintenance of the corpus luteum in some species, in contribution to progesterone secre-

HORMONES AND REPRODUCTION

tion, and in slowing of progesterone degradation. But suppression of prolactin secretion does not seriously interfere with the cycle in at least some species.

Progesterone secreted by the corpus luteum exerts actions on reproductive structures described above. Both progesterone and estrogen probably exert negative feedback inhibition of FSH and LH secretion toward the end of the ovarian cycle.

After a time characteristic for the species, corpus luteum function declines, and the structure is converted into a corpus albicans. Conditions affecting maintenance and demise of the corpus luteum are considered below.

During the luteal phase, selected follicles of at least some species begin preliminary preparation for later maturation into Graafian follicles. Follicles which have already matured beyond a certain stage but have not ovulated degenerate.

The decline of corpus luteum function leads to sloughing of superficial cells of the uterus and vagina and to reduction of steroid hormone levels to the point where negative feedback inhibition of gonadotrophin secretion ceases. This is soon followed in most species by initiation of a new cycle.

In many mammals, infertile matings can lead to prolongation of the life of the corpora lutea and induction of the condition of *pseudopregnancy*. Neurogenic pathways from the reproductive tract to the hypothalamus seem to mediate its onset; in at least some, secretion of prolactin is important.

A variety of external stimuli influence reproductive cycles; these include not only environmental lighting, temperature, humidity, and food supply, but also pheromones and other odorous substances. The pineal gland is involved in mediation of some of the effects.

EXAMINATION OF VAGINAL SMEARS^{14, 16}

Cell samples are obtained from the inner surface of the vagina by simple procedures (which are painless to the subject), suspended in saline on a glass slide, and examined under the light microscope. The technique is widely used in the laboratory for timing and study of events of the

ovarian cycle, and has also been adapted for bioassay of hormones. Clinical use of vaginal smear techniques has provided valuable information on ovarian hormones, effects of therapy, and existence of pathological conditions.

In laboratory rats, four stages are recognized:

1. During *proestrus*, the vaginal walls are thin enough to permit diapedesis of leucocytes. Small, round, nucleated epithelial cells with granular cytoplasm are easily picked up with a bit of saline-moistened cotton at the end of a toothpick (or with a small probe). Smears taken at this time contain both the epithelial cells and scattered leucocytes (Fig. 21-15A).

As proestrus progresses (over a period of 16-22 hr), the vaginal epithelial cells increase in size and undergo rapid proliferation. Barriers to diapedesis mount, and the numbers of leucocytes in the smear diminish toward the end of this stage.

During proestrus, follicles (which began maturation earlier) are approaching the preovulatory stage, estrogen is secreted in increasing quantities, and LH is released from the adenohypophysis. Estrogens pro-

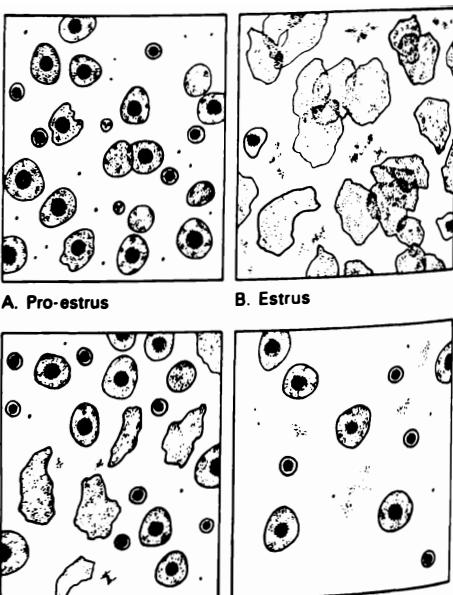


FIG. 21-15. Appearance of vaginal smears of the rat at four phases of the estrous cycle.

mote uterine hyperemia, and the uterus begins to accumulate fluid.

2. During *estrus* (which lasts 9-15 hr), the outermost cells of the vaginal lining gradually dry out as distance from the nearest capillary is increased by cell proliferation. The most superficial cells become flattened, irregular in shape and *cornified* (keratinized), and the nuclei are lost. Large numbers slough off. The estrus vaginal smear is dominated by the cornified cells which appear scale-like; no leucocytes are seen at this stage (Fig. 21-15B). Distention of the uterus, which started at proestrus, continues for a time, and the uterus appears "ballooned"; fluid is discharged toward the end of the estrus phase when secretion of small amounts of progesterone leads to relaxation of the uterine cervix.

"Behavioral estrus" is reflected in increased running activity, quivering of the ears, and sexual receptivity. Mating at this time is likely to result in pregnancy, since ovulation occurs toward the end of estrus. Ruptured follicles form corpora lutea, and the latter remain active if conception occurs. Mating with an infertile male, or artificial stimulation of the cervix, can lead to onset of pseudopregnancy. If no mating or other stimulation intervenes, corpora lutea form, but (compared with many other species) relatively little progesterone is secreted.

3. *Metestrus* starts soon after ovulation and lasts 10-14 hr. Sloughing of superficial vaginal cells continues into early metestrus, and cornified cells may therefore appear in the smear. But toward the end of metestrus, the vaginal wall has thinned sufficiently to permit some diapedesis, and leucocytes reappear (Fig. 21-15C). An occasional young (small, round, nucleated) epithelial cell and some mucus shreds may also be seen.

4. *Diestrus* is the longest and most variable phase of the cycle, lasting 3 days in some rats and 2 days in others. The vaginal wall is thin, permitting extensive migration of leucocytes, and the latter (along with mucus strands) make up the bulk of the smear. Occasional small epithelial cells may also be seen (Fig. 21-15D). Follicles begin ripening under the influence of small amounts of FSH and LH in preparation for the next cycle.

MAINTENANCE OF THE CORPUS LUTEUM^{1, 2, 8}

It has been observed that follicles which are damaged may undergo repair, but those from which the ovocyte is removed tend to luteinize. This raises the possibility that luteinization is a spontaneous process (requiring no hormones) which is inhibited by the ovocyte. Another possibility is that the ovocyte releases a *luteostatic factor* which travels to cells of the granulosa and theca interna at the time of ovulation or ovum removal. The observations do not necessarily rule out facilitation by LH.

The life span of the corpus luteum seems to depend upon certain factors in one species and on very different ones in another. In some, it may simply undergo spontaneous involution after following a genetically "programmed" series of changes.

LH is clearly needed for maintenance in several species. It has been proposed that follicles which begin development during the luteal phase of the cycle may contribute to death of the corpus luteum by "competing" for a limited supply of gonadotrophin. In the rabbit it looks as if LH does not act directly, but that it prolongs the corpus luteum by promoting estrogen secretion; in these animals (but not in most others) estrogen alone is sufficient.

PRL is apparently essential for maintaining the corpus luteum of rats, mice, ferrets, and some others; in these, the designation luteotropic hormone or LTH is therefore appropriate. Evidence against such a role is strong for humans, monkeys, guinea pigs, and rabbits; and it has been shown that administration of 2Br- α -ergocryptine (which blocks PRL secretion) does not impair ovarian cycles of women. On the other hand, there have been reports that a luteotropic effect can be demonstrated in hysterectomized but not in intact sheep, pigs, and cows. In at least some species, PRL synergizes with LH, and it enhances responsiveness to exogenous LH: but in the ferret it may function alone to maintain the corpus luteum.

PRL has been reported to increase the uptake of cholesterol and to affect activities of cholesterol esterases and of sterol-acetyl transferases. It also reduces formation of progesterone-degrading enzymes and

PUBERTY^{1, 2, 3, 6, 12}

significantly reduces the formation of 20-hydroxyprogesterone. Influences on ovarian enzyme activities have been observed not only in animals dependent upon PRL for corpus luteum maintenance, but also in some of the others. Hamsters may require FSH in addition to PRL and LH.

Hysterectomy prolongs the life of the corpus luteum in ewes, guinea pigs, pigs, hamsters, and certain others (but not in primates); in some, removal of one uterine horn affects only the ipsilateral ovary. It is believed that the uterus of some species produces a *luteolysin* which acts physiologically to terminate the activity of the corpus luteum. The concept is supported by additional studies in which uterine preparations have been shown to shorten pseudopregnancy. The uterine factor may reach the ovary via a direct lymphatic channel in those animals in which ipsilateral effects have been observed, or in which interference with lymph flow affects the responses of the ovary. In others, it may travel to the ovary via the systemic circulation. The existence of a direct blood channel from the uterus to the ovary is debated.

It seems likely that there are marked species differences which must be clarified. In some the uterine factor may act directly on the corpus luteum, and in others via influences on ovarian blood flow. Prostaglandins have been extracted from the uterus and they can promote termination of corpus luteum function; they have been implicated in reduction of ovarian blood flow, in inhibition of progesterone synthesis, and in promoting increases in formation of 20- α -hydroxyprogesterone. Some influences may involve reduction of ovarian cAMP. There are indications that estrogens can promote prostaglandin synthesis and release by the uterus. On the other hand, there is evidence that the uterine factor in at least some species is either a protein or is protein-bound.

Evidence for a uterine luteolysin in primates is questionable. According to some authors, the Fallopian tubes regulate corpus luteum life span in humans and other primates; according to others, the corpus luteum in these species is resistant to humoral factors and follows a programmed period of growth, hormone synthesis, and decline.

The timing of the onset of puberty depends upon complex interactions between genetic and environmental influences. The importance of genetic factors is best demonstrated by maintaining different strains of the same species under seemingly identical conditions, while effects of extraneous factors are most obvious when members of the same strain are raised under different conditions. Manipulations of neuronal, endocrine, nutritional and environmental factors can affect the onset of developmental changes and the numbers of germinal cells maturing at a given time; but they do not seem to influence the number of days required for production of gametes from germinal precursors.

All of the structures involved in reproductive processes seem to be capable of function long before the onset of puberty. (But they characteristically pass through a brief infantile phase during which they either respond poorly to stimulatory hormones or respond in a manner different from that seen later.)

Premature ripening of ovarian follicles and ovulation have been achieved by administration of appropriate hormones to very young animals. Precocious spermatogenesis can be induced in animals with long maturation times, but not in species in which the onset of puberty normally takes place within weeks after birth. Mature sperm cells are first seen in male rats at around 6½ weeks of age. Since the time required for development of mature sperm cells from type A spermatogonia is about 40 days, it is understandable that attempts to induce early spermatogenesis have been uniformly unsuccessful.

Responses of immature *accessory reproductive structures* to gonadal hormones are so predictable that they are widely used for hormone bioassay. *Secondary sex characteristics* can also develop ahead of time when immature animals are given hormones.

Pituitary glands of very young animals synthesize and secrete gonadotrophins, and they respond to administration of hypothalamic hormones. Immature pituitary glands implanted close to the hypothalamus can sustain reproductive func-

tions of mature animals.

Vascular channels between the hypothalamus and the pituitary gland are established early in life, as are synaptic connections between hypothalamic neurons secreting gonadotrophin release factors and other neurons of the brain.

Removal of one gonad leads to compensatory hypertrophy of the remaining one. This indicates that feedback mechanisms are operative in immature animals. Lesions placed at appropriate sites within the hypothalamus can interfere with the compensatory hypertrophy. Lesions at other sites within the hypothalamus and parts of the amygdala can advance puberty, while damage to parts of the hippocampus may block effects of such lesions.

Children with brain lesions and endocrine tumors have been known to develop at surprisingly early ages. Onset of menstruation has been observed at 6 months and growth of the penis at 5 months. There is a documented case of a 5-year-8-month-old girl giving birth to a normal infant and of a boy fathering a child before the age of 7 years. (Mature gametes are not usually produced in humans before the 12th year.)

All of the preceding is consistent with the belief that the entire reproductive system and its associated structures are capable of function long before the usual onset of puberty, and that something normally delays maturation until the appropriate age.

A widely accepted explanation for the delay is that *hypothalamic cells are especially sensitive to negative feedback influences* exerted by low concentrations of gonadal steroids. Some authors believe that the change in sensitivity which permits onset of puberty is but one aspect of more generalized, spontaneous maturation of the brain, while others believe that steroid hormones must build up to a critical level to induce the maturation. However, onset of puberty in female rats has been attributed to activation of positive feedback influences of estrogens on the hypothalamus,²³ and other species variations may be revealed in the future.

Both systemic and intrahypothalamic administration of estrogens can promote early maturation in female animals. This can be interpreted to mean that estrogens induce the necessary changes in a hypo-

thalamus that is already prepared and awaiting the hormonal stimulus. But it could also mean that a not-yet-prepared hypothalamus can be pharmacologically provoked to release gonadotrophin-releasing factors earlier than it would under physiological conditions.

A somewhat different concept is that parts of the brain outside the hypothalamus (and especially components of the "limbic system") exert strong inhibitory influences on the hypothalamus up to the time when the brain as a whole advances toward adult status. Estrogens could be affecting some function of neurons that influence those secreting release factors.

Brain lesions are the most common cause of precocious (or delayed) puberty in humans. Control mechanisms may not be identical for males as compared with females. Differences in patterns of secretion of gonadotrophins have been demonstrated in maturing animals of the two sexes. Some have been related to positive feedback influences of estrogens which may be operative in female but not in male animals. In many species the female develops earlier than the male. Among humans, disturbances in timing of onset of puberty is far more common among females. This could be related to the greater complexity of the female system.

The age for onset of puberty in human females decreased steadily over many decades for those residing in "developed" countries, but not seems to be stabilizing.¹ Evidence is strong for earlier onset of menstrual cycles in those living in urban as compared with rural regions. Opinion is divided on whether the major factor is nutritional or cultural. Experiments designed to separate out the two influences are difficult to interpret.

Nutritional factors must certainly play a role. "Well fed" infants are given a variety of protein-rich solid foods at an early age along with vitamin supplements; they grow and develop rapidly, and tend to be tall for their age and heavy for their height.

One group of investigators has advanced the concept that onset of menstrual cycles awaits attainment of a "critical body weight" which depends in part on accumulation of at least a minimal quantity of subcutaneous fat.⁷ Statistical studies have been cited which indicate that for any

given age, girls who are heavy for their height tend to undergo puberty at an earlier age, while undernourished girls who may be of normal height tend to have delayed onset of menstrual cycles. Other studies indicate that undernutrition in very young adults can lead to secondary amenorrhea which is correctable with diet. It has been pointed out that a reserve of calories (as fat) can be useful for sustaining pregnancy and lactation. There is no information on the nature of the signal to the hypothalamus associated with changes in body fat stores. The observations may be related to evidence (emanating from different laboratories) that adipose tissue contributes significantly to estrogen biosynthesis.

Other investigators have placed greater emphasis on the importance of external stimuli in maturation of the central nervous system and brain, and have suggested that bombardment of children today with a variety of stimuli not available many years ago (e.g., in the schools, from television, radio, etc.) has promoted earlier maturation.

Studies have been performed on experimental animals raised in litters of different sizes to determine whether early nutrition affects the time required for sexual maturation. Those reared in small litters grow more rapidly and develop earlier. Animals kept on minimal diets during the immediate postweaning period tend to exhibit delayed puberty, while those force-fed or given diet supplements may develop early.

The studies are complicated by the fact that animals raised in very small litters receive much more than additional food from the mother. They are licked and otherwise stimulated more often. Animals that are handled gently by the investigator during the first few days of life tend to mature earlier than non-handled siblings receiving the same treatment in every other way. But the "gentled" animals are often larger for their age. If animals are handled roughly or genuinely stressed, maturation may be delayed; a combination of reduced food intake and catabolic influences of glucocorticoids released in response to stress seems to be involved.

Prolactin may fit into the picture in ways that have not as yet been defined.²⁸ The

hormone is released in response to estrogens, but also in response to stress.

A number of "nonreproductive" hormones can influence the maturation processes. Animals deprived of thyroid hormones at an early age exhibit retardation of growth and maturation of skeletal, nervous, and reproductive systems. Later administration of thyroid hormones can induce skeletal and reproductive maturation (although the nervous system never fully recovers from deficiency of thyroid hormones at a critical stage of development). Interestingly, milder forms of *hypothyroidism* have been implicated in premature onset of puberty in human females.

There are marked species variations in susceptibility to environmental influences. When rats (which are nocturnal) are reared in darkness or blinded in infancy, puberty is often delayed. However, statistical data point to early onset of puberty in blind girls. Some species are far more affected by factors such as environmental temperature, humidity, rainfall, and presence of other animals than are others.

Removal of olfactory bulbs, and interruption of nerve pathways involved in transmission of olfactory stimuli have been reported to delay puberty under many circumstances; and olfactory denervation may exacerbate effects of blinding in rodents. Delayed puberty has been described in congenitally anosmic humans.

Influences of the pineal and thymus glands on the reproductive system are described in Chapter 25. Both delayed and precocious puberty have been found in patients with pineal gland tumors. Some clinicians believe most of the difficulties arise from mechanical pressure exerted on neighboring structures, while others attribute them to disturbances in pineal secretory functions.

MENOPAUSE^{1, 2, 6, 12}

Aging females of most mammalian species exhibit loss of fertility and cessation of regular ovarian cycles late in the life span. The changes are attributable to alterations in hormonal function. In rats and some other species examined, aging ovaries usually contain a sizable population of oocytes, and they may resume functions if transplanted to young recipients.

By contrast, cessation of menstrual cy-

cles (menopause) occurs *relatively* early (compared with total life span) in the human female and seems to result from depletion of viable germ cells. Descriptions of postmenopausal ovaries vary; in some, few or no germinal cells have been found while others seem to contain scattered ovocytes and some follicles.

There are indications that the ability to sustain a pregnancy declines earlier than the ability to produce fertilizable gametes. Gradual changes in hormone levels during the few years preceding cessation of menstrual cycles have been demonstrated. Estrogen and progesterone secretion by the ovary usually decline, and rising gonadotrophin levels have been attributed to reduction of negative feedback influences of the steroid hormones. But women exhibit considerable individual variation; in some, the interstitial cells of the ovary produce large quantities of hormones, and the adrenal cortex may increase its output. There is a tendency for increased secretion of androgens and especially of androstenedione. Sufficient estrone may be produced directly (supplemented by estrogen formation from the androstenedione) to elicit irregular uterine bleeding for years after follicles have ceased to ripen and ovulate.

The relatively early cessation of ovarian function in the human may confer several biological advantages.

It was noted above that ovocytes which mature toward the end of the fertile period in women have emerged from a state of "arrested development" in which they have been maintained for many decades, and that the incidence of chromosomal aberrations of the zygote rises sharply when the female parent is more than 42 years old. Termination of ovarian function at middle age may therefore provide protection against birth of large numbers of abnormal infants. (Problems associated with aging ovocytes do not arise in species with much shorter life spans.)

Gestation in the human is long and demanding. It is reasonable to believe that pregnancy is far better tolerated by young than by middle-aged women; the concept is supported by evidence of greater incidence of complications in women experiencing pregnancy toward the end of their reproductive years.

The period during which human infants

and children require maternal care is far greater than that for any other mammal. There is obvious need for survival of the mother for many years beyond the time of birth of the youngest child. Women past the child-bearing years have a fund of wisdom and experience which can be used to contribute substantially to the rearing of children born to younger mothers. Therefore, from a purely biological viewpoint, the relatively early cessation of ovarian function in the human may be wholly desirable for perpetuation of the species.

Cessation of reproductive activity is associated with a number of bodily changes. Some are well understood (and can be related to hormonal changes), while others are not. Certain information useful in sorting out hormone-related changes from those attributable to aging processes or psychological factors can be obtained from examination of effects of ovariectomy in younger women. (However, ovariectomy is performed because of the presence of pathological conditions, and it, too may be associated with psychological problems in young women.)

There is a thinning of the bones, and some clinicians attribute the "postmenopausal osteoporosis" to estrogen deficiency. Opinion is divided on whether estrogen therapy is useful for alleviation of the condition; transient (but not sustained) improvement has been described (see also Section IV). There is no real evidence that hormones are useful for reversal of any process associated with normal aging.

Vasomotor instability with fluctuations in blood pressure and pulse rate, and appearance of "hot flashes" are commonly seen during the menopause and after ovariectomy in younger women. The symptoms are reproducibly relieved by administration of estrogens; but in menopausal women, they most often subside after a time without hormone therapy. Surprisingly little is known of the underlying etiology of so common a symptom. The hot flashes have been attributed to high gonadotrophin levels which result from loss of steroid hormone inhibition. It is established that gonadotrophin levels do rise and that they are reduced by estrogen treatment; on the other hand, there are several clinical conditions associated with

high gonadotrophin levels in which such a symptom does not appear. Moreover, gonadotrophin levels after ovariectomy or menopause do not always fall when symptoms abate spontaneously.

Symptoms which have been labeled "psychogenic" by some clinicians include headache, fatigue, dizziness, sense of weakness, gain in body weight, and psychological depression. These, too have been reported to respond to estrogen treatment in some but not in all cases.

It is extremely difficult to make judgments about most of the conditions. It is on the one hand very common for women to experience the menopause at a time when special psychological situations arise. The endocrine changes often occur just when a woman's social life and activities are undergoing radical change because children emerging from adolescence no longer need maternal care (and the term "empty nest syndrome" has been applied to women suddenly finding themselves without a function they have performed for many years). Some women are much distressed over external signs of physical aging and possible loss of attractive appearance. Moreover, this may be the very time when husbands experiencing psychological difficulties of their own make special demands. On the other hand, the symptoms of menopause appear frequently in women quite busily engaged in careers or other activities which hold their interest.

Responses to hormone treatment also raise problems of interpretation. It is well known that humans are affected by the very presence of an individual interested in their welfare, and that administration of placebos can have beneficial effects. In some "double blind" studies in which neither the patient nor the investigator knows which patients are receiving placebos and which are receiving potent hormone preparations, there are always a few subjects who respond only to the hormones and some that respond to all types of "medication."

There are physicians who maintain that estrogens should be administered to all women who seem to benefit from them. But the treatment is not without drawbacks; e.g., some patients are troubled with water retention, nausea, and other difficulties. There are also physicians who

maintain that any symptom experienced by a large percentage of the population should be classified as "normal" and should therefore not be treated with medication. Recent statistical studies point to greatly increased incidences of endometrial cancers and hepatic tumors in postmenopausal women if the latter receive estrogen therapy.

There are lengthy discussions in the literature about whether there is a "male climacteric." While mature sperm are produced by older men, the numbers may be reduced with advancing age. Testosterone secretion tends to decline late in life, but this is offset in many individuals by reduced rates of hormone catabolism and excretion. Just as in females, there are psychological conditions which may appear at the time that hormone changes are suspected to occur. For example, as middle age approaches, some men are concerned with growing awareness that they are not as energetic as younger workers in their field, or are less familiar with new techniques accessible to those educated at a later date. Some men react strongly to age-related changes in physical appearance, or perhaps to problems with wives experiencing one kind of difficulty or another. Again, as with women, some men have responded well to administration of hormones (especially of androgens alone or in combination with thyroxine). But most do not exhibit especially favorable responses, and in many, androgen administration leads to disturbances of hepatic function.

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22. Conception, Contraception, Gestation, Parturition, and Lactation

FERTILIZATION^{1, 2, 4, 9, 16}

Site and Timing

Fertilization usually takes place within the mammalian Fallopian tube. The ovary is a normal site for a few species (*e. g.*, the hedgehog), and it is suspected that the rare ovarian pregnancies of primates result from conception within the ovary.

Ovocytes sometimes penetrate deep into the peritoneal cavity, and motile spermatozoa have been identified there after coitus. Abdominal pregnancies can originate from union of gametes near the implantation site, but it is suspected that most result from movement of the conceptus following tubal or ovarian fertilization.

Female germ cells deteriorate soon after ovulation^{24, 25}; if fertilization is delayed long beyond the optimal time, the incidence of abnormal zygotes rises sharply. There are marked species variations in duration of sperm viability; survival is shortest when the sperm are free in the

vagina, intermediate in the body of the uterus, and longest when the cells gain access to crypts of an estrogen-dominated cervix.

Human spermatozoa begin to show signs of deterioration and loss of ability to fertilize within 24 hr after leaving the male tract, although they retain full motility at that time. While normal pregnancies can result from fertilization taking place 3 days after coitus, it is suspected that "overripe" sperm contribute to formation of abnormal zygotes. Horse sperm seem to remain healthy for at least 6 days after ejaculation. Some of the bats regularly retain viable gametes for months, and survival for years has been described in snakes.¹⁴

Synchronization of coitus with ovulation is achieved in "spontaneous ovulators" with estrous cycles (Chapter 21) by timing of sexual receptivity of the female; in induced ovulators, the ovocyte release is triggered by mating stimuli. In humans and other primates (in which sexual activi-

ity can occur at any phase of the ovarian cycle), conception is most likely to occur when active sperm enter the female tract between 48 hr before and 12 hr after ovulation.^{4, 5}

Sperm Transport^{1, 2, 4, 6, 16}

Sperm may be catapulted directly into the uterine cavity (in horses, zebras, pigs, dogs, rats, and others), or they may be deposited within the vagina (as in humans and some other primates, rabbits, cows, and sheep). Passage from the vagina through the estrogen-dominated cervix to the uterus occurs within minutes. (Such findings lend objective support to the practical knowledge that postcoital douching has little influence on the probability of conception.)

Estrogens promote secretion of copious quantities of an electrolyte-rich, acellular watery secretion which favors sperm survival and transport (Chapter 21). The hydrogen ion content is optimal for sperm motility; carbohydrate and amino acid components support metabolic processes, and macromolecules afford protection against deleterious influences of vaginal fluids. The geometrical arrangement of glycoprotein filaments of cervical mucus directs orientation of the spermatozoa so that heads of some are sent toward the uterus while others are shunted into the protected regions of the cervical crypts. Sperm that are unusual in size or shape seem to be selectively excluded from entry into the uterus. When sperm of foreign species are mixed with native whole semen, their passage is preferentially retarded.

Antibodies promoting sperm immobilization and agglutination, and an antiagglutination factor which exhibits highest activity around the time of ovulation have been identified in human cervical mucus.¹ Physiological functions have not been evaluated, but it is suspected that excessive or abnormal production of antibodies and low antiagglutination titers contribute to some forms of human infertility.

The cervix of at least some of the mammals functions as a *sperm reservoir* providing for sequential release of limited numbers over a period of hours in most forms and of days in others. Activity of proteolytic enzymes which contribute to liberation of sperm from sites of entrapment

within the crypts seems to be high close to the time of ovulation.

Contraction of the cervix and vagina during coitus aid in direction of sperm toward the uterus.

Reduction of intrauterine pressure via uterine and respiratory contractions has been described when coitus occurs close to the time of ovulation; but it has not been established that this promotes "drawing" of sperm through the endocervical canal.

While estrogens favor sperm passage around the time of ovulation, progesterone secreted at a later stage of the cycle acts in several ways to reduce chances of fertilization of oocytes which have "passed their prime." Progesterone promotes formation of a highly viscous cervical secretion which is chemically hostile to sperm cells. The high acidity reduces motility; and alignment of glycoprotein filaments results in formation of interstices too small to permit free passage. Polymerization of macromolecules contributes to the mechanical impediment. Leucocytes which are abundant in progesterone-dominated secretions engage in sperm destruction through phagocytosis and release of lytic enzymes, and contraction of the exocervix contributes to the mechanical barrier. Fewer sperm enter the uterus during "unfavorable" phases of the ovarian cycle of mammals utilizing vaginal insemination; conception incidence can be increased by placement of sperm directly into the uterus.

Prolonged secretion of progesterone during pregnancy leads to formation of a relatively dry, almost rubbery cervical secretion which retards entry of bacteria as well as of spermatozoa.

Mechanical barriers to sperm passage provide a partial explanation for the low fertility associated with reduced sperm numbers. Usually a very small fraction of the ejaculated sperm proceeds through each of the junctions of the female tract; in most species not more than 0.1% gains access to the fertilization site. There are reasons to believe that substances released by heads of "accessory" spermatozoa contribute to the fertilization process. (If this is true, the situation is reminiscent of functions of "cohort" ovarian follicles and of accessory corpora lutea, Chapter 21.)

Fertilization seems to require the presence of several sperm cells in the vicinity of the ovocyte. But it is also possible that conditions (natural or artificial) leading to reduction of sperm counts lead simultaneously to other factors affecting fertilization process, and that metabolites produced by motile sperm influence the microenvironment.

The distance traveled by the spermatozoa from the cervical entrance to the site of fertilization at the ampullary-isthmic junction of the Fallopian tube depends upon the anatomy of the female tract. Observations on swimming patterns and rates, and on the time interval between coitus and first appearance of sperm in the oviduct lead to the conclusion that some process must accelerate passage in cattle and other large species, and probably also in the human (but not in rabbits). Uterine contractions are most evident around the time of estrus. Estrogens directly stimulate uterine muscle and enhance its sensitivity to other agents, and it is known that uterine contractions can promote upward movement of inert particles.

Oxytoxin can accelerate movement of particles within the estrogen-dominated uterus, and reflex release of the hormone by the female occurs in response to coitus in many species. Seminal fluid is rich in *prostaglandins*, including PGF_{2α} which is a powerful uterine stimulant. Although very little fluid gains access to the uterus after vaginal insemination, the amounts of PGF_{2α} that enter may be sufficient, since extremely small quantities are effective. Prostaglandins may also be released by the estrogen-dominated uterus.

Highly motile sperm travel rapidly, but it remains to be demonstrated whether reduced sperm motility *per se*, some alteration of the sperm surface associated with reduced motility, or an influence of sperm metabolism on the uterus account for poor fertilizing ability of slower moving sperm cells.

Progesterone tends to quiet uterine muscle during the luteal phase of the cycle, so that contractions are fewer, less forceful, and uncoordinated (Chapter 21). This hormone also reduces responses to uterine stimulants.

The *uterotubal junction* presents a mechanical impediment which may be espe-

cially important following intrauterine insemination. It can also serve as a sperm reservoir. Although small inert particles can pass through the junction, active participation of sperm cells seems to be important for physiological passage.

Once within the oviduct, sperm move upward with the help of fluid currents and coordinated contractions of the oviducal musculature. The muscles are adrenergically innervated, and hormones influence activity of both α- and β-receptors.

The oviduct does not function as a single unit. Sperm migration must be coordinated with downward movement of the ovocyte and retention of the latter at the fertilization site. Estrogens seem to promote secretion of watery fluids and contractions of the lower oviduct during the period just preceding and immediately following ovulation, while progesterone exerts inhibitory influences at a later time. Physiological concentrations of estrogens also reduce motility of the upper oviduct close to the time of ovulation (favoring retention of the ovocyte); later, estrogens enhance activity of the upper oviduct and thereby promote downward movement of the con-ceptus.

Intrinsic sperm motility may be most important for gaining access to the ovocyte and its surrounding cells; but swimming movements are random rather than directed.

Spermatozoa which fail to fertilize are destroyed, and remnants are discarded through both the vagina and peritoneal cavity. Leucocytes appear within the uterus around the time of estrus, and their entry is increased when sperm are present.

Questions have been raised about why leucocytes appear in greatest numbers around the time of fertilization; but there is no need for sperm destruction at other times in animals with estrous cycles, since the animals are not sexually receptive.

Transport of Ovocytes⁴

Movements of the Fallopian tube and associated ovarian and tubal ligaments and mesenteries bring the finger-like or fringe-like projections of the oviduct (the fimbria) in apposition with the site of follicle rupture at the surface of the ovary. The ovocyte and surrounding cells are

drawn through the opening (ostium) into the broad funnel-like infundibulum of the oviduct. The products of ovulation seem to ooze from the ovary to the tube as cilia at the surface of the fimbria exert a drawing action on the mucus coating of the cumulus cells. Contractions of the fimbria are important in some species, including primates. Ciliary and muscular movements facilitate rapid passage of the ovocyte downward into the ampullary region of the oviduct; passage from there to the ampillary-isthmic junction is slower. Retention at the junction is facilitated by sphincter-like function which is adrenergically controlled.

The presence of the cumulus oophorus and surrounding mucus contribute to the effectiveness of ciliary activity as the ovocyte is moved along the oviduct wall against the current of fluid in the lumen. Estrogens promote growth and mitosis of surface cells and cyclic increases in numbers of cilia, while inhibition of muscle activity lessens the force of fluid currents. Maximal influences on musculature of uppermost portions of the oviduct precedes in time the effects on lower portions.

Progesterone is said to increase the rate of ciliary beating. Later, influences on secretion of organic components of oviducal fluid create an environment suitable for the zygote; still later, progesterone stimulation of the oviduct muscle promotes passage of the conceptus downward toward the uterus, as fluids participate in loosening of the attachments of cumulus oophorus cells to each other and to the enclosed ovocyte.

Attractions between Sperm and Oocytes¹⁸

The ovocyte and its surrounding cells present a sizable target for spermatozoa. Sperm approach obliquely (tangentially). Attempts to demonstrate the existence of specific *chemical* attractants which draw the sperm toward the target have yielded mostly negative data for mammalian experiments.

When sperm of several species are mixed and then introduced into the rabbit vagina, more of the native sperm traverse the cervix and they also exhibit greater tendency to adhere to rabbit ovocytes. But it is possible that foreign sperm contain substances which impede their passage, and that rabbit sperm more rapidly undergo capacitation.

Egg cells of some invertebrates have specific surface mucopolysaccharides (fertilins) which combine with complementary small acidic proteins (antifertilins) on heads of sperm of the same species in an antigen-antibody type reaction. Such substances may be needed by animals breeding in waters inhabited by other species, while similar protection is unnecessary for mammals mating under normal conditions.

Animals utilizing external fertilization have developed adaptive mechanisms for facilitation of fertilization. Production of large numbers of oocytes compensates for reduced chances of contact with sperm cells, and jelly coats on egg surfaces aid in sperm adherence. Some species engage in elaborate prenuptial rituals which ensure that sperm and egg cells are cast simultaneously (or nearly so) in close proximity. Frogs engage in amplexus, in which the male grasps the female and releases sperm directly over the eggs as the latter are extruded.

Sperm Capacitation^{2, 4, 5, 20, 22}

Freshly ejaculated sperm of most species require a period of residence within the female tract before they are capable of fertilization. The time required varies with both the species and the conditions under which capacitation takes place. At least 1 hr in an estrogen-dominated tract seems to be required by the mouse, about 3 hr for the hamster, 3-4 hr for the monkey, and 5 hr for the rabbit. Evidence of need for capacitation of human spermatozoa is mostly indirect, but a period of about 7 hr has been proposed.

It is not known whether specific agents in the female tract are required, or if the process depends on mechanisms within the sperm which function only under favorable conditions. If uterine factors are required, they may be similar in different species. "Cross capacitation" has been achieved with sperm and uteri of sheep and goats and in other studies employing hamsters, rats, and mice. Rabbit sperm are most easily capacitated in rabbit uteri, but this does not necessarily indicate the presence of molecules peculiar to those animals.

Epididymal sperm incubated in nonreproductive tissues (e.g., the colon) and in sera and other body fluids develop ability to fertilize. "Capacitation" has also been accomplished by exposure of sperm *in vitro* to fluids containing certain enzymes (e.g., amylase). But it is not known if the physiological process is imitated in this way.

Since capacitation is often completed within the oviduct and is directly followed

by the acrosome reaction (see below), it is difficult to sort out events relating specifically to the capacitation process. Moreover, the changes may not be identical in all animals. Structural changes in the acrosome cap have been found in rodent but not in other sperm studied.

Capacitated sperm which have not yet undergone the acrosome reaction penetrate the cumulus oophorus and also adhere to denuded ovocytes more readily than do epididymal sperm. Oviducal fluid contains considerable quantities of bicarbonate, and the latter acts directly on cumulus cells to promote their dispersion. (Untreated sperm more readily gain access to the ovocyte if the products of ovulation are incubated for a time in oviducal fluid, and effects of the treatment can be blocked with carbonic anhydrase inhibitors.) But bicarbonate also stimulates sperm cell metabolism and may induce changes in surface properties or in release of enzymes affecting cumulus cell adhesion. Uterine leucocytes have also been implicated in events leading to enhanced sperm penetration.

Changes in the spermatozoa include accelerated oxygen utilization and glycolysis, altered membrane permeability, and modification of swimming patterns. Adenylate cyclase may be activated; some changes can be mimicked by addition of cyclic nucleotides.

Capacitation seems to involve changes in the sperm surface and exposure of receptor sites for the acrosome reaction. It is not known whether enzymes are directly liberated at this stage or whether this awaits onset of the acrosome reaction. Surface changes could result from removal of a coating surrounding the plasma membrane.

When fluorescent markers are attached to spermatozoa, they are detached during capacitation. A phospholipid that stains with malachite green accumulates on rabbit sperm as they pass through the distal portion of the epididymis; this, too, is removed during capacitation.

At one time it was believed that hyaluronidase released by several spermatozoa is necessary for depolymerization of the cement substance which holds cumulus cells together. The concept is difficult to reconcile with observations that a single spermatozoan can penetrate the cumulus and proceed as far as the oolema.

The Acrosome Reaction^{1, 2, 3}

The acrosome reaction is an irreversible process involving alterations of sperm head membranes and release of several enzymes including hyaluronidase, a trypsin-like protease, and a glycolipoprotein. The precise roles of each of the enzymes have not been fully elucidated. A *zona lysin* attached to the sperm head has been implicated in liquefaction of the zona pellucida; the fertilizing spermatozoan has been observed to burrow through a very narrow channel on its way to the oolema. A phospholipase seems to be directly involved in fusion of sperm and egg membranes.

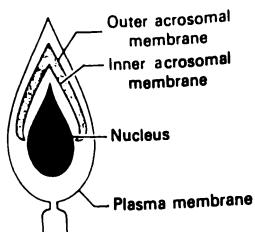
The plasma membrane of the spermatozoan fuses at several points with the underlying outer membrane of the acrosome (Fig. 22-1B), and a series of vesicles forms in the head region. Acrosomal enzymes are believed to be released through the spaces between the vesicles (Fig. 22-1C). The vesicles then detach, exposing the inner acrosomal membrane which remains associated with the remainder of the sperm plasma membrane (Fig. 22-1D). The plasma membrane makes first contact with the oolema (Fig. 22-1E); then the oolema encircles the exposed inner acrosomal membrane (Fig. 22-1F).

Responses of the Ovocyte to Sperm Penetration (Zona Reaction)^{1, 2}

The ovocyte responds rapidly and dramatically to contact with the fertilizing sperm. Fusion of the two cells depends on active participation by the ovocyte. The egg cytoplasm bulges at the point of entry to form a *fertilization cone* (penetration cone), and the sperm is drawn inward.

Polysaccharide material is extruded from granules in the ovocyte cortex, and associated permeability changes in the zona region present barriers to entry of additional sperm. The ovocyte soon resumes the meiotic division (which had been interrupted at the time of ovulation), and the second polar body is extruded to the periphery of the ovum. Egg and sperm pronuclei are formed and they later approach each other. Lysosomes within the egg cytoplasm rupture and the released enzymes initiate widespread metabolic activities. Within hours, membranes of the apposed egg and sperm pronuclei disap-

HORMONES AND REPRODUCTION



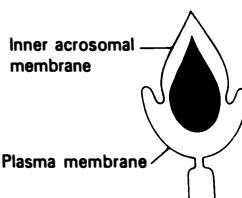
A. Before acrosome reaction.



B. Fusion of plasma and outer acrosomal membrane: formation of vesicles.



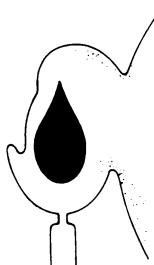
C. Release of acrosomal enzymes through spaces between vesicles.



D. Detachment of vesicles and exposure of inner acrosomal membrane.



E. Contact of spermatozoan with oolemma.



F. Fusion of spermatozoan with ovum.

pear and the genetic material is reassembled to form the zygote nucleus as the conceptus prepares for the first cleavage.

Cortical granules of the ovocyte may protect the structural integrity before fusion. Loss of such granules has been described in "overripe" ova.²⁴

This sperm actually performs two distinct functions: (1) It donates genetic material needed to restore the diploid number of chromosomes characteristic of the species. (2) It activates the relatively dormant ovocyte. The two processes are actually distinct and separable. In a few unusual

species (some worms, beetles, and fish), the sperm performs only the second function: it activates the egg, but does not donate genetic material. This strange reproductive process is known as gynogenesis. An activation function for "accessory" (nonfertilizing) sperm has been proposed for mammals.

Parthenogenesis^{1, 16} is activation of the ovocyte without participation of sperm. It occurs regularly in many invertebrates (including rotifers and insects). Some arthropod species are entirely "female" (so that reproduction is asexual); in certain wasps

there is alternation of parthenogenetically produced and fertilization-derived generations (a situation reminiscent of that seen widely in the plant kingdom). Female bees develop from fertilized eggs while males develop from unfertilized ones.

Artificial parthenogenesis has been achieved with a variety of nonspecific stimuli in sea urchins, frogs, and rabbits, and methods have been developed for nurturing the products. (In all species in which the XX/XY pattern determines sex, products of parthenogenesis are female. Parthenogenesis occurs in some birds and produces males.) The procedures are of considerable theoretical interest since they provide information on (among other things) the minimal requirements for initiation of development, and problems arising from deletion of a complete set of alleles.

Disorders of Fertilization^{1, 16}

Occasionally, in spite of protection afforded by the zona reactions, more than one sperm penetrates the ovocyte. One explanation offered for such occurrence of *polyspermy* is that the second sperm enters before the zona reaction has advanced. Abnormal ovocytes may be more receptive to fertilization by more than one sperm. Adherence of the second polar body to the ovocyte can lead to formation of a zygote containing genetic components of two female cells (polygyny).

It is possible for both the polar body and the ovocyte to be fertilized. Retention of the entire first polar body (with its double set of haploid chromosomes) has also occurred on rare occasions. *Polypliody* is a term applied to zygotes possessing multiple complete sets of chromosomes. The zygotes and their progeny have abnormally large nuclei and increased quantities of cytoplasm.

Polypliody has been extensively studied in amphibians, and it is known that salamanders with triploid, tetraploid, pentaploid, and even heptaploid chromosome patterns are capable of maturation and survival. Such animals compensate for enlarged cell size by reduction of cell numbers.

Mammalian polypliod zygotes do form in nature, and they may commence development. (Polypliod embryos have been recovered after spontaneous abortion in the

human.) Survival beyond the early stages is not possible, probably because highly complex organisms cannot tolerate reduction of the cell numbers of essential structures. Abnormalities compatible with survival in the human seem to be largely restricted to duplication, translocation, alteration, or deletion of individual chromosomes, with little tolerance for deviation much beyond the normal number.

CLEAVAGE, BLASTOCYST FORMATION, AND IMPLANTATION^{1, 4, 8, 9, 16}

Early Cleavage Stages⁸

Within hours after fertilization, the zygote prepares for the first mitotic division and the journey down the oviduct toward the uterus. At 30 hr, the human conceptus consists of two *blastomeres* surrounded by the zona pellucida (Fig. 22-2A). Although the cells may be unequal in size, separation at this stage can lead to development of identical twins.

Subsequent divisions of the conceptus proceed rapidly. The larger of the first two blastomeres is believed to divide first, giving rise to a 3-cell stage. Within 40 hr, the smaller blastomere has also divided to form the 4-cell stage (Fig. 22-2B).

Experiments on laboratory mammals suggest that each of the cells of the early cleavage stages is capable of producing an entire individual if separated from the others. However, most cases of monozygotic twinning seem to result from separation at a later stage. Armadillos consistently produce identical quadruplets by a process which involves separation after implantation of the blastocyst. (Twining and quadruplet formation are examples of asexual reproduction of a sexually derived zygote.)

As mitosis continues, the cells become progressively smaller, since cytoplasmic materials and nutrients are largely derived from the original ovocyte. At 3 days, the cluster of cells forms a small, solid ball or mulberry-like structure, the *morula* (Fig. 22-2C), still surrounded by the zona pellucida. Depending on the species, the conceptus may be sent through the ampullary-isthmic junction at this stage or a very short time later when the *blastocoel* begins to form.

During passage through the junction, mucopolysaccharide layers are added to

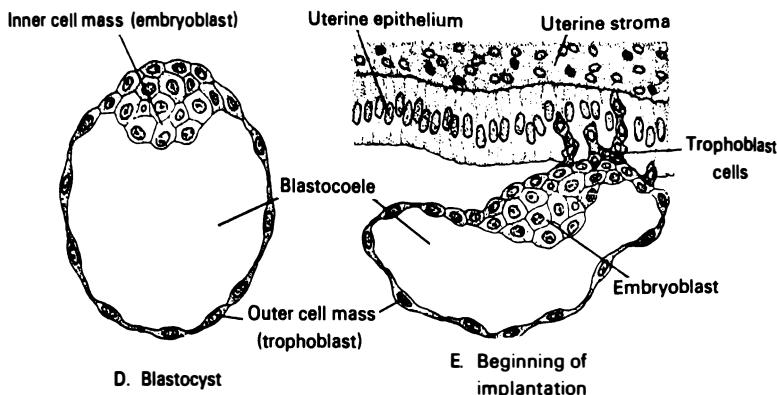
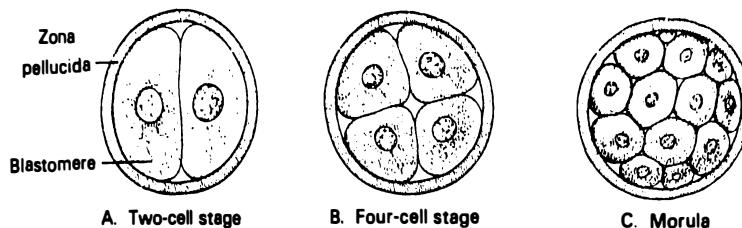


FIG. 22-2. Early development of the conceptus. (Reproduced with permission, from Langman. *J. Medical Embryology*, 2nd Ed. Williams & Wilkins Co., Baltimore, 1972.)

the external surface of the zona pellucida. Progesterone may promote secretion of the mucopolysaccharide.

The right combination of estrogen and progesterone at the right time is essential for maintaining viability of the zygote, support of early cleavage stages (beyond the first division), retention of the conceptus within the oviduct, and for later passage of the blastocyst downward at the optimal time.

Thus far it has proven easier to promote premature or delayed passage of the conceptus than to precisely define hormonal and metabolic requirements. The zygote utilizes lactate. Larger amounts of the metabolite are formed by cells of the ampullary region of the oviduct when a zygote is present, and the cells exhibit greatest stimulation in the immediate vicinity of the zygote. Cumulus cells produce and release pyruvate which can serve as a precursor. Progesterone seems to stimulate glycogen synthesis by ampullary cells and to play a role in secretion into the oviducal fluid of the amino

acids required for early development. The hormone also acts on α -adrenergic receptors of oviducal muscle cells; timing of the contractions may protect against implantation within the Fallopian tube.

Uterine Preparation for Implantation^{1, 4, 29}

Around the time of ovulation, the estrogen-dominated uterus provides a favorable environment for sperm survival and capacitation. It is not especially hostile to developing zygotes at this stage, but the endometrium does not respond to their presence.

During the time when the zygote begins development in the Fallopian tube, the corpus luteum forms and it secretes the progesterone needed to prepare the uterus for reception of the conceptus. In addition to exerting direct influences, progesterone seems to increase the availability of estrogen receptors. After progesterone has acted for a brief time (about 2 days in the rat and

slightly longer in humans and others), estrogen is again needed to complete preparations for implantation.

For a short time period the uterus becomes highly receptive. In addition to providing an environment conducive to survival and continued development of the conceptus, the uterus may secrete nutrients, metabolic stimulants, and an enzyme which catalyzes destruction of the zona pellucida.

The uterus participates actively in the implantation process. Morphologically visible changes include hyperemia, migration of nuclei of epithelial cells toward the basement membrane, characteristic alterations in appearance of the nucleoli, endoplasmic reticulum, mitochondria and microvilli, histochemical evidence for increased enzyme synthesis, and edema of the stroma leading to dispersal of stromal cells.

The blastocyst stimulates the endometrium to undergo changes collectively known as the *decidual reaction*. (But some decidualization can occur during the luteal phase of unfertile menstrual cycles.)

Permeability of uterine capillaries increases, and fluid collects within the uterine stroma. Enlarged, specialized *decidual cells* form, and they rapidly accumulate glycogen and lipids. RNA synthesis is increased, followed in time by increased synthesis of new proteins and associated cell growth. Later, DNA synthesis is increased and cells proliferate by mitosis. Decidualization can be impaired by administration of actinomycin D.

A specific protein (*uteroglobin*, *blastokinin*) known to stimulate *in vitro* development of blastocysts is secreted. Formation of copious quantities of "uterine milk" has been described in the domestic cat and some other mammals (but not in humans or rats). "Hystiotrophic nutrition" may assume special importance in the cat, pig, cow, and other mammals in which the blastocyst undergoes prolonged development before implantation. But localized increases in metabolic and secretory activities of endometrial cells in the immediate vicinity of the blastocyst suggest that some nutritive function is universal among mammals. Mucopolysaccharides (including one with properties similar to those of

heparin) are secreted; they may function in protection against immunological rejection of the blastocyst by uterine tissue.

The period during which the uterus remains in a receptive state is extremely brief, often measurable in hours. Continued exposure to endogenous progesterone leads very soon to development of a "refractory state" in which conditions for a newly arrived conceptus are at first less favorable than earlier, and later become genuinely hostile to the point of acceleration of blastocyst deterioration and death.

Development of the hostile condition occurs late in the luteal phase of normal ovarian cycles; under such circumstances, it can protect against implantation of blastocysts developing after fertilization of "overripe" ova. When the situation arises in early pregnancy, it reduces the chances of a second implantation (superfetation) while the first conceptus is being nurtured. Second implantations have been experimentally achieved by administration of estrogens either systemically or locally to the pregnant mother.

The optimal time after fertilization for arrival of the conceptus in the uterus is remarkably similar for animals with very large differences in gestation periods. Usually, it is 3 or 4 days.

Studies in which blastocysts have been removed from animals and transferred to uteri of other animals at the wrong time (e.g., a 4-day old conceptus into the uterus of an animal fewer or more than 4 days after the usual fertilization time) or to uteri of animals ovariectomized and treated with hormones, have demonstrated phenomena of *receptivity* of suitable prepared endometria, *hostility* of improperly prepared tissues, and interference with survival and implantation when excessive or inadequate amounts of estrogens are given or when progesterone is presented prior to estrogen treatment.

Blastocyst Formation^{1, 8}

The conceptus of most species is in the late morula stage as it enters the uterus. *Blastocoel* formation is initiated just before or soon afterward. Fluid collects between the cells of the morula; later the fluid-filled cavities converge to form a single vesicle. Changes in membrane permeability account in part for the rapid uptake of water;

but a hormonally regulated ionic environment is needed, and some materials may require active transport.

It soon becomes possible to distinguish an outer hollow cellular sphere or *trophoblast* and a smaller group of quite different cells, the *inner cell mass* or *embryoblast* (Fig. 22-2D). The entire structure at this stage is known as a *blastocyst*. The trophoblast is primarily concerned with attachment of the embryo to maternal structures, development of membranes needed for nourishment of the conceptus and removal of metabolic wastes, and with secretion of pregnancy hormones. Cells of the embryoblast give rise to structures of the new individual.

The blastocyst is at first surrounded by the *zona pellucida*, which probably protects against implantation within the oviduct and contributes to preimplantation survival. In species in which implantation is delayed (see below), the *zona* may be retained for extended periods.

Artificial Induction of Decidual Reactions¹⁷

The presence of a blastocyst is not an absolute requirement for development of decidual reactions. In its absence, the "receptive" endometrium responds rapidly and dramatically to a wide variety of seemingly unrelated stimuli, including localized application of pressure, pinching with a small hemostat, pricking with a needle, presentation of mild chemical irritants, insertion of threads or glass beads (which probably act through either mechanical pressure or localized irritation), and placement within the uterus of heparin, salt solutions, some oils, or even a bubble of air containing the right amount of carbon dioxide. There are, however, marked species differences in susceptibility to the stimuli. Responses elicited in the uterus are remarkably similar to those seen under conditions of early pregnancy, and the term *deciduoma* (or *placentoma*) has been applied to the conglomeration of cells which is formed around the site of stimulation. Decidualization reactions have been utilized in bioassay procedures for progestational hormones, and it has been observed that 20α -OH-progesterone is less than 1/20th as effective as progesterone

for supporting uterine changes. Uterine responses can be blocked by prior administration of actinomycin D.

Artificial induction of the decidual reaction is of considerable theoretical interest because it demonstrates the active role of the endometrium and the conditions under which the uterine responses can be altered. It also sheds some light on the nature of the stimulus presented by the blastocyst. But there is some question about whether artificial stimuli do, in fact, imitate the natural events.

One highly controversial area surrounds the question of whether the blastocyst slightly injures the endometrium and whether *histamine* released in response to the injury provides the physiological stimulus. Histamine is among the most potent of exogenous stimuli, and decidualization can be readily elicited by administration of the amine or of agents promoting its endogenous release. Estrogens are potent stimulators of histamine release and may play a role in induction of histidine decarboxylase; moreover, decidualization can be blocked by administration of a number of antihistamines. However, evidence for physiological participation of histamine is considered inconclusive by many observers. Deciduoma formation can be blocked by several agents which reduce capillary permeability without exerting specific antihistaminic actions.

Interactions between the Blastocyst and the Endometrium¹⁸

In some species, the blastocyst swells rapidly and grows large enough to exert *mechanical pressure* on the uterine wall. Additional mechanical stimuli arise from *rhythmic contractions* and dilations of the blastocyst. Vesicles containing granular material rich in RNA (Wilson bodies) have been observed to detach from the inner cell mass and to exit through the trophoblast. They may provide a *chemical stimulant* for the endometrium (although a function in embryonic development is not ruled out). Known properties of endometrial cells during the "receptive" stage are consistent with rapid absorption and incorporation of macromolecules.

Steroid hormones may alter surface properties of the blastocyst and affect its ability to interact with uterine cells. Blastocysts exposed *in vitro* to estradiol tend to implant early; it is not known whether this

results from stimulation of the blastocyst itself or from concentration of estradiol which is then released to act on the endometrial cells.

There are some intriguing questions regarding the functions of the steroid hormones. Blastocysts readily implant in many tissues which do not have estrogen priming, and embryos develop if conditions are favorable. (Abdominal pregnancies in the human have gone to full term; tubal pregnancies are usually surgically interrupted to avoid rupture of the oviduct, not because of abnormal development. Plasma levels of steroid hormones are elevated, however, since the trophoblast performs many functions comparable to those seen in normal pregnancies.) The uterus actually seems to provide an *unfavorable* site for implantation except during very limited time periods in which it has been specifically prepared by hormones.

When blastocysts implant ectopically, they are highly invasive; and trophoblastic tumors are among the most destructive known. Within the uterus, invasiveness is kept under control, and hormones may actually provide protection to the endometrial cells.

Removal of the zona pellucida seems to be an essential preimplantation step; an electronegative surface is exposed which is attracted to the more positive endometrial surface. (Denuded blastocysts stick readily to endometrial surfaces.)

Absorption of electronegative colloid substances and retention of these substances within the cytoplasm has been described for pregestational endometrial cells. The process, which has been called *athrocytosis*, may set up an electrical field which draws the blastocyst to a specific uterine site. Bicarbonate production by both blastocyst and endometrium have also been implicated in attraction of the blastocyst to a specific site in the uterine wall, and it has been shown that inhibitors of carbonic anhydrase can interfere with implantation.

All species show preferential implantation sites which are most compatible with development of the conceptus. In the human, implantation normally takes place along the wall of the corpus of the uterus, more often on the posterior surface close to the midline, and it seems reasonable to suspect operation of a localized signal. Im-

plantation too low in the human uterus (close to the cervical os) leads to the condition of *placenta previa* in which hemorrhage occurs late in pregnancy or during parturition.

Blastocysts artificially detained with the oviducts retain the zona pellucida; but after several days the outer coating is shed by a process which has been called hatching. It is possible that a uterine enzyme promotes physiological removal of the zona, while a different mechanism becomes operative at a later time if the enzyme is unavailable.

Delayed Implantation^{4, 16, 26, 29}

There are considerable species differences in the characteristic time period which ensues between entry of the blastocyst into the uterus and implantation, and there are debates concerning whether delayed implantation involves an inhibitory molecule released by the uterus, absence of a stimulatory uterine factor, insufficiency or imbalance of nutrients in the uterine environment (e.g., altered relative concentrations of regulatory amino acids), or possibly because the blastocyst fails to release a uterine stimulant. It is known that implantation can be artificially delayed if estrogen availability is reduced by ovariectomy and administration of progesterone, and that the process can be reversed by estrogen administration. (A second implantation can be accomplished in rats after the uterus develops the unfavorable environmental characteristic of pregnancy, if estrogen is injected.)

Human blastocysts implant about 8 days after conception. Four days are required by the mouse and about a week by the rabbit. True implantation in cows is delayed for 30-35 days, but a close association between the blastocyst and the endometrium is established much earlier as the conceptus undergoes development.

In many species the blastocyst survives a prolonged period of dormancy (*diapause*) before it implants and resumes development. This occurs in the deer, weasel, fur seal, black bear, armadillo, and others. Delays of up to 200 days have been described for the spotted skunk.

Martens and badgers mate during the summer. Postponement of development

ensures that the young are born at a time most favorable for survival. In these animals it is believed that *very high* levels of estrogens inhibit implantation, but very low progesterone secretion by the corpus luteum may contribute to the delay. Seasonal variations in thyroid gland function occur in these animals and may also play some role.

Very different mechanisms operate in other mammals. In some, implantation is strongly influenced by environmental clues, and the pineal gland may participate in regulation of endometrial functions of certain species.

The presence of a foreign male delays implantation in mice, and it is suspected that a pheromone released by the male induces disturbances in maternal secretion of the pituitary hormones needed to maintain the corpus luteum (Section I). Presumably, delay under such conditions facilitates establishment of a safer nesting site before the time when the mother is burdened by the demands of advanced gestation.

Lactation can delay implantation. Laboratory rats become sexually receptive and can conceive soon after parturition. If the litter contains five or fewer pups, lactation may not affect the second pregnancy; however, implantation is usually delayed if a larger number of pups is suckled. (Mice experience delayed implantation when nursing three or more young.) The survival value of such adaptation becomes apparent when one considers that a rat weighing 260 g may be called upon to provide sufficient food for up to 19 pups, with each going from a birth weight of 4 g to a weaning weight 3 weeks later of about 40 g.

It has been suggested that average intervals of 3 or 4 years between births of infants to women living in primitive societies can be related to the practice of nursing children for several years. Usually, however, it is possible to find other explanations for the birth patterns. While fertility may be reduced during lactation, the quantitative differences between rodents and humans are enormous. A mother weighing perhaps 130 pounds may provide nutrients over a six-month period to permit a 6-8 pound infant to grow to a weight of about 18 pounds. In affluent societies, lactation may have little influence on fertility when

mothers can readily obtain an enriched diet while infants are provided with solid food supplements.

In marsupials the gestation period is not very different in length from the ovarian cycle, and conception in certain species does not arrest the cycles. Some of the kangaroos go into estrus, mate, and conceive shortly before parturition while others do so soon afterward. The conceptus develops as far as the blastocyst stage; if there is a young joey attached to a teat in the mother's pouch, the blastocyst goes into a prolonged state of dormancy. If the joey stops sucking (because of injury or removal from the pouch), the blastocyst implants and continues development.

The joey is at first little more than an embryo. It may spend up to 200 days in the pouch, going through developmental stages not very different from those accomplished within the uterus of placental mammals. Separation from the pouch at the conclusion of this period is comparable in many ways to birth of a placental mammal. At that time the second conceptus implants, while the older joey returns regularly to the pouch for feeding much as the eutherian mammal nurses.

Parallels can be drawn between diapause in marsupials and lactational delay in placental mammals; but there are obvious differences. The marsupial blastocyst consists of a hollow ball of similar cells (protoderm); there is no division into trophoblast and embryoblast. The marsupial blastocyst is also protected by a shell membrane and a layer of albumin.²⁶

Advantages of the marsupial arrangement are obvious. The two matings are only about a month apart, and therefore both can take place during a single breeding season. Implantational delay is important because of limited accommodation within the pouch. But an otherwise long period of lactation is not "wasted" if the young joey is injured.

Mechanisms for lactational delay of implantation are only partially understood, and there are species variations. Prolactin secretion may be especially high during early stages. The hormone exerts powerful influences on the ovaries of some animal types, and probably plays a key role in implantation in these (and a supplementary role in others). In rodents, prolactin

affects progesterone metabolism, and it may also reduce the sensitivity of pituitary gonadotrophs to hypothalamic hormones.

The *suckling reflex* (p. 341) leads to increased prolactin secretion, but it also promotes release of oxytocin and tends to reduce secretion of gonadotrophins affecting steroid hormone secretion by the ovary. In at least some of the mammals, delayed implantation has been attributed to relative estrogen deficiency; and implantation can be induced by estrogen administration. In others there may be reduction of progesterone secretion below that needed for receptivity of the endometrium.

Implantation and Development of Fetal Membranes in Primates^{1, 2, 8, 14}

In pigs, horses, and donkeys, implantation is a superficial process in which the trophoblast and endometrium become closely associated, but the uterine epithelium retains its functional integrity. This type of implantation is designated as *epitheliocchorial*.

The *endotheliocchorial* type seen in cats, dogs, ferrets, and others involves trophoblastic invasion and some destruction of the epithelium. Close association between the conceptus and the uterine capillaries is established, but blood vessel walls remain intact.

In humans, monkeys, and some rodents invasion by the trophoblast is more extensive as *hemochorionic* implantation is established. Contact is first made between the sticky surfaces of the denuded trophoblast cells and a part of the endometrial epithelium close to a capillary. Both cell types respond rapidly, and intimate interdigitations are established.

The trophoblastic cells at the site of the initial reaction proliferate rapidly and soon form a *cytotrophoblast* close to the inner cell mass consisting of large mononucleated cells, and an outer *syncytiotrophoblast* which invades the endometrium. The outer layer does not have discernible cell boundaries; it is believed to be formed by the cellular layer. The latter is continuous around the conceptus but remains thin where contact has not yet been made with the endometrium.

Knoblike protrusions of the syncytiotrophoblast burrow into the uterus and make

their way to the stroma by pushing between stromal cells, and by destruction of superficial cells. Soon the entire conceptus sinks into the uterine wall. (Fig. 22-3A).

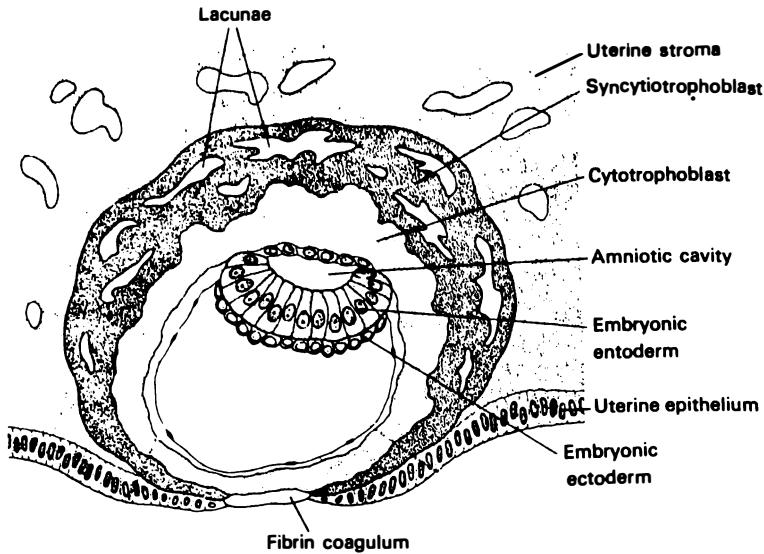
Stromal cells at the implantation site enlarge, accumulate considerable quantities of glycogen and lipids, and secrete mucus and glycogen into the extracellular spaces, as the uterine capillaries dilate. The endometrium in the vicinity develops into the *decidua basalis*. Many uterine cells become disrupted and incorporated into the syncytiotrophoblastic mass.

While trophoblastic invasion resembles phagocytosis in certain respects, and proteolytic enzymes released by the conceptus contribute to the destruction of uterine cells, interactions between trophoblastic and uterine cells have been called "cell fusion." Some of the content of the epithelial cells is preserved within the trophoblast; moreover, uterine cells provide a stimulus to the trophoblast. Since decidual cells have a very limited life span when reactions are induced (without the presence of the blastocyst), it has been proposed that decidual cells participate in their own destruction because they are "programmed" to do this.

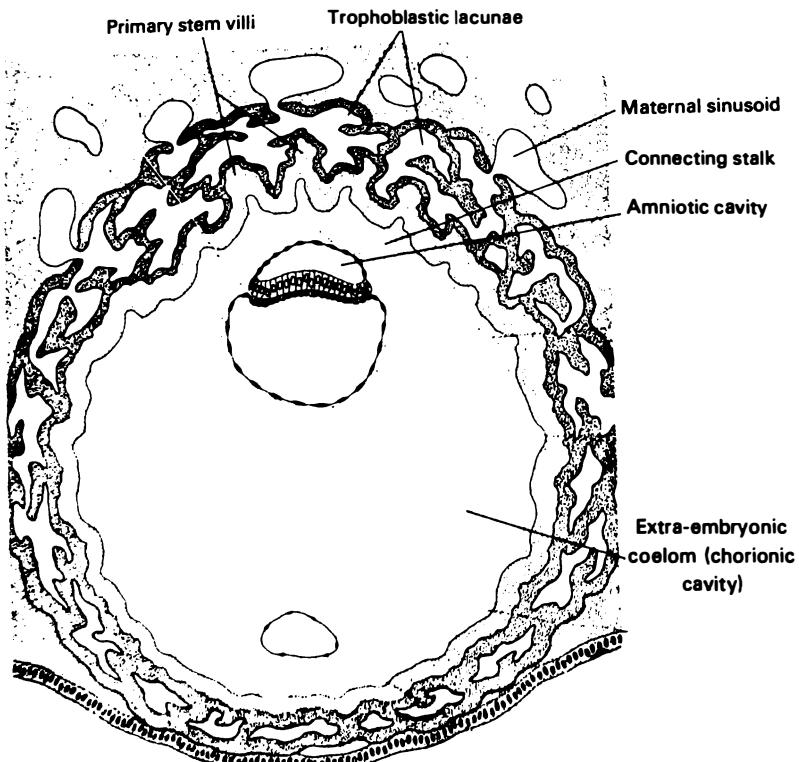
Endometrial cells at the site of the blastocyst penetration grow toward each other. The defect in the uterine wall is soon closed over, first by a fibrin clot (within 2 days), and a little later by epithelial cells. By the time the human embryo is about 13 days old, the endometrium has formed a thin *decidua capsularis* at the implantation site, and only a slight bulge can be seen from the luminal side. The implantation is therefore said to be *interstitial*. The endometrium not directly associated with the conceptus forms the *decidua parietalis*.

As the syncytiotrophoblast burrows deeper into the uterine stroma, the latter becomes markedly congested. Uterine capillaries dilate, and blood sinusoids develop from anastomoses of uterine spiral arteries and endometrial veins. There is some evidence that a factor released by the conceptus promotes vessel dilation and sinusoid formation. Nourishment at this stage is obtained from products of uterine cell destruction.

Intracytoplasmic vesicles develop within the syncytiotrophoblast; they soon fuse to form large vesicles or *lacunae* which join in an interconnecting network. As the en-



A. About 9 days after conception



B. About 13 days after conception.

FIG 22-3. Diagrammatic representation of implantation. (Reproduced with permission, from Ref. 8.)

dothelial lining of maternal sinusoids is eroded by the syncytiotrophoblast, the lacunar system of the conceptus becomes continuous with the sinusoids and maternal blood flows into the lacunae. At this stage the developing embryo proper is separated from the maternal circulation by a continuous layer of cytotrophoblast, the part of the syncytiotrophoblast in contact with the cytotrophoblast, and small quantities of noncellular matter on the uterine surface of the trophoblast.

While this is going on, the inner cell mass or embryoblast differentiates into a *bilaminar disk* comprised of a layer of entoderm in contact with the blastocoele, and a layer of ectoderm between the entoderm and the cytotrophoblast. Clefts then appear between the ectoderm and the cytotrophoblast while cells of the latter which are in contact with the cavity become *amnioblasts*. The clefts coalesce to form the amniotic cavity (Fig. 22-3B).

Within a week after implantation begins, the syncytiotrophoblast sends cytoplasmic projections into uterine tissue. *Primary villi* consisting of syncytiotrophoblast with a small core of cytotrophoblast are formed. Invasion of mesoderm converts them into *secondary villi*; finally, the *tertiary villi* are established when embryonic blood vessels grow into the core. Nutrients which diffuse in from the sinusoids are transferred via the vessels to the growing embryo. Cytotrophoblast then pushes through the ends of the chorionic villi to form a *trophoblastic plate* which is continuous except at the site of entry of maternal blood vessels into the intervillous spaces.

The uterus presents greater *resistance to invasive properties* of the trophoblast than is seen when blastocysts are implanted in other tissues. Mucopolysaccharides near the surfaces of endometrial cells in contact with trophoblast have been implicated. A fibrinoid substance fills spaces between embryonic and maternal tissues, and it is suspected of forming an immunological barrier which reduces the tendency for development of cellular immune reactions.¹ Studies in which blastocysts of one animal type have been nurtured within the uterus of another indicate that the thickness of the fibrinoid layer bears a direct relationship to the magnitude of

genetic difference between maternal and embryonic cells. But immunological tolerance between mother and fetus is certainly far from understood. It has been variously attributed to processes involved in cell fusion during implantation, to low antigenicity of the trophoblast, and to release by the trophoblast of inhibitors of immune responses. A role of chronic gonadotrophin (see below) in suppression of maternal immune responses has been both supported and refuted.¹⁸

Fetuses can stimulate maternal production of humoral antibodies capable of combination with paternal antigens; and transplantation antigens can accumulate on membranes of trophoblast cells.¹¹ It has been proposed that the trophoblast *glycocalyx* functions in the masking of antigens and that it repels lymphocytes. The amniotic fluid may also participate in protection of the fetus.

LUTEOTROPHIC HORMONES^{1, 4, 11}

During nonfertile ovarian cycles, the corpus luteum regresses at a characteristic time following its formation, and this soon leads to sloughing of the superficial layers of the endometrium (Chapter 21). If conception occurs, some mechanism is required for maintaining secretion of estrogen and progesterone. In the rabbit and some others, the corpus luteum must be maintained throughout the gestation period. In others (including humans) the corpus luteum is required during early pregnancy, but the placenta later takes over the hormonal functions.

Diverse mechanisms for termination of corpus luteum function in nonfertile cycles were described in the previous chapter. There is corresponding variation in mechanisms utilized for prolonging it during pregnancy. In guinea pigs, pigs, cows, and sheep, the blastocyst seems to suppress release of a uterine agent promoting luteolysis. In the rat, secretion of prolactin (which is luteotrophic in these animals) may be facilitated by stimuli arising in the uterus; but a factor formed by the trophoblast also seems to be important.

In humans, monkeys, and other mammals, high levels of ovarian steroid hormones exert negative feedback influences

on the pituitary, and the release of adeno-hypophysial gonadotrophins is suppressed. In these, the trophoblast assumes primary importance for maintaining the corpus luteum of pregnancy, while trophoblastic hormones may augment pituitary influences on the ovary in others.

The human trophoblast begins secretion of a *chorionic gonadotrophin* (CG) at a very early stage, possibly even before implantation. Within little more than a week after conception, sufficient quantities of human chorionic gonadotrophin (HCG) are picked up by maternal blood vessels to permit spillage into the maternal urine, and the presence of HCG can be picked up by highly sensitive assay procedures (see below). Unlike the cells of the adenohypophysis, trophoblastic cells are not subject to negative feedback influences of steroid hormones to the point where gonadotrophic hormone secretion is inhibited. Prevailing evidence favors a syncytiotrophoblastic origin of HCG.

In common with the other known glycoprotein hormones, HCG is made up of two dissimilar subunits held together by non-covalent linkage. The α -subunit resembles that of TSH and LH in many respects; its total amino acid content is similar and it contains some sequences that are identical. (But the α -subunit of HCG is less similar to that of LH than is the α -subunit of TSH.) The β -subunit shares some chemical, biological, and immunological properties with that of LH.

The carbohydrate content is variable and seems to range from 28-31% by weight of the total molecule. There is a high content of sialic acid (close to 12%); mannose, fucose, galactose, *N*-acetylglucosamine, and *N*-acetylgalactosamine are also present. The molecular weight of the circulating hormone molecule has been difficult to determine, partly because experimental data vary with extraction procedures. A value of 30,000 has been proposed for the total molecule; on the other hand, some authors have reported molecular weights of 25,000 and 40,000 for the subunits.

HCG exerts some FSH-like activity which most observers attribute to intrinsic properties rather than to contamination with other molecules. There are indications that HCG consists of a "family" of related glycoproteins whose composition changes during the course of gestation. More α than

β -subunits seem to be synthesized, and some free α -component has been detected in plasma.

PREGNANCY TESTS^{13, 16}

Older clinical procedures measure LH-like activity of HCG present in the maternal urine. Immunological tests are more sensitive and can often be performed more rapidly. Recently an exquisitely sensitive procedure employing purified hormone receptors has been developed for early detection of pregnancy.

The Aschheim-Zondek, A-Z or Mouse Test

The first of the dependable tests was developed in 1928; it is based upon ability of HCG-containing urine to promote formation of hemorrhagic follicles in the ovaries of immature mice. It can be performed early in pregnancy and is considered to be about 98% reliable, but suffers from certain disadvantages. Several (usually 6) moderately expensive animals of the right age and sex are required; they must be injected repeatedly with diluted urine. Results cannot be obtained in less than 96 hr; if the findings are inconclusive, a fresh specimen must be obtained because HCG has limited stability.

The Friedman or Rabbit Test

Since rabbits are "induced ovulators" they usually do not secrete sufficient LH to promote ovulation when they are kept in isolation. If HCG is present in the test urine, animals injected with the material form corpora lutea within 48 hr. The Friedman modification of the A-Z test therefore requires less time than the original mouse test. Rabbits are more expensive than mice, but fewer are needed. Occasional false positives result from spontaneous release of LH if rabbits are unduly stressed by the test procedure.

The Galli-Mainini or "Frog" Test

Pregnancy urine can induce spermatiation in male frogs and toads. The animals are cheaper than mammals to purchase and maintain, and test results are obtained within 2-4 hr; moreover, the same urine specimen can be used for additional tests if the first is inconclusive. The major draw-

back arises from seasonal variations in sensitivity of the animals, with a greater tendency to yield "false negatives" during the summer months.

The *Hogben Test* is loosely referred to as a frog test. It depends on HCG induction of ovulation in the African toad. Ova are released within 8 hr, and are readily detected by observers having minimal training.

Immunological Tests

Immunological methods are sensitive, do not require direct use of animals, and are quite specific. Detection of HCG can be accomplished rapidly.

Quantitative procedures have been developed for assay of most hormones and of a variety of other biological molecules. While some of the procedures are technically difficult and require considerable skill and experience, the principles upon which they are based are relatively simple.

HCG is collected from pregnancy urine, concentrated and purified. When injected into a foreign species (e.g., the rabbit), it stimulates production of specific antibodies which are then recovered from the blood plasma (Fig. 22-4A).

Purified HCG can be labeled with radioactive markers. When labeled HCG is added to anti-HCG antibody, a reversible complex is formed (Fig. 22-4B). The radioactivity of the complex can be measured.

If unlabeled HCG is then added to the complex, some of the radioactive HCG will be displaced by the unlabeled hormone (Fig. 22-4C). The greater the quantity of unlabeled hormone added, the greater the displacement and reduction of radioactivity of the complex.

The complex can be precipitated, and its radioactivity measured. Standard curves are constructed relating amount of unlabeled HCG added to loss of radioactivity of the complex (Fig. 22-4D). An alternate procedure involves measurement of the amount of labeled HCG displaced from the complex; in this case, radioactivity of the medium is measured after removal of the antibody complex (Fig. 22-4E).

Once the curve has been constructed, an estimate can be made of the HCG content of a urine sample.

It will be appreciated that the outlined methodology requires facilities for purifica-

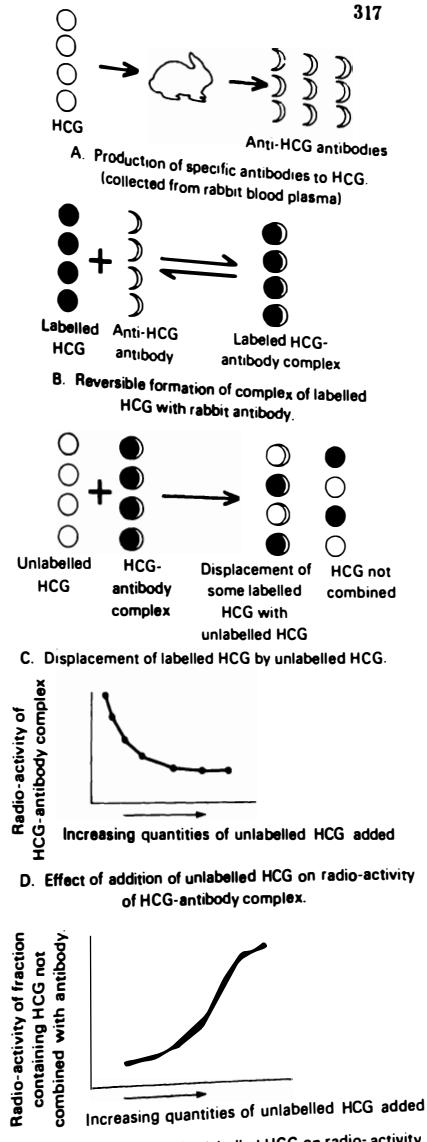


FIG. 22-4. Principle of radio-immuno-assay procedure for HCG.

tion and labeling of the HCG, for obtaining and purifying the antibodies, separation of the antibody complex, and measurement of the radioactivity. (However, kits are

available to test laboratories so that procedures there are minimized.)

A logical extension of the method is the labeling of the HCG with a dye that changes color as the HCG is displaced from its combination with the antibody. Detection of pregnancy would then involve purchase of the dye-labeled HCG-antibody complex, and observation of color change when the test urine is added. At the time of this writing, an adequate dye-labeled preparation is not available.

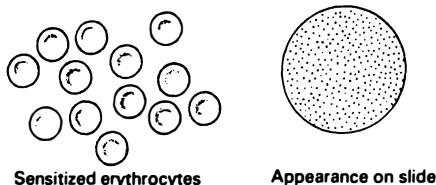
Another procedure based on immunological properties of HCG depends on the ability of HCG antibody alone (when not complexed with the hormone) to promote agglutination (clumping) of suitably prepared "sensitized" red blood cells. If urine free of hormone is added to the antibody before the mixture is added to the erythrocyte preparation, agglutination will not be affected (Fig. 22.5A). But if the urine contains HCG, the latter will combine with the antibody and reduce the amount of

agglutination. Quantitative estimates of the HCG concentration can be obtained by use of serial dilutions of urine. (The greater the dilution that is effective, the higher the HCG content of the urine.) The agglutination can be observed when the urine-antibody combination is added directly to the erythrocyte preparation on a slide (Fig. 22.5C), or by accumulation of the agglutinated cells at the bottom of a test tube.

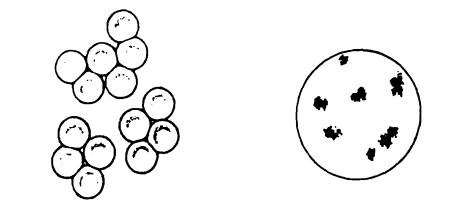
The Radioreceptor Assay²⁵

The most sensitive of all procedures for detection of HCG utilizes purified hormone receptors obtained from the bovine corpora lutea of early pregnancy. The receptors do not exhibit marked species specificity, but will react only with chorionic gonadotrophins or with LH. (They are not affected by TSH or FSH.)

Unlabeled hormone in urine or plasma can displace radioactively labeled hormone



A. Appearance of erythrocytes before addition of anti-HCG antibody.



B. Appearance of erythrocytes after addition of antibody.

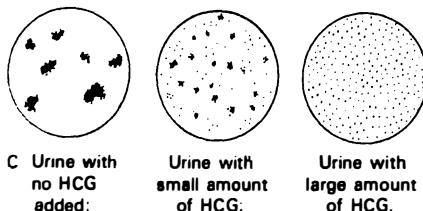


FIG. 22-5. Agglutination test for HCG.

combined with the receptors. Pregnancy has been diagnosed as early as 6 days after conception; but it is not certain whether such early tests measure HCG, LH, or a combination.

Because of the cost and the technical skill required, it is unlikely that receptor methods will be adopted for routine clinical use. They find special application in those cases in which diagnosis must be made at the earliest possible moment, and also in the research laboratory when it is necessary to measure minute quantities of hormones.

CONTRACEPTION AND CONTROL OF FERTILITY IN THE FEMALE^{2, 3, 4, 6, 9}

The explosion of information on hormonal regulation of reproductive processes has been applied to development of methods for voluntary avoidance of conception in humans, control of populations of animals which create problems, treatment of some forms of human infertility, increasing production of farm and laboratory animals, promoting fertility of rare species maintained in captivity, and synchronization of births of animals for economic and investigative purposes.

Continued research is needed for development of newer and safer methods for control of human conception, since all existing procedures have limitations and drawbacks. There is growing awareness that methods introduced on the basis of what appeared at one time to be sound theoretical principles are actually effective for very different reasons.

There are difficult problems involved in the investigation of human reproductive biology. Most kinds of information must be obtained from laboratory animals in which it is possible to perform controlled experiments utilizing procedures such as gonadectomy, hypophysectomy, ligations of various kinds, and administration of pharmacological doses of natural and synthetic hormones. In no field of biology are species variations more evident; moreover, differences are encountered between closely related animal strains, between siblings subjected to seemingly identical conditions, and within the same individual at different times.

Dosages and timing of administration of

reproductive hormones can influence the results obtained; the hormones interact in complex ways with other regulators; and data which involve subjective reactions are notoriously difficult to interpret.

Rhythm Methods of Fertility Control^{24, 25}

The regular recurrence of the human menstrual cycle, and recognition of relationships between ovarian secretory functions and menstrual bleeding, have led to the belief that the timing of ovulation can be predicted with reasonable accuracy, and that the predictions can be used to determine during which parts of the cycle coitus is most likely to result in conception.

Since it is known that the life span of the ovocyte within the oviduct, and the life-span of the sperm within the female tract are both limited, it has been suggested that conception can be avoided if couples abstain from sexual relationships for periods extending from at least 72 hr preceding through 72 hr following the predicted time of ovulation. To allow for variability of the ovarian cycle, it has also been recommended by some that the time periods in both directions be extended by 1 or more days.

While it is undoubtedly true that the incidence of conception can be reduced if the prescribed procedure is followed, the "rhythm method of birth control" suffers from serious limitations. Aside from potential psychological problems arising from the requirement of abstinence during times when many women experience greatest sexual interest, and from the obligatory association of concern over pregnancy with the sexual relationship, there are other real dangers.

Even in women experiencing regularly recurring periods of menstrual bleeding, the timing of ovulation is far from predictable. Ovulation can be precipitated at times when it would ordinarily not occur; and coitus following a period of abstinence provides a potent stimulus. Statistical data indicate that the method often fails to prevent conception when all the rules are followed rigidly. It is also known that couples are more likely to "take chances" with this than with most other procedures for fertility control.

More disquieting is the evidence that the

incidence of congenital defects among offspring of parents experiencing "failure" of the method is higher than would be predicted on the basis of the genetic background.^{1, 29} Restriction of coitus to times unfavorable for fertilization increases chances of formation of zygotes by overripe ova and spermatozoa. Moreover, fertilization too long after ovulation increases the incidence of ectopic and especially of tubal pregnancy.

Progesterone tends to elevate body temperature (Chapter 21). Since plasma concentrations of the hormone rise around the time of ovulation, it has been proposed that the rhythm method can be improved by taking daily measurements of body temperature to more accurately define the cycles. The procedure requires preparation of charts or graphs, and demands abstinence from soon after cessation of the menstrual flow until after the basal body temperature has fallen—an even longer period than recommended by the original rhythm method. Temperature graphs in different women take many forms, and most are difficult to interpret. The relationship between ovulation and body temperature is not as consistent as proponents of the concept suggest; the time required for progesterone released by the ovary to attain sufficient concentration in the vicinity of temperature-regulating neurons is affected by many variables. Thresholds of the neurons cannot be assumed to be constant; and nonreproductive functions also affect body temperature.

A recent modification of the method involves examination of the cervical mucus, which is known to be under hormonal control (Chapter 21). It has been suggested that corpus luteum function can be detected by appearance of the "progesterone type" mucus. Presumably, this will first appear at a time when the ovum has lost its ability to become fertilized; therefore coitus any time afterward and up to appearance of the menses will not result in conception.

The value of cervical mucus examination awaits the test of experience. Women who have tried to use the procedure encounter difficulty in diagnosis of the specimens (in which changes are not of an "all or none" nature). While cervical glands are in-

fluenced by hormones, they also seem to possess some autonomy; glands of experimental animals previously experiencing regular ovarian cycles have been shown to continue cyclic changes after ovariectomy.

Additional suggestions for improvement of the rhythm method include administration of agents which promote ovulation. The scant data available from women using such agents do not indicate a lower incidence of "failures." Moreover, there is no assurance that precipitation of ovulation at one time of the cycle prevents subsequent ovulation of follicles not ready at the time of administration of the agent. There have also been suggestions that combination of the original rhythm method with application of information derived from astrological charts can provide effective conception control. While most scientists are amused by such suggestions, the concept that a variety of as yet undefined external factors can influence ovarian cycles is not easily dismissed.

The Estrogen-Progesterone "Pill"^{4, 6, 10}

Negative feedback influences of estrogens and progesterone on gonadotrophin secretion have been recognized for a long time. But application of such information to fertility control was delayed because the naturally occurring hormones are rapidly destroyed by the liver soon after administration. Some potent synthetic agents which have recently become available (and are highly effective orally) were described in Chapter 21. Structures of a few now in use as contraceptives are compared with the natural hormones in Figures 22-6 and 22-7.

Procedures most widely employed utilize a combination tablet containing potent estrogen-like and progesterone-like agents. Usually the tablet is taken daily for 20 or 21 days; this is followed by 5-7 days in which either no "pill" is taken, or a placebo is ingested to maintain the routine. Highly effective control of conception is achieved.

The endometrium exhibits cyclic proliferation and specialization in response to the agents, and sloughing of superficial layers along with bleeding occurs during the intervals in which no synthetic hormone is taken. Endometrial cycles cannot

be considered to be completely "normal", since the uterus is exposed to progestagens as well as estrogens during what would otherwise be the proliferative phase of the ovarian cycle.

Exactly what is accomplished with the hormone-like preparations is not known in detail, and there are reasons to believe that responses vary between individuals and within the same woman at different times. Studies of hormone concentrations in the plasma of women taking the pill suggest that most do not ovulate. But suppression of ovulation may depend more upon a relatively constant release of gonadotrophins (so that no preovulatory surge occurs) than on total inhibition of gonadotrophin secretion.

There is evidence that some women do, in fact, ovulate (but not necessarily on a regular basis). Ovocytes may even be fertilized. However, implantation does not follow. This has been attributed to rapid extrusion of the ovocyte or conceptus. Another possibility which has been considered is that use of progestagens during the follicular phase interferes with preparation of a suitable uterine environment.

The estrogen-progestagen combination is almost 100% effective, except when the dosage schedule is interrupted. However, many women experience "side-effects" ranging in severity from mild discomfort to incapacitation or endangerment of health and survival. Concerns have also been expressed about possible as yet undetermined long range effects of chronic interference with hormonal processes, especially if initiated too soon have ovarian cycles have become established.

It has been proposed that toxicity results from pharmacological actions of synthetic agents which are in many ways different from natural hormones. But it must be recognized that if natural hormones were utilized, they would have to be taken in pharmacological dosage on time schedules very different from physiological secretory patterns. Many of the symptoms resemble ones seen during normal pregnancy.

It is sometimes helpful to change the chemical nature of the preparation or the relative dosages of estrogens and progestagens. Most "progestagens" exert weak estrogen-like actions. Some share with pro-

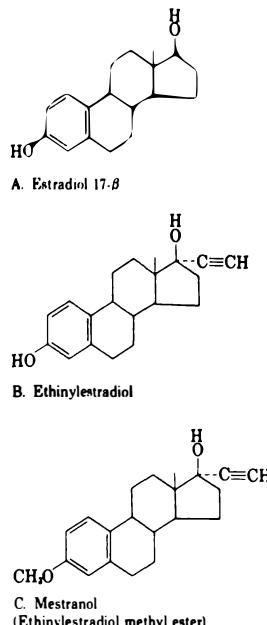


FIG. 22-6. Comparison of structure of naturally occurring estradiol with those of synthetic estrogens used in oral contraceptives.

gestrone the ability to be biologically converted to androgens; it is claimed that others (e.g., chlormadinone) are devoid of potential androgenicity. Representative drug combinations now in use are shown in Table 22-1.

Problems encountered by some (but not other) pill users, which are also seen during pregnancy, include gastric upsets, nausea (sometimes severe enough to lead to vomiting), breast engorgement that can be painful, pigmentation of the face (chloasma), and effects on the eye which make necessary a change in contact lenses that were comfortable with lesser amounts of steroids. Water retention is a common finding. In some women the symptoms subside in time, even though pill use is continued, or ways are found to cope with the problems (e.g., by taking pills at night to avoid awareness of mild nausea). In others they subside only if use is discontinued. In a few, the water retention and weight gain persist long afterward.

Estrogens enhance hepatic production of

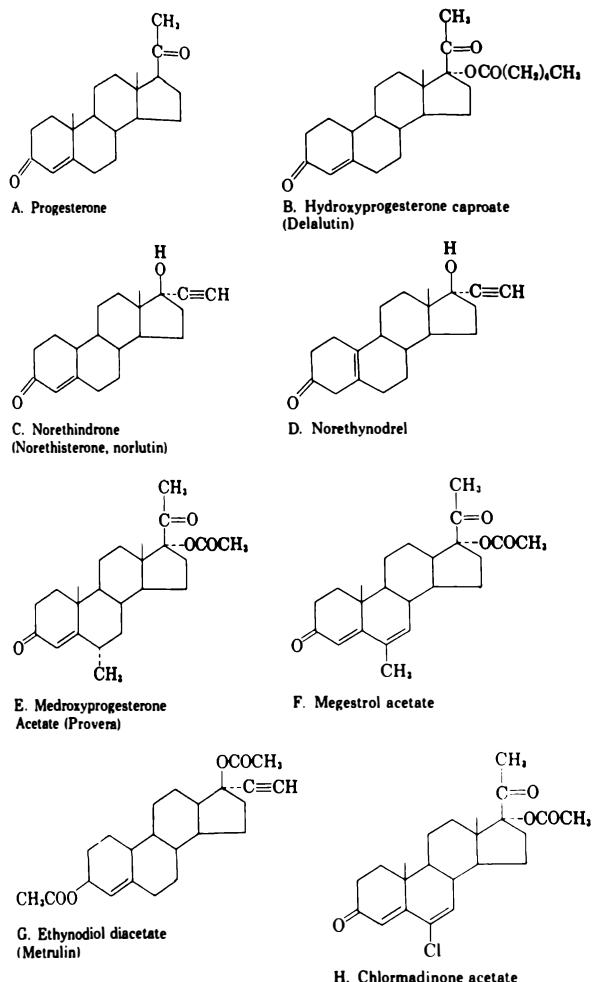


FIG. 22-7. Progesterone and synthetic progestogens.

renin substrate and elevate bioassayable levels of angiotensin II. Estrogen induction of specific estrogen receptors in the liver has been demonstrated, and the rise in hepatic content of receptor protein has been associated with elevation of renin substrate in the plasma.¹⁰ Some women develop hypertension; it is suspected that their mechanisms for regulation of renin production by the kidney are defective, or that abnormal electrolyte metabolism and water retention aggravate the problems of renin substrate increases.

Influences of estrogens on the cardiovascular system have caused the greatest con-

cern. The incidence of embolus formation and arterial and venous occlusion is higher in pill users than in a comparable population of women who do not use the agents. According to some observers, the incidence of thromboembolism and associated stroke is higher than that seen during pregnancy. No relationship has been found between duration of use of the agents or estrogen dosage and the occurrence of the problem.

Estrogens tend to induce elevations of plasma lipid levels, and the effect is most pronounced in women with preexisting problems of lipid metabolism. Fear has been expressed that chronic elevation of

TABLE 22-1
Composition of Several Commercially Available Oral Contraceptives

Trade Name	Progestagen	Estrogen
Enovid	Norethynodrel	Mestranol
Norinyl, orthonovum	Norethindrone	Mestranol
Metruulin, ovulen	Ethinodiol diacetate	Mestranol
Lormin	Chlormadinone	Mestranol
Delpregnin	Megestrol acetate	Mestranol
Provest, farlutal	Medroxyprogesterone acetate	Ethinyl estradiol
Norlestrin	Norethindrone acetate	Ethinyl estradiol

triglyceride or cholesterol concentrations in otherwise normal women may ultimately exert deleterious effects, and it is suspected that the observed vascular accidents are related to effects of estrogens on the lipids. Some estrogenic compounds also increase plasma lecithin concentrations and it is possible that lysolecithin derived from this sensitizes platelets and predisposes to clot formation. There are indications that progestagens may have opposing actions.

Some of the plasma proteins directly involved in blood coagulation mechanisms are increased during normal pregnancy (when both estrogen and progesterone levels in the blood are high), and similar increases have been found in women taking oral contraceptives.

Higher concentrations of a variety of specialized proteins have also been found in both pregnant women and pill users. These include corticosterone-binding globulin (CBG, Section III), thyronine-binding globulin (TBG, Section V), ceruloplasmin which transports copper in the plasma, and transferrin. Other influences on the liver can be detected with standard hepatic function tests; in most cases there are no associated symptoms but a few individuals develop jaundice. For unknown reasons the incidence of jaundice is higher in some European countries than it is in the United States. Suspicion has been raised that use of the pill increases the incidence of liver tumors.

Abnormalities of carbohydrate metabolism have been detected in pill users, and related changes in carbohydrate metabolism have been noted in pregnant women. Both influences on secretion of growth hormone and on responses to insulin may be involved, and progestagens as well as

estrogens have been implicated. Some physicians recommend that women with predisposition to development of diabetes mellitus use the pill with great caution, while others believe they should not use it at all.

Problems which have been directly associated with the progestagen content of the pill include psychic depression and excessive uterine bleeding during the days when the pill is not taken.

Symptoms such as depression, reduction of sexual interest, fatigue, nausea, increased appetite, and associated weight gain (or loss of appetite), are difficult to evaluate. Psychogenic origin is frequently proposed, and it has been suggested the symptoms are more likely to develop in women taking the contraceptives for practical reasons or to please their husbands but having a deep underlying wish to become pregnant. It is difficult to test the concept. There are women who switch to other methods of contraception and enjoy total loss of the symptoms. (Few women who have experienced severe nausea during pregnancy exhibit receptiveness to suggestions by male obstetricians that it is "all in the mind.")

Since estrogens have been shown to be carcinogenic under specialized laboratory conditions, removal of ovaries has proven helpful in some cases of breast cancer, and unusual vaginal cancers have been detected in children of women taking large doses of diethylstilbestrol during pregnancy, there has been concern over the possibility that long term use of oral contraceptives can predispose to development of malignancies. Recent statistical data supporting the concept are described in Chapter 21. Some observers claim that the

incidence of cancers of the reproductive system is actually lower in pill users. One explanation offered is that, unlike the woman with normal ovarian cycles, the person taking oral contraceptives has days when estrogen levels are very low. However, the incidence of pituitary tumors rises in susceptible strains of experimental animals exposed to cyclical administration of the same agents.

The possibility that chronic use of the pill may lead to infertility, or to production of children with congenital defects has also been seriously considered. There is no evidence whatever for a higher incidence of birth defects in children born to mothers with a history of years of ingestion of oral contraceptives.

Most women resume normal ovarian cycles within 2 or 3 months after discontinuing use of the pill. Some require 6 months or more. An occasional subject experiences persistent amenorrhea.

In spite of all of the problems cited above, there is a strong feeling that use of oral contraceptives poses far less danger than repeated pregnancy. However, the need for investigation of potentially superior methods of contraception is obvious.

An attempt has been made to provide a "more physiological" situation through sequential use, first of a synthetic estrogen, then a synthetic progestagen, followed by withdrawal of both. Greater toxicity and a higher incidence of conception have resulted. Interest in the sequential use of steroids has declined, and sale of some previously accepted agents is now legally restricted.

Use of Progesterone Alone

Because many of the difficulties associated with use of the oral contraceptives have been attributed to the estrogenic components, attempts have been made to use only progestagens, which are taken continuously without interruption. Fertility is reduced, often with no evidence of side effects, but the *dependability* of the method compares unfavorably with use of combination pills. The mechanism of action is believed to be quite different. Secretion of gonadotrophins by the pituitary is less affected than with the "pill." FSH concentrations in the plasma may be lower than in undisturbed cycles, but ovarian hor-

mone secretion proceeds, LH peaks have been observed, and ovulation may take place regularly. It is believed that progestagens exert their principal action on glands of the uterine cervix, promoting secretion of a mucus which in several ways interferes with sperm penetration (Chapter 21). Continuous use of progestagens may also interfere with preparation of the endometrium for implantation, with capacitation, and with sperm motility.

Preparations have been designed for local application to the cervical region (to achieve contraception without disruption of plasma hormone levels); but the effectiveness is inadequate, possibly because cervical glands exhibit some resistance to the persistent presence of progesterone, and possibly also because the limited numbers of sperm which succeed in overcoming the imposed obstacles may be sufficient to effect fertilization.

There has been some interest in *systemic* administration of progestagens alone, insufficient dosage to *disrupt gonadotrophin secretion*. It has been reported that fertility is reduced by about 50-75% below that which pertains without hormone treatment. Some interesting discussions have taken place between "social minded" males of middle age and beyond, who point out the benefits to population control as a whole without endangerment of health of the progesterone users, and women of child-bearing age who are unwilling to play games of "steroid roulette." There has been recent interest in development of a progesterone contraceptive for lactating women, since fertility seems to be reduced without impairment of milk production.

The "Morning-After" or Postcoital Contraceptive

Synthetic estrogens, and especially diethylstilbestrol (DES) in large doses promote vigorous contractions of the musculature of the female reproductive system and have been successfully used to promote expulsion of the ovocyte or fertilized ovum before implantation can take place.

Because of the high dosage required and evidence that DES can be harmful to an embryo, the procedure is contraindicated under conditions in which it is possible that pregnancy is already in progress (un-

less it is to be followed by abortion). There is also concern over possible influences of the very high doses on the pituitary gland, especially after repeated use.

A variety of other agents for postcoital use are under investigation including progestagens, antiestrogens, and nonsteroidal agents. The fully effective nontoxic preparation has yet to be developed.

Recent Advances

New approaches to fertility control now under investigation include attempts to promote endogenous production of specific antibodies to HCG which do not affect LH function, and of specific antibodies to sperm components which are harmless to the female system. Analogs of LRF providing potent anti-LRF activity have been synthesized and are being tested for use as ovulation suppressants.¹⁻⁶

The Prostaglandins¹⁻⁶

Prostaglandins, and especially PGE, and PGF_{2α}, are powerful stimulants of uterine muscle. They are effective at all stages of pregnancy, and have been used to impede implantation, induce abortion, and hasten labor. Actions on both the oviduct and uterus can promote expulsion of ovocytes and the early products of fertilization.

In at least some species, the prostaglandins are luteolytic; large doses administered at appropriate times can shorten ovarian cycles, reduce progesterone secretion, and terminate pregnancy through interference with corpus luteum function.

Early hopes that prostaglandins might provide a safe, effective means for prevention or termination of early pregnancy (possibly in a form suitable for self-treatment by untrained women) have been dimmed by the realization that it is sometimes necessary to administer repeated doses over a 48-hr period, that painful uterine contractions, nausea, vomiting, diarrhea, and shivering are common "side effects," and that cardiovascular and respiratory actions may pose genuine danger to the recipient.

Some undesirable effects have been diminished by administering the PGs locally. Intra-amniotic injection has decided advantages for induction of abortion, but can obviously be performed only under clinical

conditions when adequately trained personnel are available. Intravaginal application is often effective, but its success depends upon absorption of prostaglandins into the systemic circulation.

Development of newer techniques (e.g., vacuum aspiration) for early induction of abortion has led to diminished interest in the prostaglandins. Some obstetricians consider them valuable agents for promoting onset of labor; but others find them less dependable than oxytocin and related peptides.

Intrauterine Devices^{2-4, 8}

Legend has it that the earliest application of the idea that placement of foreign bodies within the uterus can prevent conception dates back to the time when stones were inserted into camels to prevent pregnancy during caravan expeditions. Modern resurrection of the concept was made possible by development of a variety of small plastic devices which are well tolerated by a fairly high percentage of women.

Objects of a wide range of size, shape, and texture have been shown to effectively reduce conception under conditions which permit free entry of sperm into the uterus. The obvious advantage is that a single insertion provides long-term protection. But disadvantages include pain and uterine cramps experienced by many women for weeks or months after insertion of the device, the possibility of spontaneous expulsion (which may go unnoticed), less common uterine bleeding, and rare incidences of uterine perforation. Devices that are large and stiff are most likely to cause uterine problems, while those that are small and soft are more readily expelled.

Several hypotheses have been advanced to explain the mechanism of action; they are difficult to test because of individual and species differences. The concept most widely accepted for humans is that a *foreign-body reaction* is set up within the uterus, associated with migration of leucocytes into the region, and creation of an environment unsuitable for implantation. In at least some species, intrauterine devices (IUDs) *disrupt the reproductive timetable* so that uterine preparation is no longer synchronized with blastocyst arrival. *Disturbances of motility patterns*

also lead to either premature expulsion of ovocytes, zygotes, and blastocysts, or excessively long retention of the conceptus within the oviduct. These may result in part from neurogenic stimuli arising within the uterus; but there is also strong evidence for increased release of prostaglandins. (The devices have been reported to be ineffective in women taking large doses of salicylates, indomethacin, and other agents which interfere with prostaglandin synthesis.)

The implantation hypothesis is supported by the finding that IUDs do not decrease the incidence of *tubal* pregnancies; but this could also be interpreted to indicate that IUDs alter the microenvironment of the oviduct.

It has recently been shown that addition of copper to the small devices increases their effectiveness. The copper is released slowly, and it seems to alter uterine metabolism and thereby render it inhospitable to blastocysts. It has been proposed that a suitable inhibitor of uterine metabolism could provide the ideal method of contraception because actions are confined to cells which will soon be discarded. Copper is known to adversely influence ovulation, but it is unlikely that sufficient quantities are released to exert this action.

CONTRACEPTIVE PROCEDURES DESIGNED FOR USE BY THE MALE^{1, 5}

It has been charged that male investigators have concentrated their efforts on development of contraceptives to be used by the female, because they prefer this arrangement and because males do not react favorably to procedures which "threaten masculinity."

But there are logical reasons for directing research at the female. In human females contraception can be achieved by interference with preparation or function of a single ovocyte each month, whereas males produce enormous numbers of spermatozoa in regularly recurring waves and fertilization can be achieved with only a small fraction of the numbers produced. Moreover, it is possible to retain the presence of sufficient female hormone to sustain metabolic functions and character of accessory reproductive structures and secondary sex characteristic, while interfering

only with the pattern of release. Sustained reduction of testosterone can have widespread deleterious effects in the male, and intermittent interference with release may not reliably reduce fertility. In addition, the more complicated female system provides greater opportunity for intervention.

Valves have been developed for insertion into the vas deferens, for the purpose of producing a reversible block to sperm passage. An interval of several weeks may be required before reliable contraception is achieved. Reestablishment of fertility has not been successful in almost half the cases in which it has been attempted; but this does not rule out the possibility that better devices will be developed in the future. "Vasectomy" is a term applied rather loosely to procedures designed to permanently remove sperm from the ejaculate; it often involves ligation rather than removal of the ducts.

While Leyding cell function seems to be maintained in humans and experimental animals, and sexual interest and performance are not impaired, there are concerns over possible immunological consequences of the procedures. Sperm antigens gain access to the systemic circulation, and specific antibodies have been found in the serum. Although experimental animals developing antibodies following unilateral ligation of the vas deferens have been shown to retain fertility, autoimmune damage to the testis has been demonstrated under certain conditions. While the incidence of such destruction in the human does not seem to be high, the potential danger has not been fully evaluated.

Agents toxic to spermatozoa have been tested in experimental animals and shown to impair fertility. No preparation is available at the present time which specifically affects spermatogenesis without induction of systemic toxicity in human subjects; and there is concern that agents toxic to germinal or Sertoli cells may cause development of abnormal spermatozoa and thereby promote formation of unhealthy but viable zygotes.

The epididymis requires very high localized concentrations of androgens. Attempts are being made to lower such concentrations without interfering with maintenance of plasma androgen levels. One approach is localized administration of

ciproterone, in a form (*e.g.*, Silastic capsules) which precludes rapid systemic absorption.

It is obvious that reduction of systemic androgen can have widespread undesirable influences on metabolism in nonreproductive tissues, and will also impair sexual interest and performance. Synthetic androgens have been administered to limited numbers of human subjects in dosages which maintain most hormone functions but which are sufficient to suppress LH secretion and thereby lower localized testosterone secretion within the reproductive system. The findings are encouraging, but additional investigation is needed.

ESTROGENS AND THE MAINTENANCE OF GESTATION^{2, 4, 10}

Functions

The needs for estrogens in preparation of the ovocyte for fertilization, in ovocyte transport, in regulation of functions of the reproductive tract favoring ovocyte survival, fertilization and subsequent development of the zygote, and in preparation of the endometrium for implantation, have been described in detail.

During early pregnancy, estrogens promote growth and strengthening of the myometrium. Earliest influences are exerted on cell proliferation; later, each of the new cells grows tremendously in size (and especially length) as the uterus accommodates to a rapidly enlarging embryo and fetus. Estrogens promote synthesis of contractile proteins, directly stimulate contractions, and enhance sensitivity to stimulatory influences of both hormones and mechanical stretching. During gestation, the contractions contribute substantially to development of the musculature. At the time of parturition, estrogenic influences play a role in establishment of the powerful, coordinated contractions needed for expulsion of the fetus and placenta.

Estrogens also enhance uterine blood flow, thereby increasing availability of nutrients and facilitating waste removal. There is also indirect evidence for a role of the hormones in development of the fetus. Positive statistical correlations have been found between birth weight and amounts of estriol excreted; and changes in fetuses

of experimental animals have been described following estradiol administration.

In addition, estrogens directly and indirectly inhibit pituitary secretion of FSH and LH; this reduces the chances that new follicles will mature and prepare for ovulation. (Fertilization of a new ovocyte and implantation occur only rarely during human pregnancy. However, follicular maturation, ovulation, and corpus luteum formation have been shown to occur in some other mammals.) Estrogens also contribute to preparation of the mammary gland for lactation.

Hepatic synthesis of several specific proteins which bind hormones in the blood plasma is enhanced. Since only the unbound hormones seem to participate in negative feedback influences, the titers of total hormones may rise considerably without elevation of levels of free (active) hormones in the maternal circulation. This could provide a reservoir of hormones for use by the placenta and fetus. It has also been proposed that high concentrations of transcortin restrict the availability of free glucocorticoids and thereby inhibit onset of lactation during pregnancy; the glucocorticoids can then be made rapidly available for release soon after parturition. (Lactation has been initiated in pregnant animals by injection of glucocorticoids.) A role for glucocorticoids in parturition has been demonstrated for sheep and some other mammals, and a similar role has been proposed for primates. But the steroid may originate in the fetus.

All of the described estrogen influences can be elicited with estradiol (which is by many criteria the most potent natural estrogen). Similar results can be obtained with large doses of estrone. Both are produced by maternal ovaries early in gestation.

Later (in humans and some other mammals), estriol is the major estrogen produced. The reasons for the shift are unknown; it is possible that estriol performs special as yet undefined functions. A role in regulation of activities of the cervix during pregnancy has been proposed.

Biosynthesis of Estrogens During Pregnancy^{9, 12}

As noted above, chorionic gonadotropins secreted by the trophoblast promote

HORMONES AND REPRODUCTION

transformation of the corpus luteum into a relatively long-lived structure which supplies the steroid hormones needed to sustain the early phases of pregnancy. (Ovariectomy performed early leads rapidly to abortion; but steroid hormone injection can compensate for loss of the ovary.)

Later, the placenta and fetus of humans and some other mammals develop the ability to engage in steroid hormone synthesis; but pathways utilized are not identical with those described for the gonads.

Cholesterol derived from the maternal circulation serves as the precursor. The placenta possesses enzymes which catalyze conversion of cholesterol to pregnenolone

and then to progesterone; but the placenta cannot directly synthesize estrogens.

Pregnenolone formed in the placenta travels to the fetus, in which it is rapidly conjugated to pregnenolone-sulfate (Fig. 22-8). Fetal liver may be the dominant site for the conjugation.

Pregnenolone-sulfate is transformed by the fetal adrenal gland to dehydroepiandrosterone-sulfate (DHEAS, dehydroisoandrosterone-sulfate, DHAS) via intermediate formation of 17α -hydroxypregnene-*sulfate*. The term *fetoplacental unit* denotes the functional interrelationship between the two structures.

DHAS is very rapidly metabolized by

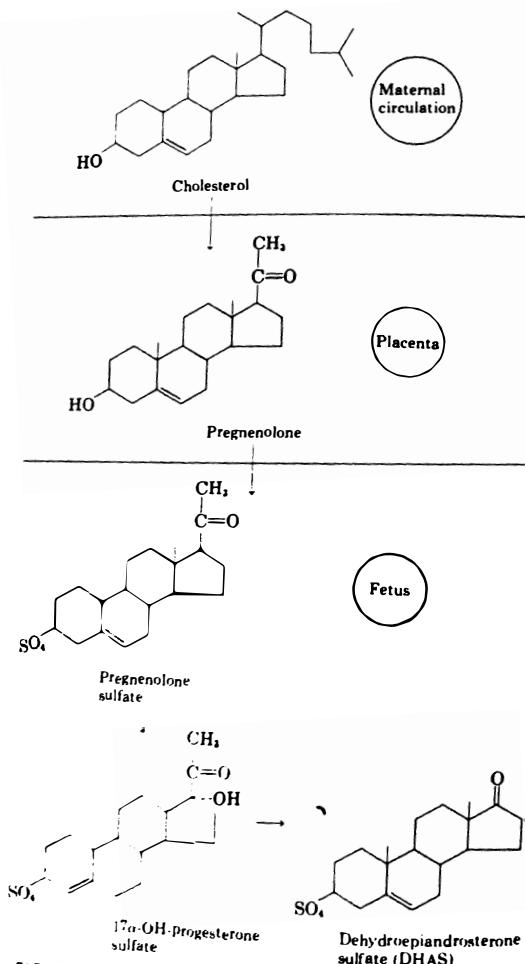


FIG. 22-8. Biosynthesis of DHAS by the fetoplacental unit.

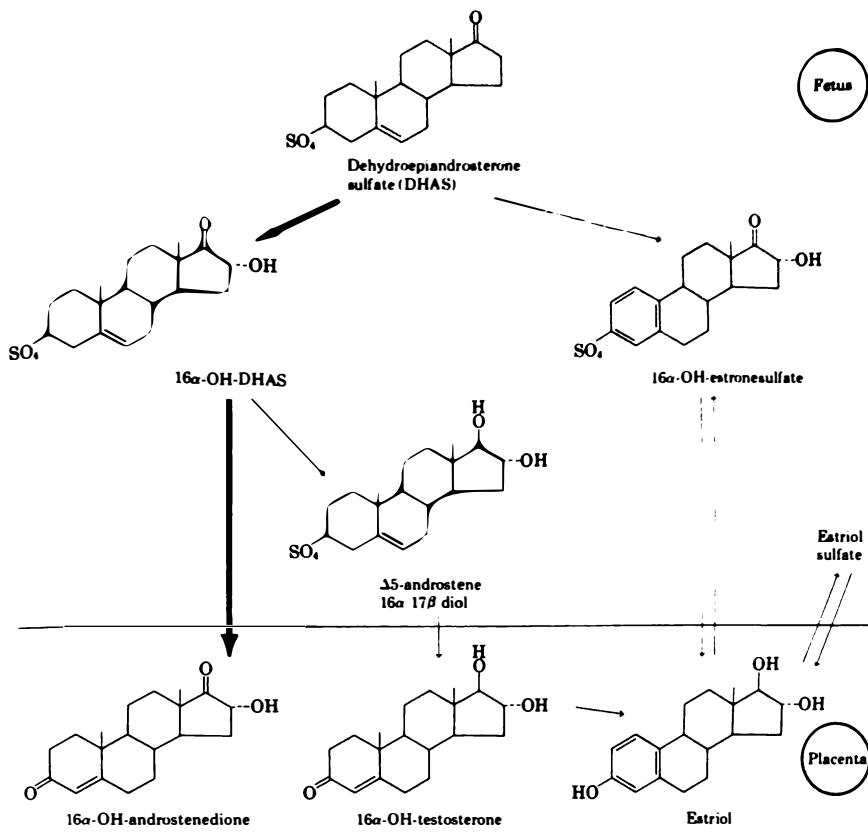


FIG. 22-9. Synthesis of estriol from DHAS.

fetal liver, and it has been suggested that such metabolism protects the fetus against excessive androgen buildup. Most is converted to 16 α -hydroxy-DHAS (Fig. 22-9). The product is returned to the placenta and is transformed (via 16 α -hydroxyandrostenedione) to *estriol*; the latter is taken up by the fetus.

A smaller fraction of the DHAS is aromatized in the fetus to 16 α -hydroxyestrone-sulfate and sent to the placenta for formation of estriol. A third pathway to estriol is via 16 α -hydroxytestosterone.

It will be noted that the fetal reactions involve hydroxylation of DHAS at the 16th carbon. Shuttling of steroids between fetus and placenta is necessary because the placenta lacks the 16 α -hydroxylase enzyme.

While estriol can pass freely to the fetus,

most enters the maternal circulation and is excreted in the maternal urine. Urinary estriol conjugates provide a measure of fetal and placental function; a sudden fall in the quantity excreted signals development of fetal problems. (However, estriol can also arise from placental conversion of maternally derived 16 α -hydroxy-DHAS.)

The more potent estrogens (estradiol and estrone) are formed via separate pathways. Some of the fetal DHAS is sent directly to the placenta (Fig. 22-10). Placental intermediates are androstenedione or testosterone.

Androstenedione and the estrogens readily enter the fetus, in which they are rapidly conjugated; but the more potent testosterone does not. Estrone and estradiol also pass rapidly into the maternal circulation.

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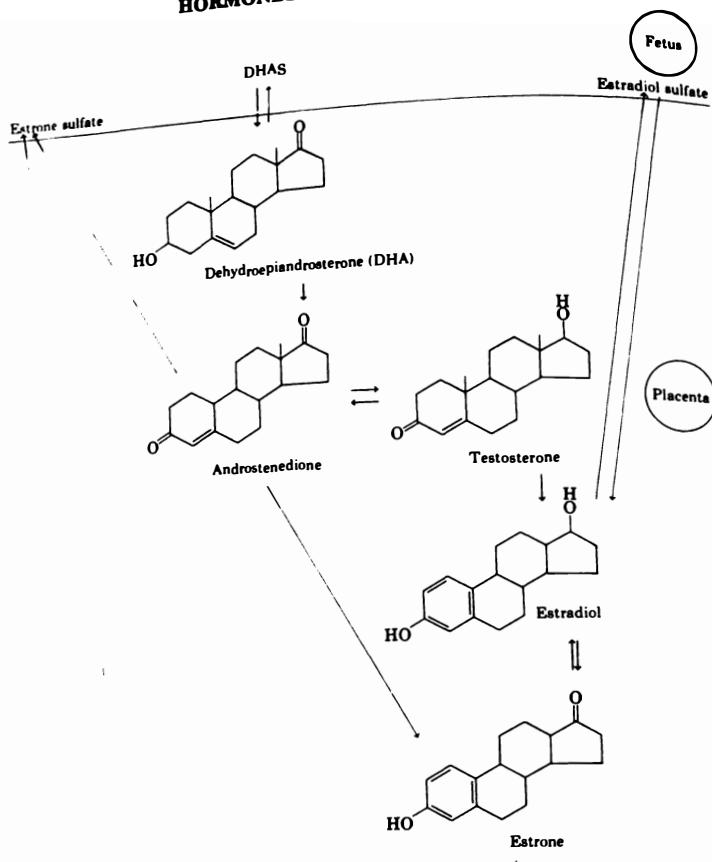


FIG. 22-10. Biosynthesis of estrone and of estradiol by fetoplacental unit.

It is apparent that once steroid synthesis has been established in the human fetoplacental unit, the maternal corpus luteum is no longer needed to provide estrogens. The same situation is seen in many other mammals, including guinea pigs, dogs, sheep, and horses. But in some (e.g., rabbits, mice, rats, and goats), ovariectomy leads rapidly to abortion at any time during the gestation period.

Estrogens, differing in structure from those described are synthesized during pregnancy by some species. For example, equilin and equilenin (Fig. 22-11) are formed in horses and zebras, and small quantities of still other estrogens are widely distributed in urines of pregnant mammals.

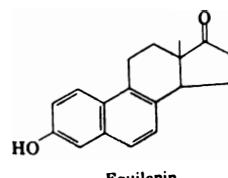
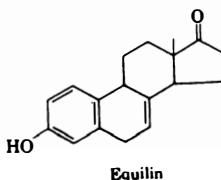


FIG. 22-11. Steroids synthesized in endometrial cups of the horse.

PROGESTERONE AND PREGNANCY⁴**Functions**

Estrogen priming prepares the myometrium for actions of progesterone. The latter contributes to growth of the myometrium and synthesis of collagen and connective tissue. But its most important function after implantation may be that of quieting the myometrium directly and protecting it against effects of estrogens and other stimulants. Influences on muscle cell membranes block transmission from the more active "pacemaker" cells so that strong coordinated contractions leading to premature expulsion of the conceptus are prevented. In addition, progesterone seems to inhibit release of oxytocin from the neurohypophysis, and also synthesis of uterine prostaglandins.

Progesterone was at one time widely used in attempts to avoid unwanted abortion. It is now recognized that the steroid is ineffective for this purpose except in the rare cases in which endogenous secretion of the hormone is inadequate. (The quantities of pregnanediol conjugates in maternal urine provide information on progesterone synthesis.) As noted (Chapter 19), progesterone can be converted to androgens, and has been known to masculinize female fetuses.

Progesterone in the quantities secreted during pregnancy may also provide effective inhibition of LH secretion. Such an action could theoretically contribute to suppression of ovulation. But the effect may be of no importance, since HCG exerts powerful LH-like activity.

Progesterone synergizes with estrogen in promoting growth of the mammary glands; but it also suppresses milk formation during the last trimester of pregnancy, apparently by interfering with production of the milk protein *lactalbumin* needed for the synthesis of milk sugar (lactose). Progesterone may also block the ability of estrogens to stimulate release of prolactin. Initiation of lactation seems to depend upon a pattern of sustained high-level secretion during pregnancy followed by abrupt withdrawal.

Progesterone induces weight gain through increased food intake in mice. Increased appetite experienced by some

women during pregnancy, or while taking oral contraceptives may be related.

The importance of progesterone in regulation of electrolyte and water metabolism during pregnancy, and implications of its ability to compete with aldosterone for receptor sites in the kidney (Chapter 13) have not been adequately investigated.

Progesterone Synthesis by the Feto-placental Unit^{4, 12}

Progesterone is directly synthesized by the placenta (Fig. 22-12). Cholesterol derived from the maternal circulation is by far the most important precursor, but some direct synthesis from acetate units is also a probability.

The hormone readily passes into both maternal and fetal circulations. Plasma progesterone is picked up by the maternal liver and conjugated with glucuronic acid either before or after reduction to *pregnanediol*. The conjugates are excreted by the kidney.

The placenta converts some of the progesterone to 15α -OH-progesterone, but no function has been found for that steroid. Fetal tissues also contain small amounts of 14-hydroxylated progesterones and of 14- and 15-hydroxylated estrogens. Some of the progesterone produced in the placenta seems to be utilized by fetal adrenals and fetal testes.

CHORIONIC GONADOTROPHINS AND PREGNANCY MAINTENANCE^{4, 12}

The functions of chorionic gonadotrophins in maintaining the corpus luteum of early pregnancy in some forms, and throughout the gestation period in others, seem to be well established. But less is known of the need for continued secretion of such hormones in species in which steroid synthesis is largely taken over by the feto-placental unit.

While most of the work has been done on the human hormone, chorionic gonadotrophins have been positively identified in several mammals; indirect evidence for their existence has been presented for some others.

Some information on the timing of the transition from luteal to placental provision of progesterone can be obtained from

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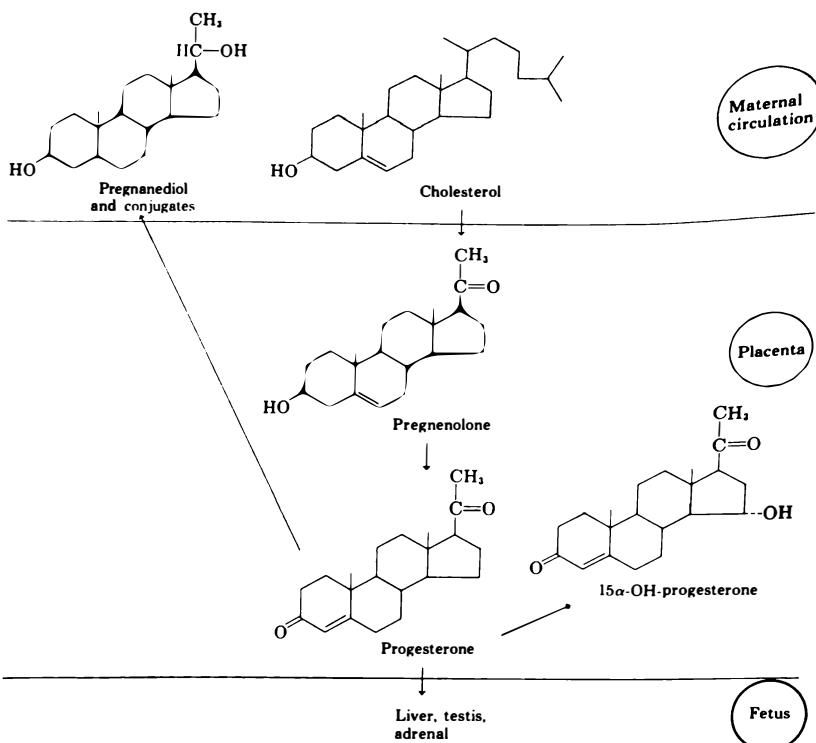


FIG. 22-12. Progesterone synthesis in the fetoplacental unit.

the 17 α -hydroxyprogesterone:progesterone ratio, since 17 α -hydroxylase activity is high in the corpus luteum but missing from the placenta. (Activity of the enzyme in fetal testis does not develop until quite late.)

The data point to a decline in the ovarian contribution during the latter part of the 2nd month of gestation in the human, and to a beginning of placental participation some time earlier than 6 weeks after conception. Maternal HCG levels rise rapidly and reach a peak at the end of the 2nd month or later; there is a gradual decline after the peak has been attained, but substantial quantities of HCG can be measured until almost the very end of the 3rd trimester. Smaller (but still potent) quantities of HCG are present in the fetus and in amniotic fluid.

HCG may promote steroid synthesis in the fetoplacental unit. The question of whether the steroid in turn affects HCG secretion remains unsettled.

The hormone has also been implicated in stimulation of fetal adrenal glands and testes, and it may synergize with other humoral regulators of fetal growth. A direct role in quieting of uterine musculature has also been proposed. A function in modulation of maternal immune responses (lessening the tendency for rejection of the fetus) has been supported by some observers and refuted by others.¹⁸

HCG may exert influences on *maternal* metabolism which favor storage during early pregnancy of nutrients needed later and during lactation. It was once believed that the hormone plays an important role in preparation of the mammary gland for lactation; but a more potent human placental lactogen (HPL) has been identified.

Some questions concerning chorionic gonadotrophin function remain difficult to answer. There are no examples of human pregnancy persisting without the hormone, and no one has devised a procedure for

removing chorionic gonadotrophin-secreting cells without disruption of other placental functions. Perhaps studies with specific anti-chorionic gonadotrophin antibodies will provide new insights.

PLACENTAL LACTOGENIC HORMONE^{1, 4, 12}

A hormone variously known as *human placental lactogen (HPL)*, *chorionic growth hormone prolactin (CGP)*, *somatotrophin (SMH)*, and *human chorionic somatomammotrophin (HCS)* has been obtained from human placenta, characterized, and shown to be present in the maternal circulation during pregnancy. Unlike the situation with HCG, concentrations of HPL have been observed to rise progressively from early pregnancy to term. The hormone is readily detected after 6-8 weeks, but secretion may be initiated by the end of the 2nd week. Peak levels are found in maternal plasma around the 35th week. Similar patterns for secretion of a placental lactogen have been described in nonhuman primates. Evidence for synthesis of a related hormone has been obtained in rats, mice, guinea pigs, goats, cows, and sheep, but there is some question of its existence in rabbits and pigs.

The structure of HPL is remarkably similar to that of human growth hormone (HGH), with which it shares a full 85% of amino acid sequences; but biological properties are in many ways more closely related to those of prolactin.

Direct actions are probably exerted only on maternal and placental physiology, but the fetus is secondarily affected. (HPL does not readily cross the placental barrier to the fetus; but low concentrations have been found in amniotic fluid and in umbilical cord blood.)

Positive correlations have been noted between infant birth weight and maternal HPL concentrations, and these are especially high in the case of underweight term infants. No similar relationships between HCG levels and birth weight have been found, but deficiencies of HCG have been related to reduced numbers of cytotrophoblast cells.

HPL seems to function more through synergism with other hormones than

through independent direct actions. It has clearly been implicated in supporting the role of HCG in maintaining the corpus luteum. It enhances lipid-mobilizing actions of growth hormone and potentiates influences of HGH on elevation of maternal plasma glucose concentrations, nitrogen retention, protein synthesis, and inhibition of glucose oxidation through resistance to insulin actions. HPL may largely account for the "diabetes-like" glucose tolerance curves often found during pregnancy in apparently normal women, and for increased insulin requirements of pregnant women with diabetes mellitus.

It has been proposed that influences of HPL on maternal lipid mobilization serve the purpose of increasing use by the maternal organism of fatty acids as a fuel, thereby conserving glucose for use by the fetus. (The fetus seems to depend heavily, if not exclusively on glucose for its own derivation of ATP energy.) Actions on maternal storage of nutrients during early pregnancy seem to be important for provision of reserves used during the later part of pregnancy (when the fetus exhibits rapid growth) and during lactation. It is interesting to note in this connection that there has been a growing tendency among obstetricians to favor a greater gain in body weight during early pregnancy than was considered optimal in the past, and that most animals having free access to food tend to store reserves during the time when embryos use relatively little.

As its name implies, HCS also promotes growth, differentiation, and protein synthesis in mammary glands. It may act in conjunction with pituitary hormones; but mammary gland development and lactation initiation can occur during the second half of pregnancy in hypophysectomized animals. HCS is luteotropic and mammotropic in rodents; and it affects pigeon crop sac in a manner similar to that found for sheep prolactin.

The view has been expressed that HPL is actually a *vestigial* hormone for pregnant women living in affluent societies. It has been pointed out that fuel needs of the fetus can be readily met from maternal food intake, that luteotropic actions of HPL are unnecessary (since HCG is present during very early pregnancy when this action is most needed), and that pregnant

women (unlike some other mammals) secrete sufficient prolactin to prepare the mammary glands for lactation.

There is a growing evidence for an important role of prolactin in regulation of fetal water and electrolyte balance. HPL present in amniotic fluid may participate in this function.

OTHER PLACENTAL HORMONES

A glycoprotein with biological properties similar to those of adenohypophysial TSH has been identified in human placenta and named human chorionic thyrotrophin (HCT). It may promote growth of the fetal thyroid during the developmental period preceding function of the fetal pituitary.

Additional peptides, including one with ACTH-like and MSH-like activities, have been detected in placental tissue. Little is known of possible functions; but fetal glucocorticoids have clearly been implicated in development of fetal lungs and other tissues.

PARTURITION^{1, 6, 14, 17}

Parturition is a complicated process in which many changes occur within a short span of time. It is possible to present hypotheses concerning mechanisms which fit with observations. However, the fact that some event regularly occurs at that time does not constitute proof that it is an essential part of the process. Moreover, the fact that parturition can be achieved after elimination of one of the associated factors does not necessarily indicate that the factor is unimportant under normal conditions.

Relaxin

Toward the end of pregnancy, *relaxin* can be extracted from, and is probably secreted by the ovary, uterus, and placenta. Relaxin is a true pregnancy hormone; it has never been found in males or in nonpregnant females. It was once thought to be a peptide of low molecular weight, but now is believed to consist of a group of quite similar peptides which differ slightly in biological as well as chemical properties.

As its name implies, this hormone promotes relaxation of the birth canal in prepara-

ration for parturition. Softening of the cervix and loosening of the ligaments of the symphysis pubis seem to result from a combination of influences on collagen, including increased hydration, decreased polymerization of mucopolysaccharides of the ground substance, reduction in numbers of cross-links, realignment of fibrils, and probably proteolysis involving increased activity of collagenases and other enzymes. Relaxin exerts its influences on tissues which have been previously exposed for considerable periods to the steroid hormones, and some special function of estriol has been proposed.

Since relaxin also seems to quiet uterine muscle, it may prevent the onset of premature labor; this concept would be compatible with a reduction of relaxin levels during the final days before parturition. Limited clinical attempts to delay onset of parturition with relaxin preparations have not been encouraging. It is possible that uterine-relaxing peptides are separable from those acting on the cervix and symphysis pubis, and that future research will provide more useful agents.

Progesterone and Parturition

Because of the described influences of progesterone on uterine muscle, the "progesterone block" hypothesis for onset of parturition was formulated. It states that expulsion of the fetus does not occur before term (in spite of capability of uterine muscle to undergo organized contractions and the presence of estrogens and other uterine stimulants) because of the depressant actions of progesterone on the myometrium; and that parturition is initiated when progesterone secretion declines rapidly during the last days of pregnancy. The reason most often cited for the decline in progesterone secretion is that the placenta is "programmed" for a limited life span, and that it ages and loses viability and secretory functions shortly before parturition. A further extension of the hypothesis states that delivery is favored by earliest loss of the progesterone block high in the uterus, so that contractions are initiated there while the lower part of the uterus is more relaxed. Loss of progesterone not only removes a direct depressant of uterine muscle, but it permits development of

sensitivity to *oxytocin* secreted in increased quantities within the last day or two of pregnancy. In addition (as previously noted), progesterone seems to exert inhibitory influences on secretion of oxytocin. A still further role of progesterone in enhancement of oxytocinase activity has been considered, but the importance of this enzyme in parturition is seriously questioned.

In support of the "progesterone-block" hypothesis, it can be shown that the amounts of free progesterone acting on the myometrium are reduced shortly before parturition, in some species because of reduced placental secretion of the hormone, and in others because of changes in metabolism of either progesterone itself or of progesterone-binding proteins. It has been possible to delay parturition in certain experimental animals (e.g., rats and rabbits) simply by administration of large doses of progesterone shortly before the expected time for onset of labor. But progesterone administration does not delay parturition in humans or other primates either at term or when onset of labor is premature; and there is no convincing evidence that progesterone levels do, in fact decline in primates.

Estrogens and Parturition

In rats, mice, rabbits, cats, and some others, parturition can be precipitated prematurely by administration of large doses of natural or synthetic estrogens. In such animals, a major factor in normal onset of labor may be a rise in estrogen:progesterone ratio. In several other species, including primates, estrogens do not hasten onset of labor even when given shortly before the expected spontaneous onset. It is suspected, however, that a rise in either estrogen concentration or estrogen effectiveness (through reduction of inhibitory influences) plays some supportive role.

Oxytocin^{16, 21}

Oxytocin is released by the neurohypophysis shortly before the onset of natural labor, and the hormone can be detected in the uterus and in maternal blood. It is believed that oxytocin functions importantly in the quite rapid birth of certain rodents and some other small mammals. In

primates and other large species, oxytocin levels continue to rise as labor progresses, and the hormone has been especially implicated in orderly and rapid termination of final processes leading to expulsion of the fetus and placenta. Oxytocin promotes release of milk from the mammary glands (see below); intermittent release of milk associated with strong uterine contractions has been taken as evidence for intermittent bursts of oxytocin secretion. Delivery can be completed in the absence of the hormone; but it may be difficult, delayed, and associated with increased incidence of fetal injury or death.

Maternal release of oxytocin is probably triggered by stimuli arising in the vagina and uterus. (Release can be promoted by artificial stimulation of those regions.) There is experimental evidence for additional release of oxytocin by the feto placental unit.

The role of oxytocinase is controversial. It has been proposed that reduction of activity of the enzyme at the time of parturition serves to increase availability of the hormone within the uterus; and reduced activity at that time has been demonstrated. However, it is known that oxytocinase is a relatively nonspecific enzyme which may have different functions, and also that other enzymes participate effectively in degradation of the hormone.

Oxytocin exerts only weak actions on the nonpregnant uterus and on the myometrium of early pregnancy. It is most effective when there has been an extensive period of estrogen and progesterone "priming" followed by progesterone withdrawal. The hormone and pharmacologically related agents are extensively used for induction of labor at or near term, but are ineffective as abortifacients.

The most important actions of oxytocin may be exerted after completion of the delivery. Because of anatomic arrangements, contraction of uterine muscle results in squeezing of blood vessels, thereby preventing postpartum hemorrhage. The uterus must continue to contract afterward, during the time when it involutes and reverts to pregestational size. It is standard procedure in modern hospitals to administer an oxytocin-like agent (usually ergonovine) during the postpartum period.

Oxytocin secretion after parturition is

triggered by the *suckling reflex* (see below). The hormone may play a subsidiary role in reduction of postpartum fertility through stimulatory actions on the myometrium, and possibly because of influences on the ovary. (Estrous cycles can be shortened in some species by injection of oxytocin.)

The Importance of Uterine Distention

The role of uterine distention in initiation of parturition is controversial. It is known that stretching uterine muscle can directly provoke contraction, and can increase secretion of oxytocin. Several kinds of animal studies suggest that parturition occurs earlier if fetuses are more numerous or oversized, and there is a definite tendency toward earlier birth of human twins and triplets. However, it is possible to apply other interpretations (than uterine distention) to such observations. There is no obvious relationship between birth weight of single human infants and length of gestation period, and there are probably genetic differences in ability of the uterus to accommodate a given volume. It seems reasonable to believe that uterine stretching plays a minor role in initiation of parturition.

The Prostaglandins⁶

Prostaglandin concentrations in the uterus, maternal blood, and amniotic fluid are sufficient to promote uterine contraction toward the end of the gestation period, and levels rise shortly before onset of parturition. Estrogens stimulate prostaglandin synthesis by the uterus, and progesterone decline seems to remove an inhibitory influence. Moreover, prostaglandins have been said to reduce placental and ovarian secretion of progesterone, and to enhance oxytocin release. Luteolytic actions may be especially important in species depending on the ovary for maintaining gestation.

The Role of the Fetus¹

There is a growing body of evidence supporting the concept that the fetus plays an essential role in initiation of labor. Proposed mechanisms include fetal contribution of prostaglandins and of oxytocin.

Activity of the fetal adrenal cortex rises sharply just before parturition in several

species, and a role for glucocorticoids seems well established in sheep. Parturition can be accelerated by administration of glucocorticoids or ACTH, and is delayed by adrenocortical insufficiency. Plasma concentrations of total glucocorticoids are elevated late in human pregnancy, and in some it has been shown that levels of the free hormone rise as well. It has been suggested that early parturition associated with multiple births in humans, and with very large litters in other species, may result from more rapid accumulation of fetal adrenocortical hormones.

Fetal adrenal glands are stimulated by ACTH released from the fetal pituitary. It is possible that maturation of the hypothalamus and its functional connections with the adenohypophysis signals the readiness of the fetus to assume independent existence. Secretion of the necessary corticotrophin release factor may occur spontaneously as part of the maturation process; or it may require a fetal trigger. Hypothalamic thermoreceptors become functional shortly before birth of some species, and it has been proposed that awareness of a rise in temperature provides the signal for release of CRF. The consequent secretion first of ACTH and then of glucocorticoids, leads to enhanced prostaglandin secretion, and the latter may initiate uterine contractions.

In some of the smaller animals (rats, mice, hamsters, etc.), hypothalamic control of the adenohypophysis is not developed until after birth; other mechanisms must be operative in such mammals.

HORMONAL CHANGES IN THE MOTHER DURING THE PERINATAL PERIOD

Throughout the entire period of pregnancy, hormonal balance in the mother is different from that of nonpregnant individuals of the same age. Attempts have been made to relate characteristic physical symptoms to specific hormonal changes. Thus, the "morning sickness" (which can progress in susceptible individuals to severe nausea and vomiting recurring over a period of months) has been attributed in part to chronically elevated estrogen levels. Persistent high progesterone concentrations may play a role in the somnolence, sense of fatigue, weight gain, and disturb-

ances of temperature regulation and of water balance experienced by some women. Altered carbohydrate metabolism has been related to release into the maternal circulation of chorionic gonadotrophins and of HPL, and to estrogen-stimulated maternal secretion of growth hormone and prolactin.

Total concentrations of adrenocortical hormones usually rise during pregnancy, and levels of free (uncombined) glucocorticoids are elevated in some women. Parturition constitutes a stress, and glucocorticoid concentrations usually rise further, along with oxytocin and often vasopressin.

There are conflicting opinions regarding the etiology of symptoms which are classified by certain observers as "psychogenic." Some women experience psychic depression during pregnancy, which they are unable to relate to emotional factors; others enjoy an especial sense of well-being in excess of that present during other periods of good health.

"Postpartum depression" is so commonly seen it has been regarded as "normal" when the condition is mild and transient. The depression is likely to be most severe in women who report feeling especially well during the pregnancy. In some with a history of previous mental illness or emotional instability (and in a few without it), the condition may be severe enough to rate the designation "postpartum psychosis." Many cases are self-limiting, others respond readily to treatment, and still others persist long afterward.

It is easy enough to find "psychological reasons" for the mood changes. Pregnant women are likely to be affected by such factors as conscious and unconscious thoughts concerning whether the child is wanted, anticipation of influences of the pregnancy and subsequent events on future economic status or life-style, reactions to physical symptoms such as nausea and fatigue and to effects of pregnancy on appearance, and attitudes of persons with whom they come in contact. During the postpartum period a woman is likely to be much fatigued by the need to attend to infant feedings which disrupt sleep, and by an endless round of small tasks (laundry, etc.) added to the usual household routine. She may react unfavorably to confinement to the home and restriction of daytime com-

panionship to that of the infant and other small children.

But there are reasons to suspect that at least some of the "psychic" symptoms are directly related to hormonal changes, and that improvement occurs as the result of (usually spontaneous) endocrine adjustments. Both glucocorticoids and estrogens are known to be taken up avidly by neurons and to affect their functions. Women taking oral contraceptives may experience many of the symptoms associated with pregnancy; and the symptoms often disappear when some other method of contraception is substituted.

Administration of glucocorticoids to nonpregnant patients often promotes development of mild to moderate euphoria, especially when the dosage is low and the treatment is of short duration. Chronic administration of high doses more commonly leads to depression. Patients with Cushing's disease characteristically exhibit wide mood swings and bouts of severe depression which are alleviated if the source of excess glucocorticoid is removed. Postpartum "letdown" is attributed by some to precipitous fall in glucocorticoid levels (sometimes complicated by disruption of thyroid function) following a period of excessive elevation during parturition.

It is possible that women who do not nurse their infants experience greater difficulty making postpartum hormonal adjustments, since it is also necessary to "turn off" the endocrine functions which have been established for lactation. Observers of animal behavior point to the active participation of the mother in nursing and describe her reactions as "consummatory." The importance of such a factor is difficult to evaluate in women, since a fair percentage of those who do not nurse their infants obtain a variety of stimuli outside the home which provide positive influences on both psychological outlook and physiological processes, while some who nurse their infants do so because of a sense of "obligation" and may suffer from adverse psychological reactions which negate possible hormonal benefits.

LACTATION^{1, 6, 11, 13, 16, 27}

Of all hormonally regulated functions, the processes of preparation for, initiation

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of, maintenance, and termination of lactation may be the most complex. A vast array of hormones is needed for both direct and supportive functions. The hormones interact with each other and with neuronal mechanisms.

The Prepubertal Mammary Gland

The human mammary gland undergoes extensive differentiation during fetal life, regresses somewhat during the first few weeks after birth, and then remains quiescent until puberty.

The beginnings of mammary gland development can be detected as early as 4 weeks after conception. Small areas of thickened skin epithelium at the sites of future glands grow downward into parts of the mesoderm which will later form the connective tissue of the lobules and the stroma of the nipple. All early growth is below the skin surface.

Later in fetal life, epithelial buds grow downward into the developing dermis to form straight, solid tubules. The latter send out branches and continue to invade the underlying tissue by a growth pattern described as arborization. As the tubules enlarge they become hollow, and the main branches enter into formation of the future milk ducts (lactiferous ducts, galactophores), while tips of the branches dilate to form the acini (alveoli). The nipple and areolar area develop toward the end of fetal life and project upward above the skin surface. Each of the milk ducts establishes a separate termination in the vicinity of the differentiating nipple. Connective tissue of the dermis separates the breast into lobes and lobules.

The epithelial cells grow quite large and develop an extensive endoplasmic reticulum, numerous mitochondria, vesicles, microvilli, and cytoplasmic extensions. Granules giving histochemical reactions for glycogen appear within and between the cells. Along the ducts, *myoepithelial* cells can be distinguished by their fusiform shape, small irregular nuclei, and cytoplasmic fibrils.

Hormonal requirements for differentiation of human mammary glands have not been extensively studied, and most of the available information is obtained by inference from studies of laboratory animals.

Insulin and *adrenocortical steroids* have been found indispensable for maintaining viability, growth and differentiation of the mammary gland cells under experimental conditions, and *somatotrophin* (STH) seems to be needed for attaining full growth and development. *Thyroid* hormones apparently play a permissive role. All of the preceding are known to be produced by the human fetus at the time when mammary development is rapid. Presumably, *trophic* hormones promoting secretion of the hormones directly involved are also needed, and some of these may be provided by the fetal pituitary. But HCG has been implicated in stimulation of the fetal adrenal cortex, human placental thyrotrophin (HCT) in stimulation of the fetal thyroid, and maternal hypothalamic hormones in regulation of the fetal pituitary.

In addition, as yet incompletely characterized epithelial growth factors may contribute substantially to the hormonal regulation. (Tissue culture studies have demonstrated that very small quantities of a peptide extracted from rodent salivary glands, and peptides present in bovine and human sera, exert powerful influences on mammary epithelial growth.)

Close to the time of parturition, the fetal mammary gland is surprisingly well developed and may even secrete small quantities of a white milk-like fluid (sometimes referred to as "witch's milk"). The maturation evidently results from prolonged stimulation by the estrogens, progesterone, prolactin, and possibly HCG, which are abundantly available. Regression of the glands after removal of maternal and placental hormones supports this concept. (Influences of placental lactogen on the fetus are questionable.)

In some of the mammals there are significant differences in appearance of mammary glands of female as compared with male siblings, and it is suspected that androgens provide inhibitory influences in the latter. Androgen secretion may be stimulated by the chorionic gonadotrophins.

Postpubertal Development of the Mammary Gland

Animals with short estrous cycles secrete substantial amounts of estrogen, but little progesterone. Development is limited during regular cycles in rats and others; but

the repeated bursts of prolactin released during *pseudopregnancy* can (through direct and luteotrophic actions) lead to induction of milk synthesis. In primates, proliferation progresses during follicular phases of the menstrual cycle, and lobuloalveolar specialization during luteal phases. Some regression takes place toward the end of each cycle. Maximal pregestational maturation takes place in humans around the 20th postnatal year. Estrogens promote connective tissue growth and fat accumulation in the human breast. (The effect is also seen in males with high estrogen levels; *gynecomastia* develops when defective hepatic function permits estrogen accumulation, or when the steroids are administered for therapeutic purposes.)

There are marked species variations in requirements for mammary gland development. While estrogens seem to act primarily on the duct system of rats, mice, rabbits, and cats, they can promote lobuloalveolar growth in ovariectomized guinea pigs, cows, and goats (but limited progesterone secretion by the adrenal cortex may play some role). Only doses of estrogen far in excess of the physiological range can induce lobuloalveolar growth in rats and mice, while no dose seems to be adequate for such action in dogs or ferrets.

In all species studied, prolactin and growth hormone enhance influences of the steroid hormones. Extensive mammary development can be achieved in the ovariectomized rabbit with prolactin alone. Lobuloalveolar growth can also be induced in ovariectomized rats if combinations of large doses of prolactin plus growth hormone are administered.

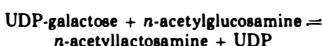
Mammary Gland Growth During Pregnancy

Development is completed under the combined influence of chronic high levels of estrogens, progestagens, and lactotrophic hormones (and probably involves additional regulators which are, thus far incompletely defined). The full extent of the changes may not be externally apparent in humans because adipose tissue is gradually replaced by epithelial components. Mitosis is most prominent during early pregnancy and seems to require the supportive influences of growth hormone and insulin (although estrogens seem to provide the major stimulus). Mitosis subsides during the sec-

ond half of pregnancy as development of the alveoli continues. Placental lactogens seem to be important for final phases in most, if not all, mammals; but high prolactin (PRL) levels are found in pregnant women. Milk synthesis seems to be inhibited primarily by the high progesterone titers. It is relatively easy to induce "precoocious" milk secretion during pregnancy in a few species such as the goat.

Milk Synthesis

Prolactin is needed for the final steps of lobuloalveolar maturation and for the production of milk sugar. Synthesis of lactose requires a *lactose synthetase system* which consists of two proteins: *galactosyl transferase* and α -*lactalbumin*. In the absence of α -lactalbumin, galactosyl transferase catalyzes the following reaction:



Therefore, free lactose is not produced. When lactalbumin combines with the galactosyl transferase enzyme, glucose is used as the substrate, and the following reaction leads to formation of free lactose:



Glucose provides the necessary precursors (Fig. 22-13).

The high levels of progesterone secreted during pregnancy (but not the lower levels present after parturition) inhibit formation of lactalbumin.

In active (but not quiescent) mammary gland, insulin promotes glucose uptake from the blood plasma, induces synthesis of enzymes needed for carbohydrate metabolism, and participates in stimulation of production of milk proteins and milk lipids.

Prolactin influences on water and electrolyte metabolism contribute to provision of water and electrolytes for the milk; but glucocorticoids are essential and it is suspected that actions of the steroid on water transport across membranes constitute an important component of their influence.

Lactation cannot be maintained after adrenalectomy except when glucocorticoid treatment is instituted soon after the surgery. Rising glucocorticoid concentrations during parturition probably play an essential role in

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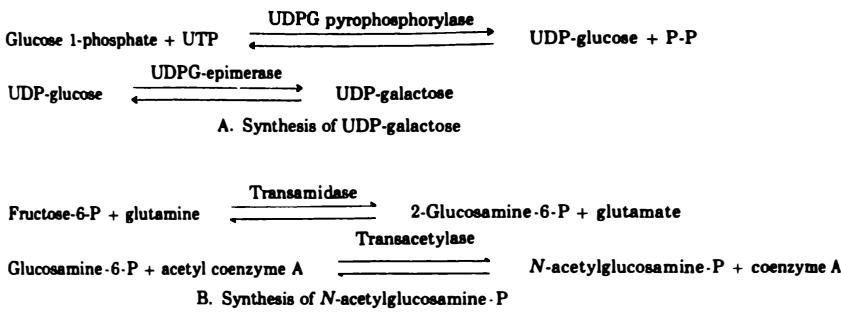


FIG. 22-13. Biosynthesis of galactosyltransferase substrates.

initiation of lactation. There are a number of experimental conditions in which the process can be started by administration of the hormones.

Milk contains substantial quantities of calcium. Maternal parathyroid hormone is needed to maintain plasma calcium concentrations. But parathyroid hormone also limits the calcium content of the milk; this seems to serve several functions. The mother is protected against hypocalcemia and excessive calcium depletion, while the ionic content of the milk is adjusted to needs of the infant.

Influences of thyroid hormones seem to be of small magnitude and may be limited to generalized regulation of metabolic processes (rather than direct influences on the mammary gland). Lactation can be maintained for a time after thyroidectomy; and administration of thyroid hormones exerts only minor influences in the intact, lactating mammal. Thyroid hormones can stimulate appetite, enhance absorption, and contribute to maintenance of glucose and prolactin concentrations in the blood plasma.

Studies of mouse mammary gland explants have provided insights into the roles of several regulators.^{16, 27} Insulin, STH, EGF, and an epithelial-epidermal growth factor derived salivary glands, promoted marked augmentation of DNA synthesis and cell replication for limited time periods, while an as yet unidentified low molecular weight, heat-labile polypeptide derived from serum sustained the stimulation. The activity was preceded by synthesis of "acidic" nuclear proteins, and was associated with methylation of the replicated DNA polynucleotides.

Estradiol-17-β in concentrations comparable to those found after puberty exerted "permis-

sive actions" and increased the numbers of cells entering the S phase, but was not sufficient of itself to promote cell proliferation. Very low concentrations of the steroid (comparable to those seen before puberty) inhibited DNA synthesis, induction of DNA polymerase, and mitotic activity.

When administered after the proliferative phase, hydrocortisone, PRL, and insulin increased the relative numbers of secretory (non-dividing) cells, and insulin pretreatment followed by hydrocortisone augmented cell content of cAMP-dependent protein kinases. But neither hydrocortisone nor PRL influenced synthesis of the nuclear proteins. PRL stimulation of synthesis of milk proteins required hydrocortisone pretreatment, and was associated with marked increases in transcription of multiple classes of RNAs and with induction of protein kinases catalyzing phosphorylation of nuclear, ribosomal, and plasma membrane proteins; PRL influences could be abolished by early administration of either actinomycin D or mitomycin C.

The findings are interpreted as consistent with a need for DNA replication and cell division for processes of derepression of specific gene sequences in daughter cells that are not transcribed in the stem cell population. PRL does not activate adenylate cyclase, and its influences cannot be mimicked with cAMP.

Initiation of Lactation

Some milk can be manually expressed from fully developed mammary glands of certain species with large udders (e.g., goats); but release of sufficient milk to nourish the young requires the actions of oxytocin. The peptide stimulates contraction of the myoepithelial cells. Functions of the hormone were first studied in animals used as commercial sources of milk, and the name *milk letdown factor* was applied.

Some oxytocin is released during parturition,

and milk may be secreted at that time. In lactating women, oxytocin release can also promote milk release in response to coitus. But the usual stimulus for oxytocin secretion is the suckling reflex. Estrogens seem to enhance sensitivity to oxytocin.

The Suckling Reflex

The neonate plays an active role in provision of its own nutrition. Influences exerted within hours after birth have long-lasting effects on maternal mammary glands.

When the newborn suckles, nerve endings in the nipple are stimulated, and impulses sent from there to the hypothalamus lead to neurohypophysial release of oxytocin. The hormone reaches the mammary gland via the systemic circulation. In addition to rapidly providing for release of preformed milk, oxytocin promotes post-partum contraction of the uterus.

Additional neuronal pathways activated by the suckling reflex lead to depletion of prolactin inhibitory factor (PIF); therefore, greater amounts of prolactin are secreted, and these provide for continued synthesis of the milk that will be required for later feedings.

It is possible to promote oxytocin secretion by mechanical stimulation of the mammary gland, and the nerve endings in the breast seem to be especially sensitive soon after parturition. Advantage is taken of this observation when it is necessary to obtain maternal milk for infants born prematurely or otherwise unable to suckle normally. While more intense stimulation may be required, a simulated suckling reflex can be produced in virgin and in male animals; and milk secretion has been induced in women at times other than after parturition.

Conditioned reflexes leading to oxytocin release are easily established. The sight and smell of the pups (without actual contact) provide effective stimuli. Release has been observed in women in response to the infant's cry, and in cows when milking machines are brought into the area. Stimuli that do not involve contact with nerve endings of the nipple are not effective for release of prolactin.

Oxytocin release can also be easily inhibited. It has been found that when women are distracted while nursing, the young infant receives less milk; but effects of the

distraction can be overridden by injection of oxytocin.

Human Prolactin (HPRL)^{11, 15}

The existence of prolactins in a variety of nonhuman vertebrates has been recognized for many years. The hormones have been extracted from pituitary glands, separated from somatotrophins, characterized and investigated for biological, chemical, and immunological properties. Several preparations (bovine, ovine) are available for experimental use, and some studies have been performed with preparations from rats, goats, pigs, and rabbits.

Problems were encountered when attempts were made to isolate a human prolactin (HPRL) distinct from human growth hormone or somatotrophin (HGH), and the question of the existence of two separate, distinct hormones was debated until only a few years ago. It is now established that the human pituitary gland does, in fact, secrete two separate hormones, and that the release of one is not necessarily linked to the release of the other.

Hindsight has provided reasons for difficulties encountered earlier. The two hormones are almost identical in size and molecular weight, and they share common amino acid sequences. Moreover, the hormones have overlapping biological and immunological properties. HGH exerts weak lactogenic activity, while HPRL mimics some metabolic actions of HGH. Both of the hormones exhibit diurnal secretion patterns with greatest release usually occurring during the night. Some acute stimuli promote simultaneous release of both.

It has long been known that patients with pituitary gland tumors can have acromegaly without galactorrhea, or galactorrhea without metabolic manifestations of growth hormone excess. And patients with growth hormone deficiency are capable of lactation.

There is good evidence for the existence of separate somatotroph and lactotroph cells in the human pituitary gland (Section VII); changes in the lactotrophs have been described during pregnancy and lactation which are not necessarily associated with changes in the somatotrophs.

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REGULATION OF PROLACTIN SECRETION^{7, 11, 23}

Hypothalamic Control

In common with other hormones synthesized by the adenohypophysis, PRL secretion and synthesis seem to be tonically regulated by hypothalamic hormones. But control is predominantly *inhibitory* in mammals, and there is little doubt that a *prolactin inhibitory factor* (PIF) is secreted.

When pituitary glands are removed from their association with the hypothalamo-hypophysial portal circulation and transplanted elsewhere within the same organism, secretions of FSH, LH, TSH, ACTH, and STH decline, while PRL output increases. Pituitary gland fragments maintained in tissue culture tend to release large amounts of PRL; this has been attributed to escape from hypothalamic inhibition. Addition of hypothalamic extracts can reduce PRL secretion. Moreover, disturbances of the blood supply to the pituitary gland and lesioning of the medial basal hypothalamus have been followed by initiation of milk synthesis in animals with suitably prepared mammary glands, and by onset of pseudopregnancy in rats.

PIF has not been prepared in pure form; but there are reasons to believe it is a small, acid-soluble peptide. Neurons secreting PIF seem to be widely distributed throughout the median basal hypothalamus and seem to make synaptic connections with regulatory neurons. It has been proposed that tonic influences are exerted by catecholaminergic neurons located close to the PIF cells.

Dopamine is the neurotransmitter most directly implicated in promoting PIF release (leading to inhibition of prolactin secretion). When infused directly into the third ventricle, it reduces PRL secretion and increases PIF activity of portal blood. Systemic injection of dopamine is ineffective, probably because the amine does not penetrate the "blood-brain barrier"; but systemic administration of L-dopa (which does penetrate) has proven useful for treatment of clinical galactorrhea.

Norepinephrine may also be important. Injection into the third ventricle, or administration of agents which increase its availability in the hypothalamus, reduces PRL

secretion. There are conflicting reports concerning influences of catecholamines on the pituitary gland. Some investigators find no effect whatever; others describe biphasic *in vitro* influences with high doses inhibiting and lower ones promoting PRL output.

A number of central nervous system depressants enhance PRL secretion. Most are believed to affect neurons synapsing with PIF-secreting cells. Nembutal (pentobarbital) first enhances and later depresses prolactin secretion; the biphasic effect seems to result from early depletion of hypothalamic PIF and late depression of pituitary gland cells.

Inhibitory influences of parasympathomimetic agents on PRL secretion have been attributed to actions on the hypothalamus. The physiological significance of the findings has not been evaluated.

PRL secretion in birds is predominantly under *stimulatory* control. The existence in mammals of a *prolactin-releasing factor* (PRF) has been proposed because the PRL release in response to suckling seems to be too prompt to be accounted for entirely by PIF inhibition. Moreover, pituitary glands receiving blood via the hypothalamo-hypophysial portal system respond more vigorously to stimuli promoting PRL release than do transplanted pituitary glands.

It has been proposed that the neurons of the medial basal hypothalamus are involved in mediation of short bursts of PRL release, and that suckling affects neurons of this region. An additional "surge" center located in the anterior hypothalamus has been proposed which effects more sustained PRL release. Moderate amounts of estrogens promote more sustained PRL release than is seen after brief stimulation of the mammary gland. It is believed that the estrogen-mediated PRL surges associated with rat estrous cycles, and the large amounts released during pseudopregnancy, depend upon the anterior hypothalamus. The center may require conditioning at a critical stage of development. PRL secretion without surges is seen in male rats and in "androgenized" females.

Stimulation of the hippocampus and amygdala indirectly affect PRL secretion, probably because of synaptic connections with neurons

more directly affecting release of hypothalamic hormones.

Serotonin, melatonin, and agents which affect serotonin metabolism (e.g., chlorpromazine and reserpine) increase PRL secretion and have been known to induce pseudopregnancy. They may promote depletion of PIF, but an influence on PRF has not been ruled out.

Thyrotrophin release factor (TRF) is a hypothalamic hormone most directly associated with regulation of TSH secretion. It is a potent stimulant for PRL secretion in humans, monkeys, cows, and sheep, and has been administered to women with inadequate milk production. The actions do not depend upon stimulation of thyroid hormone secretion; in fact, TRF is more effective in thyroidectomized than in intact animals. (Thyroid hormones do, however, affect PRL binding to target tissues.) TRF does not promote PRL secretion in intact rats and in some other species. Direct stimulation can be demonstrated on pituitary halves taken from thyroidectomized (but not from intact) rats.

TRF may contribute to regulation of PRL secretion in some species. But it does not seem to be related to PRF. There are many conditions in which changes in TSH and PRL secretions go in opposite directions, e.g., in responses to cold environmental temperatures or to the suckling reflex.

Estrogens

Effects of moderate dosages of estrogens on hypothalamic mechanisms affecting PRL secretion were mentioned above. Estrogens also affect thresholds to other stimuli, and seem to enhance PRL secretion in response to stress and to stimulation of the rodent cervix. Estrogen implants into the hypothalamus can lead to increased PRL secretion. In addition, estrogens directly stimulate pituitary lactotrophs and affect their morphology as well as secretory activity.

Large amounts of estrogens are inhibitory. Use of estrogen-progestagen contraceptives has been known to seriously impair lactation; and reduction of milk output during pregnancy has been attributed to high estrogen titers in maternal blood plasma.

High doses of estrogens have been administered postpartum to women who do not plan to nurse their infants, in attempts to suppress milk secretion and breast engorgement. The treatment is often ineffective, probably because of the endocrine milieu present at that time. There has been recent concern that such use of estrogens can contribute to the development of cancer.

Progesterone does not have consistent influences on PRL secretion. Suppression of lactalbumin synthesis was described above. While such action helps to delay onset of lactation during pregnancy, relatively large amounts taken after lactation is established have a somewhat different influence. The lactose concentration of the milk is reduced; but total milk volume may be increased to the point where total lactose production is high.

PRL plays a role in development of certain forms of mammary gland cancer. Ovariectomy is helpful in some cases, and this has been attributed to removal of a stimulus for PRL secretion. However, there is wide variation in responses of mammary tumors to hormonal intervention. Some tumors are estrogen-dependent, and PRL seems to increase formation of estrogen receptors; others regress following estrogen administration.

Prolactin and Pituitary Function

High concentrations of PRL can act directly on the pituitary gland (via an "ultrashort feedback loop") to reduce PRL secretion. Implants of PRL into the hypothalamus also lead to reduction of secretion of the pituitary hormone. Experiments demonstrating these influences have utilized very large amounts of PRL, and the physiological significance of the findings has therefore been questioned.

A reciprocal relationship between secretion of PRL on the one hand and secretion of FSH and LH on the other has been repeatedly noted. But there is no evidence that PRL directly influences the secretion of the glycoprotein hormones. The possibility that luteinizing hormone-releasing factor (LRF) is identical with PIF has been considered; but LRF does not seem to influence PRL secretion when given in doses that promote substantial increases in LH and FSH release. It is more likely that stimuli affecting PIF-regulating neurons

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affect other neurons controlling FSH and LH secretion. Dopamine has been observed to promote gonadotrophin secretion and seems to do so by enhancing LRF release.

Fertility is reduced during lactation. As noted above, this has been variously attributed to PRL influences on the ovary, and to effects of oxytocin on reproductive tract musculature and possibly also on the ovary.

MAINTENANCE AND TERMINATION OF LACTATION

Once lactation is initiated, it can be maintained for long periods if the suckling stimulus is sufficiently intense and is repeated on a regular basis. Evidently, only small amounts of PRL are secreted in response to each stimulus; it is believed, therefore, that mammary gland stimuli affect the medial basal hypothalamus rather than the proposed "surge" region in the anterior hypothalamus.

There are indications that the very high levels of PRL secretion seen early in lactation decline gradually as lactation is successfully maintained. If the suckling stimulus is withdrawn, the mammary glands soon involute and milk secretion ceases. The mammary cells become reduced in size; this is followed by cell autolysis (autophagocytosis) and phagocytic destruction by macrophages (heterophagocytosis). The processes may be initiated by mechanical pressure resulting from milk accumulation; but reduction in PRL secretion is probably also important. PRL levels in the plasma fall below those required to sustain lactation as regressive changes associated with lysosomal activity proceed in the pituitary lactotrophs. Once involuted, the mammary glands do not again secrete milk until subjected to long term influences of steroid hormones and prolactin.

Recent development of methods for measurement of oxytocin concentrations in the plasma have made possible a closer scrutiny of the suckling reflex.²¹ In lactating women and in cows, there is not a consistent elevation of plasma oxytocin in all individuals at the beginning of the nursing or milking period. And there is no quantitative relationship between oxytocin levels and milk production. It has been considered that while oxytocin is clearly needed

to establish lactation, a rise may not be required each time nursing is started once the neuronal pathways involved become fully operative. Others have suggested that ability of mammary gland tissue to bind oxytocin may provide a better index of lactation function than can be obtained from determination of plasma concentrations of the hormone.

A fascinating question that remains unanswered is, how do some kangaroos manage to produce a milk of one composition for an immature joey attached to the teat while milk of very different composition is provided for an older joey on foot, when both sources of supply receive blood containing the same hormone concentrations?

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VII

HYPOPHYSIS AND HYPOTHALAMUS

23. The Pituitary Gland

GENERAL NATURE OF PITUITARY GLAND FUNCTIONS^{4, 7, 11}

The hypophysis or pituitary gland is a very small structure which secretes at least nine distinct hormones and thereby influences virtually every known biological function of higher animals. Because of its role in regulation of growth, maturation, reproduction, lactation, the cardiovascular system, organic metabolism, water balance, pigmentation and behavior, it was once called the "master gland." But the pituitary is also the most *regulated* of all structures; many of its components shrivel if deprived of hypothalamic stimulation; and adrenals, thyroids, gonads, and other endocrine organs exert mostly inhibitory controls. Since it mediates diverse hypothalamic influences, it has also been called an "endocrine transducer."

Hypophysectomized animals can survive in a controlled environment without hormone replacement. But they are delicate, defenseless animals, ever poised on the brink of disaster. They cannot withstand even limited periods of food or water deprivation, or rapid changes in environmental temperatures; and they succumb quickly to a variety of noxious stimuli that are easily tolerated by intact or hormone-treated controls. Such animals may, however, fare better than others deprived of a single regulator (e.g., insulin) since hormones with opposing actions are simultaneously lost.

Examination of the problems of pituitary-deprived subjects brings into sharp focus the primary function of the endocrine system: it facilitates *adjustment* to chang-

ing environmental conditions and internal needs.

Hypophysectomized animals can eat, and can utilize food to maintain plasma glucose concentrations; but ability to accumulate reserves or to mobilize them when fasting, is defective; and weakened jaws limit the intake of certain kinds of food. They can drink and absorb liquids; but they have difficulty resisting waterlogging when they do and dehydration when they do not. Adults maintain the skeletal musculature well enough to engage in limited activity; but they lack the ability to make the necessary cardiovascular, respiratory, and metabolic adjustments utilized by intact animals in their natural habitat for activities involved in acquisition of food and flight from predators, or to reverse those changes and institute proper reparative processes during times of rest. Synthetic mechanisms are inadequate to support growth of the young, and adaptations required for reproductive functions are lost.

DEVELOPMENTAL ANATOMY^{4, 5a, 11, 12}

The pituitary gland is a complex structure. Examination of its embryological origins and of the anatomical associations established early in the course of development provides insights into interrelationships between the component parts and between the hypophysis and the hypothalamus. Developmental stages of a "typical" mammalian pituitary gland are summarized in Figure 23-1. As with all aspects of endocrinology, marked species variations are encountered. A few of the more

obvious aspects of comparative morphology are considered below.

In the very young embryo (within 6 weeks after conception in the human), the ectoderm of the roof of the mouth (the *stomodeal* or *buccal* ectoderm) lies close to the neural ectoderm which forms the floor of the third ventricle of the brain (Fig. 23-1A).

The buccal ectoderm grows upward and evaginates to form a saclike *Rathke's pouch*, while the neural ectoderm grows

downward as a funnel-shaped closed tube, the *saccus infundibulus* (Fig. 23-1B).

Continued growth of the buccal ectoderm leads to emergence of a vesicle which retains attachment to the roof of the pharynx for a time (Fig. 23-1C) but eventually becomes completely pinched off in most species. (Growth of the embryonic mesoderm near the lower part of the vesicle probably contributes to the separation.)

The vesicle enlarges and proliferates in a mostly anterior direction to form the *pars distalis* (Fig. 23-1, D and E); in time, several cell types can be distinguished, each associated with synthesis of a specific hormone. Meanwhile, the *saccus infundibulus* elongates downward, forming the funnel-like *infundibulum* (Fig. 23-1D) while the upper part of the neural ectoderm retains permanent attachment to the floor of the third ventricle and is actually part of the hypothalamus. The portion of the hypothalamus that forms the floor of the third ventricle and extends from the optic chiasma to the mammillary bodies is known as the *tuber cinereum*.

The neural ectoderm seems to provide an inducer which promotes assembly of a *pars intermedia* by cells of buccal origin with which it comes in contact (Fig. 23-1, D and E). An anatomically distinct *pars intermedia* does not form in species in which the contact is not ordinarily made, or in others if barriers are artificially interposed.

The cavity of the original vesicle may persist as the *residual lumen* or *hypophysial cleft* separating the *pars intermedia* from the *pars distalis*. Rathke cysts can form within the vesicle, partially or completely occluding the cleft in the mature gland.

The most ventral part of the neural ectoderm proliferates and forms a bulbous structure sometimes called the *infundibular process*. Some authors use *neural lobe* synonymously with *infundibular process*, and this terminology is followed here. (Others use *neural lobe* to designate the entire neurohypophysis.)

The cavity of the infundibulum may extend into the neural lobe as the *hypophysial (infundibular or ventricular) recess* (Fig. 23-1E). The upper expanded portion which becomes vascularized is the *median eminence (ME)*. In rats and some others it

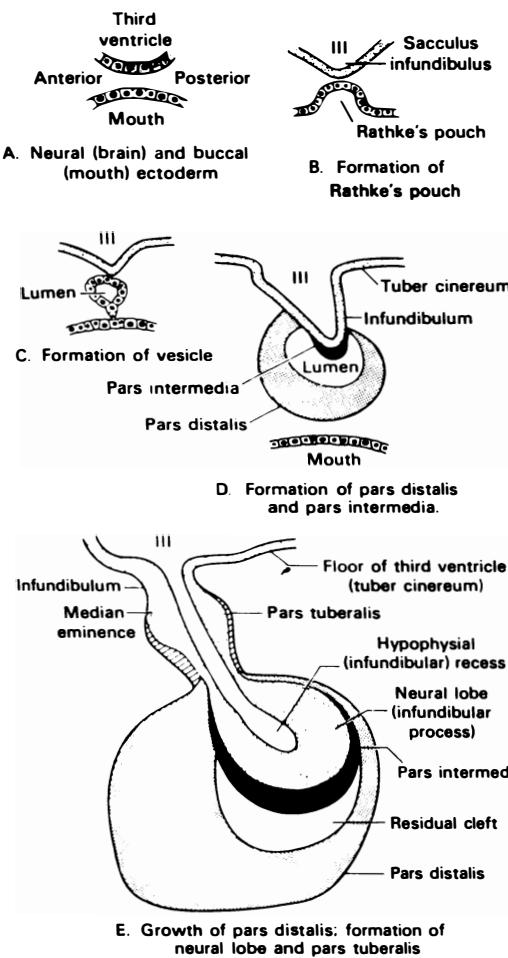


FIG. 23-1. Diagrammatic representation of hypophysial development.

forms a mostly anterior protrusion, but in others it makes more extensive contact with the infundibular surface close to the tuber cinereum. The posterior median eminence is more prominent than the anterior in humans.

In some vertebrates the part of Rathke's pouch giving rise to the pars distalis is obviously divisible into *oral* and *aboral lobes*, and these develop respectively into the *rostral* (cephalic) and *caudal* regions of the mature pars distalis. In others the oral lobe is vestigial or reduced to a small, anterior remnant, and the pars distalis differentiates almost entirely from the aboral part.

Small, lateral projections of the vesicle (often arising between the oral and aboral lobes) form the *pars tuberalis* (pars infundibularis). The projections usually grow upward and fuse to form a collar around the saccus infundibulus; it may extend over most of the tuber cinereum or terminate more ventrally (Fig. 23-1E). When fully developed, it carries blood vessels and small nerve fibers. The term "pituitary stalk" refers to the infundibulum and its associated nerves and blood vessels; it includes at least part of the pars tuberalis. Neural tissue extending from the tuber cinereum which enters into the formation of the infundibulum is called the *nerve stem*; it is partially covered by the pars tuberalis.

SUBDIVISIONS OF THE HYPOPHYSIS, AND CONNECTIONS WITH THE HYPOTHALAMUS^{1, 9, 12}

The Adenohypophysis

The adenohypophysis or "glandular" portion is derived entirely from the buccal ectoderm. When fully formed it consists of the relatively large *pars distalis*, the *pars intermedia*, and the *pars tuberalis*.

Cell types of the pars distalis are named for the hormones synthesized (Table 23-1). When a distinct pars intermedia is present, it secretes the melanocyte-stimulating hormones; otherwise, those hormones may be produced elsewhere in the pituitary gland.

While certain secretory functions have been associated with the pars tuberalis, the latter is more generally regarded as a site for *transport* of regulators from the hypothalamus to the adenohypophysis.

The Neurohypophysis

This part is derived exclusively from the neural ectoderm. It consists of the *neural lobe* (which usually retains contact with the pars intermedia), the *infundibulum* by which the neural lobe is suspended from the floor of the third ventricle, and the *median eminence* (the enlarged upper portion of the infundibulum which is actually part of the hypothalamus). The term *pars nervosa* is sometimes used synonymously with neurohypophysis.

Neurons whose cell bodies reside within the hypothalamic nuclei send long axons through the infundibulum to terminate in the neural lobe. The axon bundles form the *hypothalamo-hypophysial nerve tracts*, which are named for their site of origin. The prominent *supraopticohypophysial tracts* contain intermingled fibers originating in the supraoptic and paraventricular nuclei. Peptide hormones synthesized within the neuron cell bodies are packaged into granules along with specific proteins and are sent down the tracts for storage in the neural lobe. After appropriate stimulation, the peptide hormones are released into the systemic circulation. Secretion of *antidiuretic hormone* (ADH, vasopressin) is most closely associated with cells of the supraoptic nuclei of mammals, while *oxytocin* seems to originate mostly in the paraventricular nuclei.

Some fibers of the supraopticohypophysial tracts terminate in the vicinity of the median eminence and at the pars intermedia. But less well defined *tuberohypophysial tracts* which arise from the cells of the median basal hypothalamus supply most of the fibers that reach the upper infundibulum. The neurohypophysis consists mainly of axons, scattered neurons, and several kinds of cells which have been classified as neuroglial.

"Anterior" and "Posterior" Pituitary^{1, 4}

Since the terms appear in the older endocrinology literature and are still used in general biology and elementary texts, they are defined (but will not be further used).

When attempts are made to physically separate the hypophysis into anterior and posterior portions, the break usually occurs

TABLE 23-1
Secretory Functions Most Commonly Attributed to the Adenohypophysis

Cell Type	Associated Hormone
Thyrotroph	Thyroid-stimulating hormone, TSH; thyrotrophin
Corticotroph	Adrenocorticotrophic hormone, ACTH
Somatotroph	Somatotrophin, STH; growth hormone, GH
Lactotroph, Mammatroph	Prolactin, PRL; lactotrophic hormone, mammatrophin (Luteotrophic hormone, LTH in rodents)
Gonadotrophs	
Folliculotroph	Follicle-stimulating hormone, FSH
Interstitiotroph	Luteinizing hormone, LH; interstitial cell-stimulating hormone, ICSH
A. Hormone-synthesizing cells of the pars distalis and associated hormones	
Melanotrophs	Melanocyte-stimulating hormones, α -MSH, β -MSH, intermedins
B. Hormone-synthesizing cells of the pars intermedia and associated hormones	

at the weakest point, *i.e.*, in the vicinity of the hypophysial cleft. The "anterior" segment then contains all of the pars distalis and a portion of the pars tuberalis, while the "posterior" pituitary consists of the pars intermedia, the neural lobe, most of the infundibulum, and part of the pars tuberalis. Hormonal functions of the pars distalis have therefore been assigned to the "anterior lobe" while the "posterior lobe" contains peptides stored in the neural lobe and also those of the pars intermedia. If the latter can be separated from the posterior segment, it is then called the "intermediate lobe." The last term has also been applied to a bit of tissue that can be separated from the neural portion which contains not only intermedin cells but also other types that have grown into that region.

The Blood Supply to the Hypophysis^{1, 6, 7, 22}

The system of portal vessels supplying the hypophysis is most highly developed in warm-blooded vertebrates. The literature contains descriptions of species variations, in which diverse terms are applied to analogous structures.

The internal carotid artery of mammals gives rise to branches usually called *superior* and *inferior hypophysial arteries* in upright creatures, and *anterior* and *posterior hypophysial arteries* in others.

The *infundibular* and *peduncular* arteries of the rat correspond respectively to the anterior and posterior hypophysial arteries of other

mammals. Additional blood supply to the hypophysis of humans derives from the *basilar arteries* formed by union of the vertebral artery branches of the subclavian arteries. The basilar arteries divide into right and left *posterior cerebral arteries*; these are joined by communicating arteries to the *anterior cerebral arteries* from the internal carotid to form the *circle of Willis*. The *loral* (trabecular) artery is a small branch of the superior hypophysial artery that contributes to the blood supply of the lower portion of the human infundibular stem.

The superior or anterior hypophysial arteries go to the median eminence region and infundibular stem. The terms *vascular tufts*, *spikes*, and *gomitoli* have been applied to complex formations of the arterioles which supply the neural tissue and penetrate as far as the ependymal lining of the third ventricle.

Blood from the upper regions of the median eminence drains into *long portal vessels* which travel mostly to the upper and anterior parts of the adenohypophysis, while blood from lower portions of the stalk drains into *short portal vessels* leading to the lower, more posterior part of the adenohypophysis. In humans, monkeys, and some others, a separate branch of the anterior hypophysial artery supplies most of the lower infundibulum and therefore passes into the short vessels; but some mixing of the circulation may take place.

The portal vessels break up into a second set of capillaries in the pituitary gland. They carry not only oxygen and nutrients

but also substances released by hypothalamic neurons. The entire adenohypophysial blood supply is derived from such portal vessels in most of the mammals. In a few (e.g., the rabbit), small branches of the superior hypophysial arteries may directly enter the pituitary gland.

Cells of the adenohypophysis receive a rich capillary blood supply. The capillaries have a discontinuous, fenestrated epithelium, a continuous basement membrane, and a perivascular connective tissue space. The structure is consistent with permeability to proteins of the size associated with adenohypophysial hormones. The earlier literature contains numerous references to "sinusoids"; but electron microscopy supports the concept that the dilated vessels seen are true capillaries.⁴⁸

While most of the blood is carried downward, there is functional evidence that materials can travel from the pituitary to the hypothalamus, to provide for operation of "short feedback control" of hormone secretion. (Materials injected at the pituitary end have been observed to reach the hypothalamus; the studies have been criticized by some on the basis of the artificially high perfusion pressures utilized.)

The hypothalamo-hypophysial circulation passes through the pars distalis. Nerve endings in this region may be exclusively vasomotor.

Posterior (inferior) hypophysial arteries lead right into capillaries of the neural lobe. Some limited mixing with the supply from anterior vessels seems to occur.

Layers of capillaries have been described in the median eminence region. The most superficial vessels of the "mantle plexus" course directly into the portal veins. Deeper capillaries may communicate with the fluid of the third ventricle.

After supplying the adenohypophysis, blood drains into intracranial venous sinuses which lead to systemic veins.

The pars intermedia is mostly avascular; exchange by diffusion from vessels of the median eminence traveling toward the neural lobe may provide the major source of nutrients. The embryological origins, component parts, and circulatory features of the two parts of the hypophysis are compared in Table 23-2.

Since much of the early work on the

pituitary gland was carried out by independent laboratories, some structures are known by two or more names, and a few terms have been used by various authors to describe different structures. A set of definitions is therefore presented in Table 23-3.

COMPARATIVE MORPHOLOGY OF THE HYPOPHYSIS^{4, 5a, 5b, 7, 11, 12}

One encounters not only *species* variations in hypophysial morphology but often striking differences within the same species (e.g., among different breeds of dogs) and among *individuals* of the same strain. The descriptions given above should therefore be regarded as merely "representative" or "average" for vertebrates and especially mammals.

Large numbers of pituitary glands of readily available species (rats, rabbits, cats, dogs, cows, frogs, etc.) have been studied in detail. Information on the more exotic varieties is understandably limited to a few individuals or sometimes only one. Some indication of the kinds of differences found are cited.

The Pars Intermedia

A pars intermedia is clearly present during developmental stages in most vertebrates (including the human fetus). In man, gorillas, and chimpanzees it tends to merge with the neural lobe, and only scattered cells may persist at the site of merger (Figure 23-2A); in other mammals, cells with similar appearance and staining properties have been identified in the pars distalis. Fusion results in formation of a *neurointermediate lobe* in adult frogs, elasmobranch fishes, and many of the reptiles (Fig. 23-3C).

The pars intermedia is very much reduced in size in the baboon, gibbon, flying lemur, and opossum (Fig. 23-4B) and seems to be completely missing in the pangolin and nine-banded armadillo. There is reason to believe that it never develops in porpoises and whales (Fig. 23-4A), since connective tissue separates the adenohypophysis from the neurohypophysis; but the associated hormones have been identified in the pars distalis of the whale and armadillo. No evidence for a distinct pars intermedia has ever been found in birds; but scattered cells with typical staining properties are present.

On the other hand, this part of the adenohypophysis is especially large in the camel and llama (in which it may account for a full one-third of the gland volume, Fig. 23-2B). It is well developed in the rabbit, shrew, rat, and mouse (Fig. 23-2F) and also in most reptiles. In

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TABLE 23-2

Some Comparisons of the Adenohypophysis with the Neurohypophysis

	Adenohypophysis	Neurohypophysis
Embryological origin	Roof of mouth, (buccal ectoderm)	Floor of third ventricle (neural ectoderm)
Component parts	Pars distalis, Pars intermedia Pars tuberalis	Median eminence Infundibulum Neural lobe
Associated cavities	Residual lumen, (hypophysial cleft)	Infundibular or hypophysial recess
Blood supply	Anterior or superior hypophysial arteries via hypothalamo-hypophysial portal system	Posterior or inferior hypophysial arteries
Principal cell types	Thyrotrophs, corticotrophs, somatotrophs, lactotrophs, gonadotrophs, and others	Axon endings, neuroglial cells
Chemical nature of associated hormones	Glycoproteins, proteins, large peptides	Small peptides

the rat it occupies about one-fifth of the gland volume.

A direct relationship between the size of the pars intermedia relative to the rest of the pituitary, and ability to withstand water deprivation has been proposed. The concept is thought provoking and consistent with information on melanocyte stimulating hormone (MSH) participation in regulation of water and electrolyte metabolism, and with terminations of some supraopticohypophysial tract fibers on the pars intermedia. It also fits with the abilities of camels and llamas to cope with high altitudes and to survive in the desert, moderately good resistance of rodents to water shortage, and ability of snakes to go for long periods without drinking. The concept is weakened by some observations on birds; and it is debatable whether the aquatic existence of whales and porpoises can be used for support. Hormones other than the intermedins participate importantly in water-balance regulation, while intermedins perform other functions. Direct relationships between MSH-producing cells and pigmentation changes have been cited in poikilotherms.

The shape and location of the pars intermedia can vary as much as the size. It grows caudally and dorsally, completely encircling the

neural lobe in dogs, wolves, polar bears, opossums, cats, giraffes, and horses (Figs. 23-2C and 23-4B). In the dog it exhibits rostral folds, while in the polar bear columns of pars intermedia cells extend into the neural lobe. There is a dorsally located lumen within the pars intermedia of the wolf, mink, and polecat, while the domestic cat, wild grey cat, miercat, and mongoose have a caudally located double layer partially encircling the hypophysial cleft (Fig. 23-2C). In tortoises and salamanders the intermedia is thickened laterally.

In most species the pars intermedia is separated from the pars distalis by the hypophysial cleft. But in some (including a few cited above), cells may be found on both sides of the cleft; in others the cleft is completely obliterated while the intermedia persists. The intermedia may contain cysts lined with ciliated epithelium and streaks of colloidal matter in addition to "typical" glandular cells.

The cone of Wulzen is a structure seen in pituitary glands of sheep, oxen, and a few others (Fig. 23-2D). It is derived from the posterior wall of Rathke's pouch and extends forward from the pars intermedia into the pars distalis. It is of interest because the cells somewhat resemble those of the pars distalis in spite of the location in which pars intermedia cells would be

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TABLE 23-3
Definitions of Terms Describing Components of Adenohypophysis and Neurohypophysis

Aboral lobe of Rathke's pouch	Embryonic structure which gives rise to the pars distalis of most pituitary glands, the caudal lobe of some, and the mesoadenohypophysis of fishes.
Anterior pituitary	(Sometimes used synonymously with <i>pars distalis</i>) In glands retaining the hypophysial cleft, the anterior pituitary breaks off from the posterior pituitary under mechanical stress. Contains all of pars distalis and parts of pars tuberalis.
Cephalic and caudal lobes	Subdivisions of the pars distalis in species in which the embryonic oral lobe persists to form the cephalic portion (typical of birds). In most mammals, only the aboral lobe develops and is more closely related to the caudal lobe.
Cone of Wulzen	Cone-shaped mass of specialized cells derived from posterior wall of Rathke's pouch. May be in contact with pars intermedia but differs in cellular structure. Seen in sheep and a few other species, but not in most animals.
Hypophysial cleft	Residual lumen; between pars distalis and pars intermedia; remnant of lumen of Rathke's pouch.
Hypophysial recess	Same as infundibular or ventricular recess.
Infundibular process	Used by other authors to designate the bulbous end of the neurohypophysis which stores and secretes peptide hormones. This structure is referred to as the neural lobe in this text.
Infundibular recess	Extension of the cavity of the third ventricle into the neurohypophysis. Same as hypophysial recess.
Infundibular stem	Neural tissue extending from the tuber cinereum to the neural lobe. Same as neural stem.
Infundibulum	Used here synonymously with infundibular stem. Also used to designate the funnel-shaped cavity of the infundibular stem of the pituitary stalk.
Intermediate lobe	Clearly demarcated region of adenohypophysis that secretes MSH. Often used synonymously with pars intermedia.
Median eminence	Expanded upper, superficial portion of pituitary stalk. Contains blood vessels of the portal system.
Neurohypophysis	Used here to designate all of the hypophysis derived from the neural ectoderm, and the associated blood vessels of the median eminence. Includes the neural lobe and infundibulum. Some authors include parts of the hypothalamus which synthesize neurohormones.
Neural lobe	Bulbous lower expansion of the neurohypophysis that stores and releases neurohypophysial peptides. Also called infundibular process. Some authors include the entire neurohypophysis.
Neural stalk	Structure leading from tuber cinereum to neural lobe; includes neural stem and median eminence. May contain infundibular recess; sometimes used synonymously with infundibulum.
Neural stem	Neural tissue extending from tuber cinereum to infundibular process (neural lobe). Neural (internal) component of neural stalk.
Neurointermediate lobe	Structure formed by fusion of pars intermedia with neurohypophysis. Typical of amphibians and fishes.
Oral lobe	Anterior portion of Rathke's pouch which degenerates in some animals; in others it gives rise to the cephalic, or rostral lobe, zona tuberalis, or proadenohypophysis.

TABLE 23-3—Continued

Pars anterior	Sometimes used synonymously with pars distalis, especially in species in which location is entirely anterior to the neurohypophysis. Occasionally used to mean anterior pituitary.
Pars distalis	Largest, expanded portion of adenohypophysis; secretes all of the established adenohypophysial hormones except MSH. Older usage to designate anterior pituitary is being discarded. (Anterior pituitary also includes parts of the pars tuberalis).
Pars infundibularis	Used here and elsewhere to designate the part of the pars tuberalis (externa) within the median eminence and extending for a varying distance along the tuber cinereum. Used by others to designate the pars intermedia.
Pars intermedia	Often used synonymously with intermediate lobe. May be used to designate MSH-secreting cells even when no anatomically distinguishable intermediate lobe is present. Part of the "posterior" pituitary.
Pars nervosa	Used by some authors to designate the entire neurohypophysis, and by others to designate the neural lobe.
Pars tuberalis	Derivative of lateral portions of Rathke's pouch; carries blood vessels of portal system. Also called pars infundibularis.
Pars tuberalis externa	The larger portion of the pars tuberalis that carries the portal blood vessels. In most mammals, only the externa is present, and the term is used synonymously with pars tuberalis.
Pars tuberalis interna	A usually small, ventral part of the pars tuberalis present in some species.
Pituitary stalk	Entire structure by which the neural lobe is suspended from the tuber cinereum, including the infundibular stem, the median eminence and parts of the pars tuberalis. Same as neural stalk.
Portotuberal tract	Strands of cells accompanying the portal vessels from the pars tuberalis externa to the pars tuberalis interna. Seen mostly in egg-laying mammals, birds, and reptiles, in which the pars tuberalis externa does not lead directly to the pars distalis.
Pro-, meso-, meta-adeno-hypophysis	Subdivisions of the adenohypophysis, typical of fishes. The mesoadeno-hypophysis seems to be most closely related to the pars distalis of mammals or to the caudal lobe (see above), the proadrenophyophysis to the cephalic lobe, and the meta-adenohypophysis to the intermediate lobe.
Rathke's pouch	Embryonic structure formed from roof of pharynx which gives rise to the adenohypophysis.
Recessus hypophyseus	Hypophysial or infundibular recess.
Residual lumen	Hypophysial cleft.
Sacculus infundibulus	Cavity formed as neural ectoderm of embryo grows downward from floor of third ventricle. Later becomes hypophysial recess.
Saccus infundibuli	Same as sacculus infundibulus.
Sacculus vasculosus (saccus vasculosus)	Folded, vascularized structure in posterior floor of third ventricle of fishes.
Tuber cinereum	The part of the floor of the third ventricle that enters into formation of the infundibular stem. It extends from the optic chiasma to the mammillary bodies.
Ventral lobe	A structure extending forward from the pars distalis of elasmobranchs; believed to be formed from the pars tuberalis.
Zona tuberalis	A defined, anterior portion of the pars distalis of some animals, believed to arise from the oral lobe of Rathke's pouch. Also called rostral zone. Present in humans, giraffes, oxen, etc.

HYPOPHYSIS AND HYPOTHALAMUS

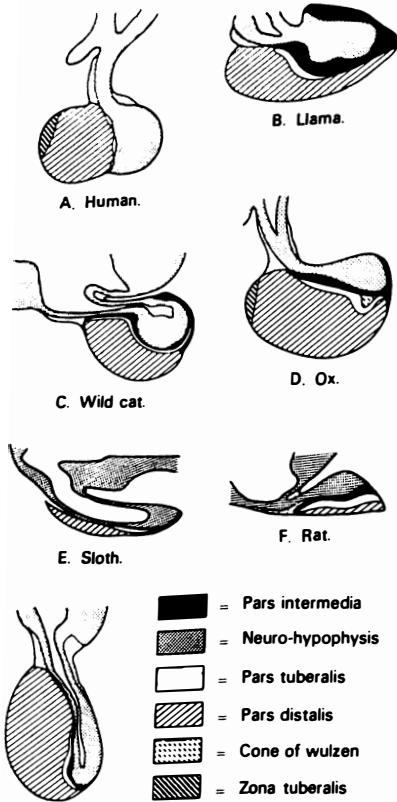


FIG. 23-2. Diagrammatic representation of adenohypophyseal morphology of some mammals. (Redrawn with permission, from Ref. 5a.)

expected to develop. No special function has been assigned to this structure.

The Pars Tuberalis

The "typical" pars tuberalis arises from lateral projections of Rathke's pouch which grow dorsally and caudally to form a collar around the infundibulum while the lower end maintains contact with the pars distalis. In some of the mammals it appears to be continuous with the pars intermedia but clearly differs from the latter in structure.

The collar may extend as far as the optic chiasma (in ferrets), completely cover not only the entire infundibulum but much of the tuber cinereum (in giraffes), or appear to be totally missing (in sloths).

In egg-laying mammals (spiny anteaters, platypuses), some marsupials (including the

wallaby), in the domestic cat, all known birds, and most of the reptiles (but not snakes), the pars tuberalis consists of a chief part or *pars tuberalis externa* which resembles the pars tuberalis of other mammals, and an additional *pars tuberalis interna* which tends to grow rostrally to fuse with the anterior pars distalis as a solid strand or as isolated nonvascular cells

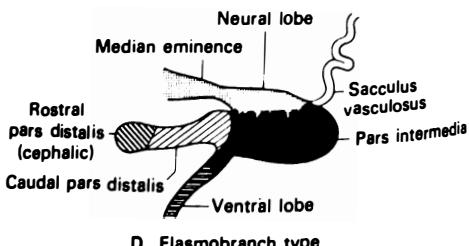
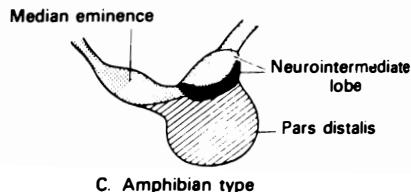
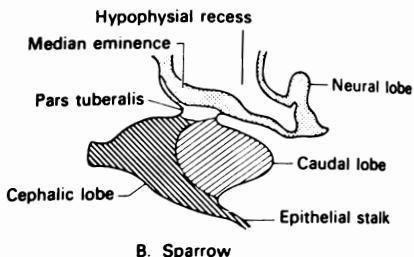
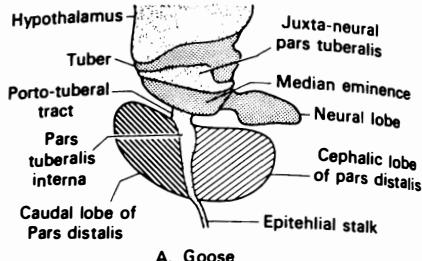


FIG. 23-3. Diagrammatic representation of some nonmammalian pituitary glands. Redrawn with permission, from Refs. 5b (Parts A and B) and 11 (Parts C and D).

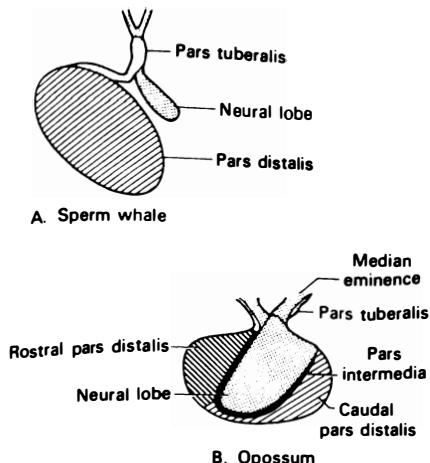


FIG. 23-4. Some unusual mammalian pituitary glands. Redrawn with permission from Ref. 5a.

accompanying the portal vessels forming a *portotubular tract* (Fig. 23-3A).

The pars tuberalis is most highly developed in *birds*, in which it may consist of a *tuberalis interna* closely associated with the pars distalis, a *juxtaneurial portion* extending along the tuber cinereum, and a connecting *portotuberal tract* (Fig. 23-3A). A prominent portotuberal tract is also widely found among *reptiles*.

In those mammals (*opossum*, *giraffe*, *ox*, and *man*) in which there is a histologically distinguishable rostral region of the pars distalis known as the *zona tuberalis*, the *tuberalis interna* extends into the region. Some authors believe that the *zona tuberalis* is an extension of the *tuberalis interna*; but most trace its origins to the anterior part of Rathke's pouch. The pars tuberalis may contain a lumen or cysts lined with ciliated cells.

In eutherian anteaters, pangolins, lungfishes, and bony fishes, the pars tuberalis is well defined during development but disappears with maturity; it probably never appears in snakes. In the elasmobranch fishes, ventral growth of the pars tuberalis leads to formation of the *ventral lobe* of the adenohypophysis (Fig. 23-3D), a structure identified only in this group of vertebrates.

The Pars Distalis

The pars distalis accounts for 96% of pituitary gland weight in whales (Fig. 23-4A), and for close to 80% in most other species. But the neural lobe and pars distalis of the rhinoceros are approximately equal in size, while the neurointermediate lobe of toads is larger than the pars distalis.

This division of the adenohypophysis may be restricted completely to the rostral part of the hypophysis (as in tigers, lions, and camels (Fig.

23-2G)), or it may extend ribbon-like in a horizontal position as far as the caudal end of the neurohypophysis as in rats, hedgehogs, sloths, shrews, and mice (Fig. 23-2F). In dogs, bears, sea lions, horses, and giraffes it grows dorsally to partially or completely surround the neural lobe; a similar arrangement is seen in the opossum (Fig. 23-4B). In amphibians the pars distalis occupies a mostly caudal position (Fig. 23-3C).

In snakes the hypophysis is asymmetric, with the pars distalis on one side and the neurointermediate lobe on the other. Both left and right side locations of the pars distalis have been found.

Cephalic (rostral) and *caudal* (proximal) lobes of the pars distalis are clearly discernible in the more primitive mammals and in birds and reptiles. The subdivision of the pars distalis is highly developed in the opossum (Fig. 23-4B).

The *zona tuberalis* in the rostral part of the pars distalis of the human, bovine, and giraffe pituitary is believed to be embryologically related to the cephalic lobe, taking its origin from the *oral lobe* of Rathke's pouch. Individuals of many species retain a connection between the hypophysis and the pharynx, extending as a remnant of the oral lobe. The remnant may later separate from the pars distalis and persist as a *pharyngeal hypophysis*.

The adenohypophysis of cyclostomes and fishes consists of a *pro-*, *meso-* and *meta-adenohypophysis*. The meta portion is embryologically and functionally related to the pars intermedia of other vertebrates. The mesoadenohypophysis or *proximal pars distalis* resembles the pars distalis of higher mammals and the caudal part of that structure in reptiles and birds. Most authors associate the proadenohypophysis (also known as the rostral pars distalis) with the cephalic region of birds and reptiles, although a homology with the pars tuberalis has also been proposed.

The pars distalis of many fishes and of cyclostomes is a diffuse structure which interdigitates with the neurohypophysis and may receive axons from hypothalamic nuclei. (Therefore, functional interrelationships between parts of the hypophysis and between the hypophysis and hypothalamus are understandably different from the usual situation in tetrapods and birds.) The adenohypophysis of cyclostomes develops in close association with the olfactory organ, and an *epithelial stalk* continuous with the proadenohypophysis persists.

The Infundibulum

The infundibulum is long, thin, and hollow with a T-shaped cavity in most reptiles, birds, and egg-laying mammals (Fig. 23-3B). It is also long in some eutherian mammals, such as the lion (Fig. 23-2G); but in others (camel, deer,

manatee), it is extremely short and difficult to define. In some it is compact for most of its length (pig, rabbit, deer, llama, seal, walrus, badger); but in others, the hypophysial cleft is well developed (as in the polar bear, anteater, cat, tiger, lion, and sloth).

The mammalian infundibulum is typically surrounded by the pars tuberalis; but the avian pars distalis is separated from the neurohypophysis by a cleft bridged only by the prototuberal tract, and the pars tuberalis may extend far outside the median eminence.

Snakes have a median eminence but no parts tuberalis. An undifferentiated portion of the infundibulum, the *pars oralis tuberis*, may separate the median eminence of some reptiles and birds from the third ventricle.

In fishes the median eminence develops along the ventral wall of the saccus infundibulus; but a structure peculiar to fishes, the *soccus vasculosus* (Fig. 23-3D) forms from the dorsal wall. The latter retains neural connections to other parts of the brain.

The Neural Lobe

The neural lobe of most vertebrates contains *pituicytes* which are modified neuroglial cells, and axons which bring in secretory granules from neuron cell bodies of the hypothalamus. In cyclostomes, however, the entire neurohypophysis is nothing more than a slight thickening of the floor of the third ventricle; secretions may be poured directly into the ventricle. The neurohypophysis of fishes is diffuse; it interdigitates with the adenohypophysis into which it sends neurosecretions, and it shares a blood supply with the pars intermedia.

The neural lobe of terrestrial vertebrates is more highly developed than that of aquatic forms. In mammals it may be surrounded by the pars intermedia, sometimes partially or completely surrounded by the pars distalis as well (as in dogs, bears, lions, and giraffes); it can even be deeply embedded within the pars distalis (Fig. 23-4B). But the blood supply is largely independent of that going to the pars distalis. In reptiles and in some of the more primitive mammals (e.g., anteaters), the neural lobe is simple and hollow, and a similar arrangement is seen in the sloth (Fig. 23-2E). It may be completely separated from, or intimately associated with the adenohypophysis.

The axis can be horizontal (Fig. 23-2F), vertical (Fig. 23-2G), or bent (Fig. 23-2, B and C) and may slant in either an anterior or posterior direction.

The Pharyngeal Hypophysis

A remnant of Rathke's pouch often develops into a small structure within the submucosa of

the posterior pharyngeal roof behind the nasal septum. This epithelial stalk remnant becomes anatomically separated from the hypothalamus by the sphenoid bone, but extensions of the portal blood vessels can penetrate the bone. The structure is quite consistently found in humans but there are few reports of its presence in other species.

Both STH and PRL have been found there in human cadavers. The structure often enlarges after menopause, and it is a relatively frequent site of formation of STH tumors.

Size of the Hypophysis

The weight of the gland ranges from 0.1 mg in the shrew to more than 50 g in the whale, and averages 10 mg in the rat and 600 mg in men. It tends to be considerably larger in females and enlarges further during pregnancy and after estrogen administration. It may weigh more than 1 g in women toward the end of pregnancy.

MICROSCOPIC ANATOMY OF THE PARS DISTALIS: SOME PROBLEMS AND SOME SOLUTIONS^{sc, 12}

The pars distalis contains several "cell types" which differ from each other in size, shape, granularity, and staining properties. It is reasonable to suspect that each type produces a specific hormone which is packaged into granules prior to secretion. (There is also an implicit assumption that hormones produced by one cell type are not actively picked up and stored by another.)

Cell fractionation procedures followed by chemical or biological assay of the derived fractions support the concept that the granules do, in fact, contain hormones; further support comes from observations relating morphologically visible changes in granulation to hormone content. (The studies do not rule out the presence of additional unpackaged hormone within the cytoplasm.)

The existence of different cell types does not constitute proof for the one-cell, one-hormone hypothesis. Cells are known to undergo functional changes which are associated with variations in size, shape, granule content, and dye affinity. And the staining characteristics can be affected by cell microenvironment, by the chemical nature and also the duration and manner of presentation of the fixative, and by numerous factors associated with the stain-

ing procedure. When pituitary gland slides are stained and examined under the light microscope, and then decolorized and re-stained, they can look very different the second time.

Much useful information has been gained from studies in which *specific antibodies* to the hormones have been prepared, labeled, and injected into animals before sacrifice, to determine binding sites within the pituitary gland. The principle of the method is simple. But antibodies do not always readily gain access to the appropriate cells. They can attach to hormones with different functions if common binding sites are present; and they can attach to nonhormonal macromolecules. The extensive washing required for some fluorescent antibody procedures has sometimes led to leaching out of the hormone-antibody combinations; but fewer difficulties of this kind are encountered with the newer peroxidase techniques.

Information on relationships between morphological appearance and cell hormone content have been obtained in several other ways. In some species, cells of a particular morphological type occur in clusters within localized regions of the glands; it is then possible to compare hormone contents of the separate zones.

Animals have been subjected to a variety of pretreatments known to influence pars distalis functions, and glands have been compared with those of controls. For example, "*target organs*" have been excised to remove feedback inhibition of specific pituitary cell types, *hypothalamic hormones* have been administered to stimulate specific cell types, and *target organ hormones* have been injected to suppress pituitary functions.

Data obtained from such studies require careful interpretation. Procedures which inhibit pituitary hormone *secretion* can have unpredictable influences on hormone *content*. The latter often rises first as release is affected; usually hormone synthesis declines later.

Any perturbation of the endocrine system can have far-reaching consequences. Thyroidectomized animals develop enlarged "thyroidectomy" cells. But thyroid hormone deficiency induces changes in somatotroph structure and function. It also tends to depress appetite and secretion of

other hormones, and all of the influences have secondary effects on the pituitary gland.

Adrenalectomy has been performed to remove feedback inhibition exerted by glucocorticoids. But greater changes in pituitary morphology are seen in animals developing electrolyte imbalance than in those given either salt supplements or mineralocorticoids.

Thyrotrophin release factor stimulates the thyrotrophs; but it also affects lactotrophs of some species. Somatostatin reduces growth hormone secretion; but it also affects release of insulin and of glucagon.

It has been possible to *dissolve out one hormone* in some cases, and to examine staining reactions after this has been accomplished. When removal is relatively specific, this can be a useful technique.

Animals with *congenital defects* of the endocrine system provide additional information. For example, there are dwarf mice with pituitary glands devoid of cells implicated in STH secretion; parallel comparisons of pituitary hormone content with those of healthy members of the same strain strengthen conclusions drawn. Human autopsy material provides much of the knowledge of the species peculiarities; but hormone-secreting tumors can be very different in appearance from normal cells secreting similar hormones.

Electron microscopy reveals details not discernible by light microscopy. It is possible to determine sizes, shapes and numbers of secretion granules, morphology of the endoplasmic reticulum and of the mitochondria, cell shapes, proximity to capillaries, and localization of labeled antibodies and of radioactively labeled hormone precursors. The chief drawbacks are the time and skill required for preparation of the specimens, and the small fraction of the gland which can be examined. Studies employing thick sections for light microscopy in conjunction with adjacent thin sections for electron microscopy have been especially useful.

Frustrations are encountered when attempts are made to apply data collected from one species to understanding the pituitary gland of other species. Differences are found in *zonation*, in the *numbers of cell types*, *cell morphology*, *staining reactions*, and *magnitude of re-*

sponse to perturbation of the endocrine system. The hormones with related functions differ in chemical makeup; and non-hormonal components of the secretion granules affect the stain affinities. It is possible to find cells performing like functions giving different tinctorial reactions (because the hormones are different), and cells performing different functions giving similar reactions (because of similar granule components).

Seemingly small differences in fixation and staining techniques used by different investigators can markedly affect the results obtained. Moreover, the stains are often complex mixtures of unknown composition which can vary substantially from one batch to the next.

INFORMATION OBTAINED FROM LIGHT MICROSCOPY¹. Sc. 7, 8d, 12, 16

Some representative findings are summarized; a comprehensive discussion of available information on staining of the pituitary gland could fill several volumes.

Chromophils and Chromophobes

Routine histological procedures reveal the presence of *chromophils* or cells with granules that readily take up dyes, and *chromophobes* in which staining of granules cannot be readily demonstrated under light microscopy. (Staining of *nongranular* components is disregarded in this separation.)

Chromophils are evidently cells which actively synthesize, store, and secrete hormones. Chromophobes may be active cells with granules too small to be visible under the light microscope, with hormone content recently discharged, or with cytoplasmic modifications which temporarily affect dye uptake by the granules. They could also be resting, immature, or degenerate secretory cells, or ones which do not package hormone into granules. Chromophobes can also be pituitary components which perform functions other than hormone synthesis.

Histochemical studies have revealed that many of the chromophobes are metabolically active and contain large quantities of specific enzymes and of ribonucleic acid.

Acidophils and Basophils

When adenohypophysial sections are stained with any one of several mixtures of acid and basic dyes under specified conditions, granules of some of the cells preferentially take up acid dyes and are therefore called *acidophils*, eosinophils, or oxyphils, depending on the investigator and the dye mixture used. Cells whose granules preferentially take up the basic dyes (and appear blue with most of the combinations) are called *basophils* or cyanophils. Acidophils secrete the protein hormones STH and PRL, while the basophils produce the glycoprotein hormones FSH, LH and TSH. The use of the periodic acid-Schiff stain (p. 360) further defines the differences. Cells secreting melanocyte-stimulating hormones also pick up basic dyes; although the hormones are peptides, the secretory granules contain carbohydrates that affect staining reactions.

Early confusion was generated by reports that the same cell can be stained with either acidic or basic dyes if the technique is varied, and that intermediate-staining cells (amphophilic) are also present. Recognition that *mitochondria* can be mistaken for acidophilic granules, and subsequent modification of fixative procedures to avoid mitochondrial staining proved useful. Further advances resulted from recognition that *cytoplasmic basophilia* (unrelated to granule content) is largely attributable to RNA content and that such staining can be eliminated by pretreatment with RNase. However, although the original designation provided important guidelines for subsequent research, it is known that cells now classified as acidophils or basophils on the basis of their hormone content contain granules which do not exhibit absolute affinities for just acid or just basic dyes of all kinds under all conditions.

Use of Differential Staining Procedures to Distinguish between Somatotrophs and Lactotrophs

Evidence that Acidophils Secrete STH and PRL. It is possible to separate the relatively large acidophilic granules from basophilic ones by differential and gradient centrifugation, and to demonstrate by bioassay that the protein hormones are contained within the fraction containing acidophilic granules. (The separation can be improved by interposing a filtration through millipore membrane between the

centrifugation procedures.²¹ Starch and polyacrylamide gel electrophoresis have been used for separation of granule proteins.)

In many species the acidophils occur within relatively discrete zones of the pars distalis; the zones can be dissected out and shown to be rich in protein hormone content.

Specific antibodies to the protein hormones have been shown (with the use of fluorescent or peroxidase markers) to attach to the acidophil cells.

The Question of the Existence of Two Distinct Cell Types. The assumption that somatotrophs and lactotrophs are distinct cell types which are not interconvertible has not been uniformly accepted. Certain tumors which appear to consist of a single cell type secrete both STH and PRL; and others which originally produced one hormone have developed the ability to also secrete the second. Observations of this kind support the concept that the microenvironment can influence secretory functions. They also raise the possibility that all acidophils secrete both hormones but that the relative amounts vary. Objections raised to these arguments center around knowledge that tumor cells can be very different from normal hypophysial residents.

Two morphologically distinct acidophil cell types have been repeatedly described, and in some species they occur in separate parts of the pars distalis. Differences in hormone content of the zones have also been found. (These observations do not rule out the possibility that microenvironments in the two zones account for the variations.)

The fact that one morphological type is absent or deficient in congenital dwarfism, and that this deficiency is specifically associated with reduced or nonexistent growth hormone concentrations in the blood in the presence of normal prolactin concentrations, has been taken as evidence for the existence of specific STH-secreting cells. Further support derives from observations that cells of this type are increased in number and hypertrophied in individuals with high growth hormone levels and symptoms of acromegaly. In addition, cells assumed to be lactotrophs have been found to increase in size, number, and granula-

tion in late pregnancy and during lactation, and characteristic morphological changes have been associated with degeneration of lactotrophs soon after cessation of lactation. Once again, it can be argued that the microenvironment of acidophilic cells is markedly affected by hormonal changes associated with pregnancy, lactation, and lactation suppression, and that individuals with congenital dwarfism have metabolic defects within the pituitary gland which interfere with normal expression of acidophil functions.

Antibody studies have not fully resolved the difficulties. Only a fraction of the acidophils of the type expected to respond actually picks up the antibody; this could be related to proposed existence within cells of hormones with diverse molecular configurations or states of aggregation. Cross-reactivity is not unexpected since STH and PRL share long common amino acid sequences.

Orangeophils and Carminophils. In many of the mammals (including humans), birds, reptiles, amphibians and fishes, two cell populations which differ in the relative affinity of the granules for the dyes orange-G and azocarmine. Cells which appear orange-colored or golden are called *orangeophils* (or aurantophils), while those that avidly take up azocarmine and appear red are *carminophils*. If erythrosin is substituted for azocarmine the cells are called *erythrophils*.

Diverse staining procedures have been utilized, some of which depend upon staining first with one of the dyes, and later with the second, so that the end result depends on the ability of the second dye to displace the first. The order in which the dyes are presented is therefore important. Because of the differences in techniques, the carminophils of one laboratory may be identical with the orangeophils of another.

The approach is useful because consistent results can be obtained when the procedure is followed properly, and it is simply necessary to specify which method has been used.

Determination of the hormone associated with the cell type is based on the following kinds of observations: Somatotrophs are the cells which remain relatively stable in size, number, and granulation throughout the reproductive cycles and

during pregnancy and lactation (while lactotrophs exhibit the expected changes). Cells of tumors secreting large quantities of growth hormone in patients or experimental animals with symptoms of acromegaly resemble the somatotroph type, and cells of this kind are the ones that are sparse or absent in cases of congenital dwarfism associated with deficient growth hormone secretion. In some of the birds, "broody" cells which become prominent in the rostral pars distalis have been identified with lactotrophs.

There are troublesome species differences; using the same procedures, the carminophils are lactotrophs in certain animals and somatotrophs in others. Some authors have applied the term α -cell (or "ordinary acidophil") to the somatotroph regardless of stain affinity, while others used the term carminophil to describe red-staining cells of either function. The term ϵ -cell has been applied to the form of lactotroph seen under most conditions, and the term η -cell or *pregnancy cell* to the larger lactotrophs which become prominent during pregnancy and lactation. Some authors use the term ϵ -cell for all lactotrophs, while others have adopted the term ϵ - η -cell. There are still others who call all orangeophils ϵ -cells, regardless of function.

Sheep secrete large amounts of PRL and have numerous lactotrophs in the nonpregnant condition. The granules of their two cell types stain orange-red and red-orange; distinction may be impossible.

Procedures most widely used for human glands give orange somatotrophs and red lactotrophs. Differential staining has been combined with specific antibody studies.

The preovulatory LH surge is triggered in many species by estrogens, and the latter also promote PRL release. Some reports that LH-secreting cells are acidophilic may derive from confusion with PRL cells; other descriptions of acidophilic LH cells are less easily dismissed.

Differential Staining of Basophil Cells

The Periodic Acid-Schiff Stain. The single most useful method for demonstration of the basophilic cells as a group under light microscopy, depends on the strong response of the granules to the periodic

acid-Schiff (PAS) reagents. Apparently a direct reaction with the glycoprotein hormones (FSH, LH, TSH) is involved. The PAS procedure will also stain polysaccharides, mucopolysaccharides and mucoproteins. Problems usually arise only from reactions with glycogen, and these can be avoided by pretreatment with glycolytic enzymes. The MSH cells secrete a peptide hormone; the PAS reaction in such cells has been attributed to carbohydrate-containing nonhormonal components of the secretion granules.

The term *mucoprotein* has been used to describe the cells giving a PAS reaction, and the term *seroprotein* has been applied to acidophilic cells. There is close agreement between PAS staining and the basophilia described above.

Observations on numbers, sizes, locations, granulation, and other characteristics of the PAS-staining cells have been made after pretreatment of animals in several ways. For example (as noted above), animals have been thyroidectomized or overdosed with thyroid hormones to facilitate study of thyrotrophs, and morphological studies have been combined with studies of pituitary content of TSH. Similarly, animals have been subjected to gonadectomy and to administration of gonadal steroids, and females have been studied at various times of the estrous cycle and during pregnancy.

The Existence of Distinct Basophil Types. As with the acidophils, morphologically distinct basophilic cell types have been described, and in some species distinct zones especially rich in one type are found. There are also tumors that secrete a single kind of glycoprotein hormone and others with histologically homogeneous appearance which "learn" to secrete other glycoproteins after repeated transplantation to new hosts. TSH, FSH and LH have α -subunits which are chemically closely related; therefore, immunological studies are most useful when antibodies are made against the β -subunits.

Use of Aldehyde Fuchsin. Cells which avidly take up aldehyde fuchsin (AF) under specified conditions have been designated as AF+, AF 1, and β -cells and biologically identified as thyrotrophs, while those with far less tendency to take up the dye are known as AF-, AF 2, or

δ -cells and are gonadotrophs. Distinctions of this kind have been made in large numbers of mammalian species, and also in birds, amphibians, and fishes.

Exceptions have been encountered. For example it has been reported that hamster thyrotrophs are AF-, while gonadotrophs of these animals are AF+.

In the more "favorable" species, the conditions under which distinctions between AF+ and AF- cells can be made must be rigidly specified. It has been shown that a highly potent (concentrated) preparation of AF will stain all chromophils (including even PAS-negative acidophils), and that increasingly greater dilutions of the dye preparation can yield, successively, mixtures that will stain (1) all basophils, including pars intermedia cells but no acidophils, (2) thyrotrophs and pars intermedia cells but not gonadotrophs, (3) just thyrotrophs, or (4) no cells. Pretreatment of cells with chromate reduces, while permanganate enhances affinity for AF. In species in which MSH-secreting cells have been identified in the pars distalis (including humans), the melanotrophs responding to (2) but not to (3) are called δ -cells, while those staining also with (3), i.e., thyrotrophs, are called β -cells by some authors. (Others apply the δ -designation to either "neutrophils" or chromophobes which in some cases seem to be corticotrophs.)

Thyroidectomy is a useful tool for demonstration of thyrotrophs in rats and others, but some species do not form obvious "thyroidectomy cells" when feedback inhibition is disrupted.

Differential Staining of Gonadotrophs. It is easier to differentiate between gonadotrophs as a group and other basophilic cells, than to identify specific gonadotroph types. Morphological separation is more readily achieved in some species than in others, and there are conflicting interpretations of the observations.

In the bat, advantage is taken of seasonal changes in reproductive cycles, of zonation, and of differential staining reactions. It has been possible to subdivide the basophils on the basis of affinity for the dye *Alcian blue* (Al B). Thyrotrophs take up the dye most avidly and are called *Al B1 cells* (or *cyanophils* since they appear blue); *Al B2 cells* or *amphophils* with intermediate affinity appear purple and are believed to be *folliculotrophs* since they seem to be most active when ripe follicles are present in the ovary, and they disappear after ovulation; *Al B3 cells* with the

weakest affinity for the dye appear brick red when dye mixtures are used, are most evident during pregnancy, and are believed to be *interstitiotrophs*.

Similar tinctorial reactions have been described for the dog pars distalis; but evidence supporting relationships between staining and hormone function is less convincing.

Cat gonadotrophs responding strongly to the PAS procedure have been classified as *G1 cells* and are believed to be folliculotrophs. The magenta-colored granules of G1 cells can be distinguished from the purple ones of *G2 cells*. The latter respond only weakly to the PAS and seem to be interstitiotrophs.

δ -Cells are easily found in the hypophysis of adult humans but are infrequently seen in children. They have been clearly associated with gonadotrophin secretion, but no definitive separation into folliculotrophs and interstitiotrophs has been accomplished.

In the rat all gonadotrophs of the male and nonpregnant female have similar staining properties. For reasons cited below, centrally located gonadotrophs are believed by some to be interstitiotrophs, while more peripheral cells may be folliculotrophs. The granulation of the interstitiotroph is described as finer and more evenly distributed within the cytoplasm. There are suggestions that solvents can be used to preferentially remove one of the gonadotrophins and effect associated changes in the appearance of one type of gonadotroph, while leaving the other type and its hormone content relatively intact.

Some investigators have reported success with special staining methods based on the relatively more intense PAS reaction of the central gonadotrophs. Thus, with a combination of PAS and methylene blue, the central cells stain red and the peripheral cells purple. With PAS and Alcian Blue at pH 2, the interstitiotrophs are said to stain red and the folliculotrophs violet, while at pH 3 the cells appear green and violet, respectively.

Advantage is taken of known relationships between the gonadotrophins and reproductive functions, and of known influences of exogenously administered hormones on pituitary hormone content.

Castration leads to hypertrophy of the gonadotrophs and an early rise in both FSH and LH content of the hypophysis. The term *castration cell* has been applied

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to all enlarged gonadotrophs regardless of location within the gland. Some of the cells accumulate a hyaline substance and assume an appearance which has led to the designation, *signet ring cell*. According to certain authors, all gonadotrophs can show the arrangement, but according to others, only folliculotrophs do so. Distinctions have also been made between "true" signet ring cells, peripherally located and presumably folliculotrophs, in which granules are coarse and confined to the cell edges, and "filigree" type castration cells that are more centrally located and contain more uniformly distributed, finer granulation.

The tendency to form signet ring cells is far greater in some species than in others. Such cells are frequently encountered in pituitary glands of castrated rats, but have also been found in glands of intact animals. They are occasionally seen in human specimens but are rare in the castrated mouse.

When distinctions between the two gonadotroph types are difficult to make in the intact animal, differences are said to be accentuated by castration. Soon after surgery in rats, both FSH and LH content of the pituitary gland are increased. Later, LH content may be relatively higher than FSH content, and this can be associated with enlarged, centrally located gonadotrophs.

However, castration affects the size and weight of the adrenal glands of most species; this has been attributed to influences on ACTH secretion. Estrogen levels affect not only corticotrophs but also lactotrophs. And gonadotrophs are affected by a wide variety of conditions including nutrition, chronic illness, and alterations of thyroid function, while pregnancy affects the entire endocrine system.

The centrally located gonadotrophs become prominent just before the onset of sexual maturation in the rat, and are rapidly degranulated just before ovulation. They also show evidence of increased activity during pregnancy, and after estrogen injection. All of the preceding are consistent with the view that centrally located gonadotrophs are interstitiotrophs. Further evidence is obtained from injection of female rats with testosterone propionate, which promotes a rise in FSH content and increased granulation of peripheral gonad-

otrophs, and a fall in LH content with degranulation of the central gonadotrophs.

Some authors interpret findings of this kind to mean that there are, in fact, two separate and distinct types of gonadotrophs in the rat, but differences between them in normal males and nonpregnant females are of a nature which makes differentiation by staining procedures difficult. Others favor the concept that there is just one kind of gonadotroph that can be influenced by the microenvironment. It has been noted (Section VI) that a single hypothalamic peptide promotes both FSH and LH secretion, and that the two gonadotrophins are usually released simultaneously during estrous and menstrual cycles. Some *antibody* studies have revealed attachment of both anti-FSH and anti-LH to the same cell. Further complications arise from indications that the chemical nature of FSH can vary with the endocrine state. Immunoelectron microscopy studies do not support the concept that FSH and LH cells are confined to different parts of the pituitary gland.

Evidence for the existence of a single type of gonadotroph is convincing in some of the fishes; but others seem to have two types. Differences between folliculotrophs and interstitiotrophs have been described in reptiles and amphibians.

Attempts to Identify ACTH-Secreting Cells by Light Microscopy

Differential centrifugation studies have demonstrated that ACTH activity can be found in the *small particle* (rather than the acidophil or basophil granule) fraction. In some species, ACTH activity has also been found in zones rich in chromophobic cells, and in tumors which do not seem to have either acidophilic or basophilic granules. Chromophobic adenomas have been described in patients with Cushing's disease (in which there is an excess of glucocorticoids), and chromophobes have been reported to increase in size and number after adrenalectomy.

These, plus other kinds of experiments which indicate that ACTH is *not* secreted by typical somatotrophs, lactotrophs, thyrotrophs, or gonadotrophs, and the fact that after one assigns functions to all of the

other cell types of the adenohypophysis and is left with chromophobes and ACTH unassigned, all point to the chromophobe cell as the corticotroph. Evidence for this seems especially strong in the cat.

On the other hand, very few chromophobes are found in the hypophyses of certain species (e.g., the pig) which seem to have adequate adrenocortical function, and ACTH activity has been found in basophilic zones located in the more anterior portions of the pars distalis. The clear *Crooke's cells* of patients with high plasma corticosteroid concentrations do not take up ACTH antibody. Basophilic tumors have been found at autopsy of some patients with Cushing's disease, and vacuolated basophils have been described in pituitaries of adrenalectomized dogs.

Immunofluorescent studies are difficult to perform, partly because ACTH is easily lost during preparative procedures. But some studies suggest localization of the antibodies within basophilic cells. All of the preceding, plus the close chemical similarity between ACTH and the MSHs put out by basophilic cells, point to the basophil as a source of ACTH. It should be noted, however, that ACTH may function as a "pro-MSH," and that some antibodies used in early studies combine with amino acid sequences common to both hormones.

Manipulations of the endocrine system can add confusion, since adrenocortical steroids exert widespread metabolic influences. Hypophysial changes associated with adrenalectomy are minimized when animals are maintained in metabolic balance without hormones; and sodium administration is especially effective. Glucocorticoids also directly affect melanotrophs; hyperpigmentation is a characteristic finding in human adrenocortical insufficiency. It has also been recognized that pituitary gland abnormalities of patients secreting excessive quantities of ACTH must be carefully interpreted; while some changes can reflect hyperactivity of ACTH-secreting cells, others are clearly attributable to chronic high glucocorticoid levels resulting from such activity.

The picture is further confused because recent evidence indicates that the concentration of ACTH is as high in the pars

intermedia of the rat as in the pars distalis, but changes in steroid hormone levels influence the concentration in the pars distalis, while the pars intermedia cells respond to neurogenic stress.²² Human ζ (melanotroph) cells may secrete some ACTH.

Exposure of animals to stress leads consistently to activation of the pituitary-adrenal system. But catecholamines, PRL, STH, and ADH can also be released in response to many of the stimuli employed, and TSH secretion may be enhanced by cold exposure. All of these affect metabolic process which secondarily influence the pars distalis; morphological effects on gonadotrophs and somatotrophs have been reported.

In the human hypophysis, a γ -cell has been described which in some respects resembles the chromophobes, but differs from the "true" chromophobe in exhibiting PAS+ vesicles. γ -Cells have been associated with ACTH secretion because they have been observed to increase in size and number after adrenalectomy and to decrease after prolonged administration of corticosteroids.

In the dog, cells which have been called *neutrophils* may be corticotrophs; but neutrophils may be identical with ζ -cells of other species. The rostromedial portion of the adenohypophysis of teleost fishes contains numerous acidophilic "X" cells which have been implicated in ACTH secretion. In different vertebrates, ACTH has been found in basophilic cells of the pars tuberalis. Electron microscopy has provided some insight into the difficulties encountered.

The Melanotroph and the Pars Intermedia

In those species in which a distinct pars intermedia develops, MSH has been localized to the region. Typically, *type I or light cells* and *type II or dark cells* are found centrally located; but these are not separable with all fixatives. Nongranulated chromophobes have also been described in the central part. Cells quite different in appearance and often ciliated are seen along the borders of the hypophysial cleft.

Type I cells are more abundant and only weakly responsive to the PAS procedure. It

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is not known whether the staining reactions point up different functional states of the same cell type (or whether there are two distinct populations). ACTH has been most often associated with the dark cells.

In the fishes, melanotrophs are found in the meta-adenohypophysis, which seems to be the equivalent of the pars intermedia of tetrapods. In some species, two morphologically distinct types of melanotrophs have been related to secretion of two different hormones affecting pigmentation.

The β -cells of humans and others which do not have a distinct pars intermedia were described above. Such cells have been found in the neurohypophysis as well as the pars distalis and have been implicated in MSH secretion. But they may also secrete ACTH; and γ -cells (corticotrophs) may participate in MSH secretion. Changes in both types have been described in response to altered glucocorticoid concentrations.

In birds (with no pars intermedia), the MSHs are concentrated in the rostral zone of the pars distalis which contains orangeophils, thyrotrophs, and probably corticotrophs. Attempts to define a κ -cell (or melanotroph different from a "V" cell, or corticotroph) have been made in the duck.

Pars intermedia cells are typically arranged in layers, and there is usually a lining layer bordering the hypophysial cleft. (By contrast, pars distalis cells of most species are arranged in clumps, while follicular arrangements are encountered in some.) The very sparse blood supply has prompted some observers to suggest that the pars intermedia does not function as an ordinary endocrine gland in mammals. Nerve fibers have been positively identified, and evidence has been presented for the accumulation of neurosecretory substances of neurohypophysial origin.

Alcian Blue has been used at different pHs to separate out basophil types. One technique involves prior oxidation with performic acid and classification of basophils into R (performic acid resistant) and S cells (with cysteine sulfur-containing granules). Aldehyde thionine has also been used because a progressive increase in the number of granules staining can be obtained related to the degree of oxidation preceding application of the stain. A terminology sometimes used which is at variance with the one described above designates folliculotrophs as β -cells,

interstitiotrophs as γ -cells, and thyrotrophs as δ -cells.

ELECTRON MICROSCOPY OF THE PARS DISTALIS^{2, 4, 6, 7, 20, 21, 22}

The high magnification, and the development of techniques of high resolution autoradiography, immunoelectron microscopy, and electron microscope histochemistry have provided information not obtainable from light microscope studies. Alterations in fine structure associated with manipulations of the endocrine system correlate well with cytological changes described earlier. In some cases, differences in staining reactions of cells with similar function in diverse species have been related to differences in fine structure.

In addition to visualization of cell contours, relationships of cells to each other and to the capillaries, differences in sizes, shapes, and electron density of granules as well as their distribution, details of endoplasmic reticulum and Golgi structure, sizes, shapes, and numbers of mitochondria, and features of the nucleus, electron microscopy has revealed the presence of cilia (which have been implicated in both nutritive and receptor functions) and of lysosomes (which seem to participate in destruction of excess hormone under special conditions, e.g., at termination of lactation). Mitotic figures have been observed especially in glands of young animals and the existence of follicular cells has been demonstrated.¹³

A major source of controversy centers around the question of whether granule size can be used as a major criterion for identification of cell type. Studies combining cell fractionation, bioassay, and electron microscopy support the hypothesis that cells containing the largest granules are lactotrophs, those with somewhat smaller (but still large) organelles are somatotrophs, while ones with granules of intermediate size are gonadotrophs. The smallest granules have been associated with thyrotrophs and corticotrophs. But some investigators have found considerable overlap in hormone content of fractions separated on the basis of particle size, and have described up to 4-fold variation in granule diameter within a single cell type.^{20, 22, 21} At least in lactotrophs,

immature granules are smaller than mature ones.

Somatotrophs

The somatotrophs seem to be the most resistant of the granular cells to post-mortem deterioration. They are usually the most abundant of pars distalis types, accounting for up to one-half the population in normal adult pituitaries. They tend to occur in groups of 3, 4, or 5 cells, often situated around sinusoids. In the rat (and in others), they may be concentrated near the periphery but can be found in all parts of the pars distalis except in areas adjacent to the pars intermedia.

Somatotrophs tend to be of medium size, rounded, oval, or polygonal in contour, with a somewhat eccentrically located nucleus. Round or nearly round electron dense granules of fairly uniform size are scattered throughout the cytoplasm. (Diameters reported by various investigators range from extremes of 100–450 μ ; but much smaller ranges are reported in individual papers, and most values fall between 300 and 350.) The endoplasmic reticulum may form a narrow band, but it is well developed and exhibits characteristic parallel arrays. Occasional cilia have been described by several authors.

Changes in appearance of somatotrophs after thyroidectomy, administration of propyl thiouracil (which impairs thyroid hormone synthesis), castration, and adrenalectomy support conclusions drawn from light microscopy studies. In addition, somatotrophs have been reported to show hypertrophy and hyperplasia in a strain of diabetic mice known to secrete large quantities of growth hormone, and to be absent from glands of a strain of dwarf mice. Predicted responses to administration of hypothalamic preparations containing growth hormone-releasing activity, and to stressful stimuli associated with STH secretion have been described; and atrophy has been seen after prolonged food deprivation. Stability of the cell throughout the reproductive cycles supports the concept that the somatotroph is a distinct cell separable from the lactotroph.

Lactotrophs⁶⁴

Cells classified as lactotrophs are large and numerous in pregnant and lactating

females, but are otherwise encountered only occasionally in females and even less frequently in males.

They are considerably larger than the somatotrophs, usually occur singly, have oval or irregularly shaped granules which vary greatly in size and shape, and are most abundant near the cell periphery. Granule size has been reported to range between 400 and 900 μ with averages around 700 and occasional granules as small as 250 μ . The endoplasmic reticulum is broad and exhibits the parallel arrays also seen in somatotrophs. The cells may be scattered throughout the pars distalis; many are close to a capillary or send cytoplasmic projections extending toward one. Pericapillary cells are usually polygonal.

The presence of PRL in the pituitary lactotrophs has been confirmed with peroxidase-labeled antibody. Changes in appearance of the cells during reproductive cycles, pregnancy, and lactation support the concept that they differ from somatotrophs. They also exhibit responses to administration of estrogens, and to cessation of lactation which agree with known physiological functions. Lysosomal destruction of granules (crinophagy) following removal of suckling young has been described in detail. Tumors secreting large quantities of PRL have been observed to contain cells identified as lactotrophs, and similar cells have been found in PRL-secreting pituitary explants.

Thyrotrophs

Thyrotrophs are usually the least abundant of the pars distalis cells (except for lactotrophs in males). They are almost always located in close proximity to blood vessels, and are near the center of the gland in many species.

Thyrotrophs are recognized as small (but variable in size), angular cells with sparse cytoplasm. Granules are very small compared with those of other pituitary cells and are confined to the periphery. Most are within the 100–200 μ range, but some as small as 40 μ are encountered. After thyroidectomy or propyl thiouracil administration, the cells tend to become degranulated, and may enlarge to sizes ranging between 2 and 10 times those seen in normal animals.

Gonadotrophs^{14, 20}

The gonadotrophs are rounded or oval cells closely associated with blood capillaries. Granules are intermediate in size, and less electron-dense than those of acidophils; most are between 150 and 250 m μ in diameter, but some are considerably smaller (down to 75 m μ). Considerable variation in size and density occurs within the same cell.

The mitochondria are elongated. Chains of vesicles are usually present in the Golgi region. Structural changes have been found related to reproductive cycles, persistent estrus, and sex steroid hormone deficiency. Descriptions of signet ring cells of castrated animals support similar findings from light microscopy studies.

Folliculotrophs are said to have prominent endoplasmic reticulum vesicles containing a material which appears greyish in electron micrographs, pale nuclei, and evenly distributed granules ranging in size from 150 to 200 m μ . Interstitiotrophs, on the other hand, have smaller, electron-lucid cisternae, granules with diameters of 200–250 m μ aggregated into clumps, and a sparse, flocculent precipitate in the cytoplasm. The cells have been described as having a "filigree" appearance. Known or suspected shifts in relative contents of FSH and LH have been related to changes in size and granulation of the specific types.

There is not universal agreement on the separation of gonadotrophs into two distinct groups,^{14, 21} and attempts to accentuate differences by manipulation of the hormonal system have lead to conflicting results. Some authors have described cells intermediate in appearance between the "typical" folliculotrophs and the "typical" interstitiotrophs. As already noted, antibodies to both FSH and LH may attach to the same cell.

Corticotrophs¹⁹

The electron microscope has provided some explanations for the difficulties encountered in attempts to distinguish ACTH-secreting cells. Corticotrophs are highly irregular cells which send out long processes that sometimes completely encircle neighboring cells and extend toward the capillaries. Nuclei are frequently lo-

cated far from the ends of the projections and associated blood vessels. Some cells are U-shaped and others stellate. Granules are numerous, highly variable in size and density, and mostly small (between 50 and 200 m μ in diameter). Some authors describe them as having clear peripheries or "halos," but others attribute this to artifact arising during tissue processing. It is suspected that their staining characteristics as well as size have led to confusion of corticotrophs with chromophobes; but "true" chromophobes or cells devoid of granules are visible in electron micrographs and are described below. Mitochondria tend to be long and narrow, and often appear bent or twisted.

Changes in corticotrophs have been observed following adrenalectomy and administration of glucocorticoid hormones; but metabolic influences of the hormones and interactions with other parts of the endocrine system (some of which have been described) have presented special difficulties for study of the corticotrophs. Some controversies concerning identification of the cells remain unresolved.

Melanotrophs

Electron microscopy has confirmed the existence of two types in rats and other species. Type I cells are said to be more abundant, cuboidal in outline, with granules 300–350 m μ in diameter. Type II cells are angular or stellate with smaller granules. Changes in both types have been described following adrenalectomy. The "Crooke's cells" seen in Cushing's disease and after glucocorticoid administration are believed to be hyalinized, enlarged melanotrophs. In species with no pars intermedia, melanotrophs have been observed to occur widely distributed in the pars distalis and neural lobe.

Chromophobes¹⁴

Several types have been described which differ in size, shape, and location but share the common property of being devoid of secretory granules. In many species they have been termed *follicular cells* since they surround colloid-filled follicles into which they send microvilli and occasional cilia. (These have been described in humans,

cattle, dogs, and rats). Clusters of "stellate" cells in species which do not have follicles (rabbits) may have similar properties. The term *interstitial cell* has been used to describe them.⁶⁰ Evidence has been presented for phagocytic activity but nutritive functions have also been proposed. A relationship to ACTH secretion has also been considered.⁶⁰ (Follicular and interstitial cells should not be confused with folliculotrophs and interstitiotrophs.)

Since chromophobes are more abundant in glands of young animals (accounting for up to 40% of the population, compared with 25% for adults), and numbers may decrease under conditions when other types increase (e.g., after adrenalectomy), it has been suggested that at least some function as "stem cells" or undifferentiated chromophil precursors, while others may be degranulated chromophils. However, young pituitary glands seem to contain large numbers of especially "dark" cells which are often granulated¹³; these could represent a different type or different stage of differentiating chromophil. Granular cells in culture have been observed to undergo mitotic division.

MICROSCOPIC ANATOMY OF THE PARS TUBERALIS

The pars tuberalis seems to function primarily in transport of materials between the hypothalamus and the adenohypophysis. The capillaries have a flattened, fenestrated appearance which suggests high permeability to molecules the size of known hypothalamic hormones. Cords of seemingly undifferentiated epithelial cells occupy spaces between the blood vessels. There are large intercellular spaces, and sometimes follicles into which microvilli and occasional cilia project.

It has recently been demonstrated that chromophilic cells, mostly basophilic, occur widely in this region. Some authors speak of "invasion" by neighboring pars distalis cells; others believe that the pars tuberalis regularly performs secretory functions which supplement or regulate those of the pars distalis. The existence in this region of trophic hormones of a unique, as yet undefined nature, has also been proposed.

Histochemical (enzyme) and antibody

studies, investigations of the effects of pars tuberalis extracts, and examination of the influences of manipulation of the endocrine system on pars tuberalis cytology point to the presence of ACTH, LH, TSH, and possibly smaller amounts of STH and PRL within granular cells of this region.

MICROSCOPIC ANATOMY OF THE NEURAL LOBE^{1, 7, 12}

Dilated axon endings of hypothalamic neurons terminate close to the abundant capillaries of the neural lobe. They are filled with granules 100–200 μm in diameter and contain numerous small (20–40 μm) synaptic vesicles.

The granules of mammals contain ADH and oxytocin, while those of other vertebrates contain oxytocin, vasotocin, and related peptide hormones (Section III). Staining reactions of the granules have been attributed to both the hormones and the associated neurophysin proteins.

Rapid degranulation has been demonstrated under conditions requiring hormone release; and slow degranulation follows interruption of the hypothalamohypophysial tracts.

The pituicytes are highly variable in size and shape, and usually have long processes extending between the axon endings and terminating in the perivascular spaces. They are often filled with pigment granules which react with silver stains. While a nutritive and supportive function is generally attributed to the pituicytes, some ascribe to them the ability to regulate release of neurohypophysial hormones.

CHEMISTRY, FUNCTIONS AND REGULATION OF THE PITUITARY GLAND HORMONES

Hormones Described Elsewhere in the Text

The chemical nature of most of the hormones of the pars distalis and of the neural lobe, and factors regulating their secretion are described in appropriate sections throughout the text (see index); for example, STH is considered along with carbohydrate metabolism, PRL with lactation, and TSH in the section on metabolic rate. Some information on hypothalamic control is also presented in Chapter 24.

Melanocyte-Stimulating Hormones¹⁰

Functions.^{10c, 18} The MSHs derive their name from stimulatory influences on pigment cells usually known as melanophores in poikilotherms and as melanocytes in homeotherms (Chapter 25). The hormones share common amino acid sequences with the ACTHs (see below) and the latter exert weak MSH-like activity.

Hyperpigmentation in Addison's disease and following adrenalectomy are attributed to loss of the feedback inhibition normally exerted by glucocorticoids on functions of melanotrophs and corticotrophs. Striking increases in pigmentation have been observed in patients bearing MSH-secreting tumors, with changes lessened or reversed following removal of the source of excessive hormone. Transient increases in skin pigmentation have also been seen following administration of MSH preparations.

Studies of hyperpigmentation in black rats following adrenalectomy suggest that other cell types participate in regulation of melanin synthesis.^{15, 17} Deoxycorticosterone (which is a mineralocorticoid devoid of typical glucocorticoid activity and no known influences on either corticotrophs or melanotrophs) can lessen pigmentation in such animals. Rats have also been observed to exhibit marked increases in pigmentation for several weeks following hypophysectomy; MSHs of hypothalamic origin have been implicated.

Poikilothermic vertebrates have melanophores with movable pigment granules, and MSHs can promote dispersion of the granules (Chapter 25). The camouflage value of such mechanisms is obvious. Since the skin of homeotherms is covered with fur, hair, or feathers, no use for movement of granules within the underlying skin can be visualized; and pigment cells of mammals and birds differ from those of cold-blooded animals.

Studies on evolution indicate that while the chemical nature of a hormone tends to remain fairly constant, functions are likely to change. For example, thyroxine is present in fishes, amphibians, and reptiles; but an influence on metabolic rate seems to have developed only in homeotherms. PRL (or a closely related hormone) is present in most vertebrates; but only in mammals

does it affect milk synthesis. And progesterone is present in most vertebrates, but it affects the endometrium of eutherian mammals.

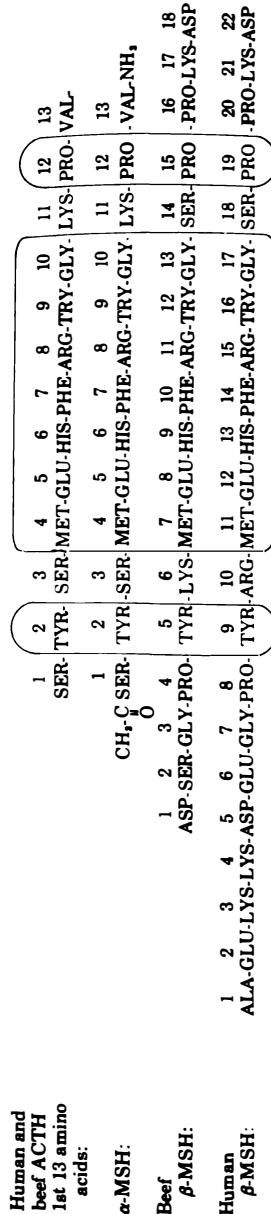
Loss of melanocyte granule dispersing function in homeotherms, and absence or reduction of an anatomically distinct pars intermedia in birds and certain mammals, could mean that MSHs are less important in those vertebrates. But it could also mean that the hormones have taken on new functions; and there are reasons to believe this is the case.

Actions on movements of pigment granules involve transmembrane movements of sodium; and MSHs have clearly been implicated in regulation of electrolyte and water balance in mammals (Section III). A relationship between pars intermedia size and ability of mammals to withstand water deprivation was mentioned earlier in the chapter. It was also noted that some fibers of the supraopticohypophysial tract end at the pars intermedia. Interruption of the tract has been reported to lead to hypertrophy of the pars intermedia in rabbits, rats, ferrets, dogs, and monkeys, with hypertrophy even more marked in the goat. (But not all studies involved reliable quantitative measurements.) Changes in pars intermedia size have also been described in direct association with dehydration or induction of diabetes insipidus.

Color responses of poikilotherms are often associated with characteristic changes in behavior. It has been reported that MSH injection leads to stretching and yawning in dogs, restlessness in mice, a sense of anxiety in women, and changes in the electroencephalograms of rats. Both MSH and ACTH are known to influence development and extinction of conditioned reflexes in rats. It has also been found that MSH potentiates submaximal monosynaptic potentials in the spinal cord of cats.

Perhaps there is some connection between observed influences on neurons and the fact that pigment cells are developed from the embryonic neural crest (which also gives rise to sympathetic ganglion cells and to the adrenal medulla). Participation of the hormones in adaptive responses to stress has been proposed.

In high concentrations, MSHs can activate adenylate cyclase enzymes of many cell types. And cAMP has been implicated

FIG. 23-5. Comparison of structures of α - and β -MSHs and of beef ACTH.

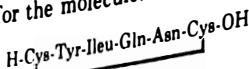
in MSH regulation of pigment granule movement. Some described actions of the hormone, e.g., promotion of lipolysis in fat cells, could result from pharmacological stimulation of adenylate cyclases.

Chemistry.³ Two peptides have been identified: α -MSH, which seems to be similar in structure in all vertebrates studied (but which may not be secreted by some of the mammals, such as the human), and β -MSH's which show some species variation. The structures are compared with that of ACTH in Figure 23-5. It will be noted that all 13 amino acids of α -MSH are identical with the first 13 amino acids of human and beef ACTH, and that most of the β -MSH structure is similar to both the α -MSH and the beginning of the ACTH molecules. The pars intermedia of the rat also contains a peptide which seems to be identical to amino acids 18-39 of the ACTH molecule. No function has as yet been assigned to the peptide which has been named CLIP (corticotropin-like intermediate lobe peptide).¹²

Regulation.^{13, 14} The presence in the pars intermedia of nerve endings, fluorescent reactions indicating the presence of norepinephrine and dopamine, inhibitory influences of those catecholamines on pigmentation responses in some vertebrates, and increased pigmentation seen in some animals following separation of the pars intermedia from the hypothalamus, are all consistent with *direct neuronal inhibition* of the pars intermedia cells. But hypothalamic peptides synthesized in magnocellular parts of the hypothalamus and released from axons of the hypothalmo-hypophysial tracts seem to exert important influences.

A tripeptide, *H-Pro-Leu-Gly-NH₂*, has been obtained from hypothalamic extracts and shown to exert potent inhibitory influences on MSH secretion. The peptide is the "side chain" of oxytocin; it has been suggested therefore that oxytocin functions as a prohormone, and that splitting may occur during or after passage of oxytocin down the axons en route to the neural lobe. In the belief that the tripeptide is the humoral regulator of MSH secretion, the name melanocyte hormone inhibitory factor (MIF) has been given by some authors. Others use the designation *MIF-I* because other peptides seem to participate in the regulation.

The ring structure of the oxytocin molecule, also known as *tocinoic acid*, has also been shown to have MSH-inhibiting actions, and the name *MIF-II* has been proposed for the molecule:



(The molecule is referred to as a pentapeptide by those who regard the cysteine moieties as belonging to a single cystine unit.) Under some conditions, MIF-I may exhibit up to 1000 \times greater potency, but in other systems very low potency has been attributed to rapid destruction of the tripeptide by widely distributed enzymes.

A pentapeptide derived from oxytocin and having the following structure has also been found in hypothalamic extracts. It is a potent stimulator of MSH secretion, and has been called *melanocyte hormone release factor* (MRF).



Melatonin exerts influences on melanophores which antagonize those of MSH (Chapter 25). Proposed influences of melatonin on MSH and MIF secretion, and related aspects of interaction are described in Section VIII.

Inhibitory influences of glucocorticoids on MSH-secreting cells were described earlier in the chapter. Additional influences of MSH on its own secretion have also been suggested.

Adenohypophysial Lipotrophic Hormones^{3, 4}

Two peptides with fat-mobilizing and MSH activity have been isolated; both share amino acid sequences with ACTH and MSH. β -*Lipotrophin* has 90 amino acids, while γ -*lipotrophin* consists of the first 58 amino acids of the β molecule.

Interest in the molecules is related to a search for possible unknown factors influencing lipid metabolism and body weight regulation. But no special actions of the peptides not shared by other pituitary principles have been demonstrated; and no specific function has been assigned. The cell type involved in synthesis remains to be discovered, as do factors which control the synthesis and possible release of the peptides.

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24. The Hypothalamus

GENERAL NATURE OF HYPOTHALAMIC FUNCTIONS^{2, 5, 6, 8, 9, 10}

The hypothalamus is appropriately included among the endocrine organs. It produces several hormones that regulate adenohypophyseal functions (Table 24-1) and releases them directly into the hypothalamo-hypophyseal blood vessels. Other hormones (described in Sections III and VI) are synthesized by hypothalamic cells and sent down the long axons of the hypothalamo-hypophyseal nerve tracts for storage in the neural lobe and subsequent release into the systemic circulation. Hypothalamic cells are in turn affected by the secretory products of peripheral endocrine glands.^{2, 5, 6, 8, 10}

But the hypothalamus is also a most important part of the brain. The cells that produce the hormones are "proper" neurons; they develop action potentials, have synaptic vesicles, and are dependent upon neuroglial elements. An extensive fiber system provides for rapid communication between one part of the hypothalamus and another, and also between the hypothalamus and other parts of the central nervous system.

Because of its location and anatomical associations, the hypothalamus provides the major link between the endocrine system and the receptors that sample the internal and external environments. Neuroendocrine mechanisms confer versatil-

ity of response and provide for rapid adjustments to changing needs. Stimuli from diverse sources can influence a single endocrine gland, functions of separate parts of the endocrine system are coordinated, and nonhormonal mechanisms are recruited to support endocrine functions.

It was believed at one time that the hypothalamus is made up of highly localized regions with very discrete functions, and that perfection of techniques for precise placement of microelectrodes (or for production of minute, circumscribed lesions) could make possible the pinpointing of anatomic loci for each type of activity. Terms such as "feeding center," "satiation center," "heat-regulating center," "sex behavior center," "sympathetic center," and so forth, were introduced with this concept in mind.

The idea came from observations that stimulation of minute regions can provoke responses such as voracious eating by previously fed (and overfed) animals, refusal of biologically needed food or water by hungry or thirsty animals, yawning, urination, defecation, ovulation, attack or withdrawal reactions, and others. The responses were found to be reproducible under specified conditions, and to be impaired or abolished after destruction of specific sites within the hypothalamus.

The picture of hypothalamic function is colorful, and it reflects the wide range of

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TABLE 24-1

Known and Proposed Hypothalamic Hormones Affecting Adenohypophyseal Cells

Name of Hormone	Proposed Functions	Comments
Thyrotrophin release factor (TRF), thyrotrophin-releasing hormone (TRH)	Stimulation of thyrotrophs, secretion of TSH; stimulation of PRL release in some species	Tripeptide; also identified in brain outside hypothalamus; Sections V, VI, VII.
Corticotrophin release factor (CRF), corticotrophin releasing-hormone (CRH)	Stimulation of corticotrophs, secretion of ACTH	Structure not established; possibility of more than one peptide proposed; vasopressin may participate in ACTH regulation; Sections II, III, VII.
Somatotrophin release factor, growth hormone release factor (SRF, GRF), growth hormone releasing-hormone (GRH).	Stimulation of somatotrophs, secretion of STH	Decapeptide structure proposed; controversies concerning structure; Sections II, V, VII.
Luteinizing-hormone release factor (LH-RF, LRF)	Stimulation of interstitialtrophs, secretion of LH	Decapeptide structure proposed; controversies concerning structure in some species and over whether there is separate hormone controlling FSH secretion
Follicle stimulating-hormone release factor (FSH-RF, FRF)	Stimulation of folliculotrophs, secretion of FSH	Controversy concerning structure and whether there are distinct folliculotrophs and a hormone separable from LRF controlling FSH secretion; Sections VI, VII.
Prolactin release factor (PRF)	Stimulation of lactotrophs, secretion of PRL	PRF activity established in birds; controversy over its existence in mammals; Sections VI, VII.
Prolactin inhibitory factor (PIF)	Inhibition of PRL secretion	Strong evidence for its existence; structure not established; Sections VI, VII.
Somatostatin, growth hormone inhibitory factor (SRIF, GIP)	Inhibition of STH secretion	Tetradecapeptide; also inhibits secretion of insulin, glucagon; Sections II, V, VII.
Melanocyte hormone release factor (MRF)	Stimulation of melanotrophs, secretion of MSH	Pentapeptide structure proposed; Sections VII, VIII.
Melanocyte inhibiting factor (MIF)	Inhibition of melanotrophs and of MSH secretion	Tripeptide structures proposed; pentapeptide structure proposed for additional factor; Sections VII, VIII.

activities affected by a relatively small part of the brain. But it does not fit all available information."

Many of the "specific" responses can be elicited through stimulation of more than one discrete hypothalamic site; and the loci often appear in widely separated clusters. Most responses cannot be classified as "pure"; e.g., hypothalamic influences on feeding behavior are accompanied by changes in motor activity and hormone function not directly related to feeding. Attempts to map control sites reveal complex patterns of overlap.

The nature of the response depends upon the conditions under which the stimuli are presented, and on the past history of the animal. It bears some relationship to the "lifestyle." When feeding activity is invoked, what the animal does is affected by the kinds of food made available, what the animal must do to get the food, and also by the amount it has eaten for some days before the experiment. Certain types of "aggressive" behavior that can be elicited in carnivores may not be seen when anatomically related parts of the hypothalamus of a herbivore are stimulated. Sexual activity responses are directly related to past experience and to patterns characteristic of the species studied.

The ability to respond to hypothalamic stimulation (or injury) requires functional integrity of many structures outside the affected region. And certain of the "typical" responses can be elicited in animals with extensive hypothalamic destruction.

It seems more reasonable today to look upon the hypothalamus as a site for (1) reception of a wide variety of internal and external stimuli, (2) processing of the derived information, and (3) transmission of signals to highly divergent structures including the integument, the cardiovascular, respiratory and renal systems, reproductive organs and mammary glands, the gastrointestinal tract, and muscles involved in voluntary movement. Sensitivities and responses of the hypothalamus are subject to continuous, dynamic modification; and each function is intimately associated with several others.

Conclusions derived from older studies have been reevaluated because it is now recognized that destruction of even minute portions of the hypothalamus can interrupt

axons passing through, and can therefore disturb activities not directly dependent on neurons whose cell bodies reside within the hypothalamus. Stimulation (electrical or chemical) can spread to remote regions. And application of potent pharmacological agents can yield negative data when penetration to otherwise sensitive cells is not achieved.

FUNCTIONAL ANATOMY OF THE HYPOTHALAMUS: OVERVIEW^{2, 6, 7, 10}

Species variations in morphology and functions of various components of the endocrine system have been cited throughout the text. In Chapter 23 some of the many differences in pituitary gland structure were described. It will be appreciated that both pituitary gland and brain peculiarities will be reflected in the highly complex functional anatomy of the hypothalamus.

A generalized summary of hypothalamic features of the more widely studied mammalian species follows.

Location and Boundaries^{3, 6, 11}

The hypothalamus is a bilaterally symmetrical structure which forms the walls and floor of the lower part of the third ventricle of the brain. It includes the *optic chiasma*, the *tuber cinereum*, the *infundibulum*, and the *mammillary bodies*. The upper border is formed by the *hypothalamic sulcus*, a usually horizontal groove which is prominent in some species and inconspicuous in others; the sulcus demarcates separation from the thalamus. The lateral boundaries are formed by the cerebral ganglia, the subthalamus, and the optic tracts.

The *tuber cinereum* is the bulging part of the floor of the third ventricle which extends downward toward the infundibulum. The expanded upper part of the infundibulum and the lower part of the tuber cinereum are richly supplied with superficially located blood vessels which course through the pars tuberalis of the adenohypophysis of most species (Chapter 23). The *median eminence* is the expanded portion containing the blood vessels.

Some authors include all of the infundibulum, and others only the uppermost portion. It is generally agreed that the

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neural lobe (which is part of the neurohypophysis) should be excluded.

Opinion is divided on classification of the *preoptic area*, which is regarded by most as anatomically part of the telencephalon (forebrain) but is functionally closely associated with the hypothalamus. This region is included in the discussion which follows. Demarcations of the hypothalamus from the anteriorly located olfactory regions and the posteriorly situated central grey matter and tegmentum of the midbrain are indistinct.

Longitudinal and Transverse Zones.^{3, 6, 11}

For descriptive purposes, it is often useful to divide the hypothalamus into segments. The sections are not cleanly separable on either a morphological or functional basis.

Longitudinal Divisions. The *periventricular zone* occupies the portion closest to the third ventricle; it contains mostly small, diffusely arranged cells, some of which are directly associated with the ventricle. But the zone also contains prominent cell clusters or *nuclei* which are named below.

The *medial zone* contains mostly neuron clusters, with scattered neuroglial cells. Small nerve fibers connect the medial and lateral zones. While most of the information seems to travel in a medial direction, reciprocal innervation has been described and implicated in regulation of feeding behavior (Section V) and other functions. Larger axons provide for afferent and efferent communication with more distant parts of the brain.

The *lateral zone* is dominated by ascending and descending longitudinal nerve fibers which form part of what has been termed the *limbic forebrain-mesencephalic reticular formation circuit*. Fibers collectively known as the *medial forebrain bundle* (MFB) provide for functional connections among the hypothalamus, the forebrain, the midbrain, and the pineal gland. But the lateral zone also contains some distinct cell clusters.

Transverse Divisions. The hypothalamus may also be divided into *rostral* (anterior, preoptic), *middle* (tuberal, infundibular), and *caudal* (posterior, mammillary) portions. Some of the terms are

more appropriate in certain species than in others.

Component Parts

It is also possible to describe the hypothalamus in terms of the structures visible under histological examination.

Nuclei.^{3, 6, 7, 8, 10, 11} The major clusters of neuron cell bodies are described in later sections of the chapter. Two types are recognized, the *magnocellular nuclei* consisting of mostly large, dark-staining cells with prominent Nissl bodies, organized into fairly well circumscribed groups, and synthesizing the neurohypophysial hormones; and the *parvicellular nuclei* containing mostly smaller cells with somewhat diffuse arrangement, implicated in synthesis of hypothalamic release hormones and in transmission of information to cells secreting such regulators. The term *area* is applied to more loosely organized cell body groups.

There is considerable species variation in size, shape, location, and sharpness of demarcation of the nuclei. Figure 24-1 shows "representative" locations of neuron cell body clusters of the more medial portions of the hypothalamus. The diagram is useful for purposes of orientation, but it is necessarily distorted since it is not possible to visualize all the groups shown within a single plane. (It does not portray true sizes and shapes.) Table 24-2 lists the nuclei found in the zones described above.

Nerve Tracts.^{3, 6, 7, 8, 9, 10, 11} The prominent *hypothalamo-hypophysial nerve tracts* are made up of bundles of unmyelinated axons taking origin in the magnocellular nuclei and functioning in transport of neurohypophysial hormones to the neural lobe. Some of the fibers terminate in the vicinity of the pars intermedia, while a few end in the median eminence.

The *tuberohypophysial* (tuberoinfundibular) *nerve tracts* are comprised of mostly finer fibers which originate in the ventromedial hypothalamus (and especially the arcuate nucleus), and terminate in the vicinity of the blood vessels of the portal system. The region occupied by the tracts is rich in catecholamines, with species variations in relative amounts of dopamine and norepinephrine. The neurons participate in the regulation of secre-

TABLE 24-2
Locations of Hypothalamic Neuron Cell Groups

Region	Hypothalamic Nuclei or Areas
Preoptic	Preoptic periventricular nucleus Medial preoptic nucleus Lateral preoptic nucleus
Anterior hypothalamic, rostral	Supraoptic nucleus Suprachiasmatic nucleus Retrochiasmatic area Paraventricular nucleus Anterior hypothalamic area
Middle, tuberal, infundibular	Dorsomedial nucleus Ventromedial nucleus Arcuate nucleus Tuberomammillary nucleus Lateral tuberal nucleus Lateral hypothalamic Nucleus
Caudal, posterior, mammillary	Posterior hypothalamic nucleus or area Premammillary nucleus Medial mammillary nucleus Intermediate mammillary nucleus Lateral mammillary nucleus Supramammillary area
Periventricular	Preoptic periventricular nucleus Suprachiasmatic nucleus Paraventricular nucleus Arcuate nucleus Posterior hypothalamic nucleus or area
Medial	Medial preoptic nucleus Ventromedial nucleus Dorsomedial nucleus Anterior hypothalamic area Premammillary nucleus Lateral tuberal nucleus Retrochiasmatic Area
Lateral	Lateral preoptic nucleus Lateral hypothalamic nucleus Tuberomammillary nucleus Supraoptic nucleus

tion of hypothalamic release factors, and provide a link between the neuronal and vascular systems. There is strong functional evidence for synaptic linkage between neurons of the tracts and afferents arising in the preoptic regions of the hypothalamus.

Myelinated fibers of the *fornix* travel from the hippocampal formation to the mammillary bodies (and especially the medial mammillary nuclei) via *hippocampo-hypothalamic tracts*. (Additional fibers of the fornix go to more lateral parts of the hypothalamus, the anterior peri-

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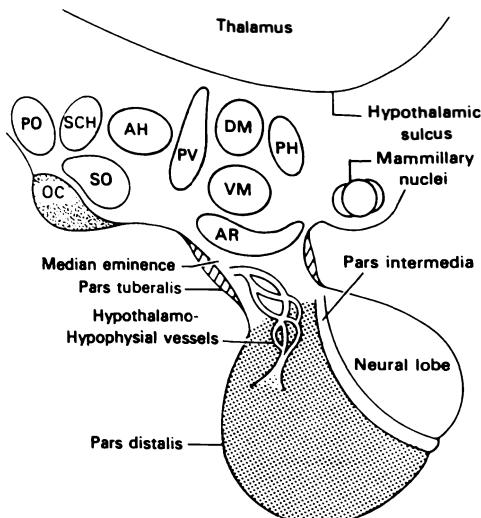


FIG. 24-1. Diagrammatic representation of hypothalamic nuclei. AH, anterior hypothalamic; AR, arcuate; DM, dorsomedial; OC, optic chiasma; PH, posterior hypothalamic; PO, preoptic; PV, paraventricular; SCH, suprachiasmatic; SO, supraoptic; VM, ventromedial.

ventricular region, and the arcuate nuclei.) The prominent *mammillothalamic* and *mammillotegmental tracts* connect the hypothalamus with the anterior thalamic and midbrain tegmental regions respectively.

An extensive *periventricular system* comprised of mostly fine, unmyelinated fibers, courses through the length of the hypothalamus close to the ependymal lining of the third ventricle. It receives much of its input from the posterior and dorsal hypothalamic nuclei, with additional axons from the tuberal and supraoptic regions. Fibers running up and down connect the hypothalamus with the ventricular and dorsal parts of the thalamus above, and with the brain stem below. Some fibers are known to terminate in the tectum of the midbrain, but others may descend in the reticular formation to the medulla oblongata. (Connections between the hypothalamus and the spinal cord seem to be multisynaptic.) The periventricular system also connects one part of the hypothalamus with another. It is not organized into grossly visible tracts.

The *medial forebrain bundles* (MFB) are

highly complex collections of mostly longitudinal fibers that occupy much of the lateral hypothalamus. Despite the name, the region contains diffuse components; it is better developed in "lower" than in higher mammals. Formaldehyde-induced fluorescence techniques have demonstrated that the bundles carry large numbers of catecholamine-containing, and lesser numbers of serotonin-containing fibers from the midbrain to the forebrain.

The MFB receives input from the basal olfactory regions, the periamygdaloid regions, the septal nuclei, and also from the magnocellular, arcuate, and ventromedial nuclei. *Amygdalofugal fibers* from the pyriform cortex and olfactory tubercles pass through the amygdala, enter the MFB, and send efferents to the anterior hypothalamus. Fibers from the MFB provide the major pathway between the "limbic system" and the midbrain; others travel into the medial regions of the hypothalamus. The MFB also makes functional connections with the retina and the pineal gland.

The *amygdaloid nuclear complex* contains grey masses located in the dorsomedial part of each of the temporal lobes

of the forebrain. Although it has extensive associations with olfactory components of the brain, it evidently is also importantly involved in non-olfactory functions, and has been especially implicated in regulation of CRF secretion and in mediation of some kinds of behavior. In addition to sending out the amygdofugal fibers to the MFB, it gives rise to the *stria terminalis* which goes to the medial preoptic and anterior hypothalamic nuclei of the right and left hypothalamus; connections to the ventromedial and arcuate nuclei have been traced in some species. Septal nuclei have both afferent and efferent connections with the amygdaloid complex; they also receive from fibers the midbrain reticular formation which ascend via the *mamillary peduncle* and the MFB and from the hippocampal formation (via the fornix).

By contrast with "olfactory" portions of the forebrain, the hippocampal regions are most highly developed in humans and in certain anomastic aquatic mammals (whales and porpoises). Antagonistic influences of hippocampal and amygdaloid stimulation on CRF secretion and other functions have been observed. Hippocampal components have also been implicated in learning processes which require visual discrimination, in memory of recent (but not past) events, and in establishment of conditioned reflexes.

Corticohypothalamic fibers travel directly from the frontal lobes of the forebrain to the suprachiasmatic nuclei; some passing through the suprachiasmatic region continue on to the arcuate nuclei. Other corticohypothalamic afferents may reach the hypothalamus indirectly via the MFB.

It has long been known that environmental lighting exerts important influences on hypothalamic functions, and there has been considerable conjecture concerning which indirect pathways might carry information from the retina to the hypothalamus. Recently, evidence has been obtained for the existence of *retinohypothalamic fibers* with efferent connections to medial hypothalamic nuclei. The suprachiasmatic nuclei have been especially implicated in both generation of circadian rhythms and in synchronization of these (and longer rhythms) with environmental cues. Some influences of environmental lighting are

also mediated via the pineal gland (Chapter 25). Olfactory cues can strongly affect responses to light; but it has been proposed that they exert their effects through changes in threshold excitability to other stimuli or to actions of humoral substances released by the pineal gland.

It is evident from the preceding that placement of a minute lesion within the hypothalamus can lead to widespread consequences which may be difficult to interpret.

Most of our knowledge of neuronal pathways has been obtained from studies of axonal degeneration following placement of discrete lesions. Myelinated fibers are more easily detected by such procedures, but methods have been developed for demonstration of unmyelinated axons. It is usually not possible to pick up the very smallest fibers in this way, or to distinguish between neurons having direct connections with the hypothalamus and those "passing through."

Microelectrodes have also been inserted for purposes of detecting sites affected by application of discrete stimuli. "Spread" of the stimulus to adjacent cells cannot always be avoided.

Retinohypothalamic projections were never visualized by such techniques. However, it has been shown that neurons of the retina will pick up radioactively labeled amino acids, incorporate them into labeled proteins, and pass them down the axons. Radioactivity has been detected in the suprachiasmatic nuclei following intracocular injection of labeled amino acids.

Considering the wide variations in hypothalamic and pituitary morphology and function, it is not surprising that species differences in neuronal pathways have been encountered.

Blood Vessels. These supply the hypothalamic neurons and carry hypothalamic secretory products to the pituitary gland. (See also Chapter 23.)

Neuroglia.^{2, 4, 5} In addition to the usual kinds of supporting elements found in association with nervous tissue, the hypothalamus contains neuroglial cells which seem to perform specialized functions. *Ependymal* cells form the lining of the central canal of the spinal cord and the ventricles of the brain. They develop from embryonic precursors which send long

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processes outward to the neural tissues. During the course of development, most ependymal cells retract the processes. The term *tancyte* refers to those ependymal cells lining the ventricles which, in the mature state, have long projections; some terminate in the vicinity of the perivascular spaces, while others establish intimate contacts with neurons. Specialized neuroglial elements lining the brain ventricles form complex associations with neuronal and vascular components, and the term *circumventricular organ* (CVO) has been applied to the histologically recognizable entity.

The lining of the median eminence is included among the mammalian circumventricular organs. Attention has been directed to specialization of ependymal components of this region. The cells are devoid of cilia found elsewhere in the hypothalamus. They form "tight junctions" which bar entry of cerebrospinal fluid via the intercellular spaces; therefore transport can be directly controlled by the cells. Intimate connections with axons of the magnocellular nuclei are suggested by extensions of the cells into the fibrous region. The ependymal cells of this area are also rich in microfilaments; a supportive function has been proposed.

The neural lobe contains cells collectively known as *pituicytes* which are variable in size and shape and have been said to exhibit characteristics in common with those of astroglia, microglia, and oligodendrocytes. At one time they were believed to participate in synthesis of neurohypophysial peptide hormones. Although the structural features are consistent with secretory capacity, this belief is no longer held. Proposed functions include a role in regulation of peptide hormone release from the neural lobe, removal of excess hormone stored there, and possibly performance of activities which have not yet been disclosed.

THE SUPRA-OPTIC NUCLEI^{3, 4, 5, 7, 8, 10}

Location and Structure

As the name implies, the supraoptic nuclei straddle the rostral and lateral surfaces of the optic tracts. They are farther from the third ventricle than most hypo-

thalamic nuclei implicated in hormone synthesis. The size, shape, number of perikaryon clusters, and the sharpness of demarcation from other elements, vary with the species.

The major cell clusters are easily recognized in histological sections because the cells are large, contain a clear area around the prominent, eccentrically located nucleus, and have large, dark-staining Nissl granules along the cell periphery. The nucleoli are conspicuous, and distinct pores are visible in the nuclear membrane. The Golgi complex and the endoplasmic reticulum are well developed.

In addition to the fairly well circumscribed collections of neurons, scattered cells of a similar kind are found bridging spaces between the large cell groups and extending toward the paraventricular nuclei. It has been proposed that the supraoptic nuclei originate as discrete cell groups, but that development of the optic tracts leads to separation of the clusters.³

As noted above, axons of the nuclei travel in groups toward the neural lobe as the supraopticohypophysial nerve tracts. The fibers intermingle to a varying degree (depending on the species) with those of the paraventricular nuclei.

Functions^{5, 12}

In mammals, antidiuretic hormone (ADH) and neurophysin proteins are produced in the perikarya, packaged into granules, and sent down the nerve tracts for storage in the neural lobe. The chemistry and functions of the hormone were described in Section III, and it was noted that the ADH of most mammals is *arginine vasopressin*. (A few, including members of the pig family produce either *lysine vasopressin* or both peptides.)

The paraventricular nuclei (described below) are most commonly associated with synthesis of the hormone oxytocin. Because of intermingling of axons arising from the two nuclei, the distribution of scattered magnocellular elements, and certain technical difficulties involved in staining procedures, it has not been possible to determine whether some ADH is consistently produced by paraventricular cells, or whether substantial amounts of oxytocin are formed in the supraoptic nuclei of all

species. Most investigators favor the concept that some of each peptide is produced in both groups of nuclei, but that no one cell synthesizes both hormones. Submammalian vertebrates produce related peptides, and especially arginine vasotocin (Section III).

It has been recognized for some time that staining reactions of the neurosecretory granules can be attributed to the presence of proteins in addition to the peptide hormones. The name *van Dyke protein* was given to what was once believed to be a single type of molecule.

The peptide hormones are associated with species-specific proteins rich in cysteine, proline, and glutamic acid, which can be separated from each other by electrophoresis. The presence of neurophysins I, II, and C have been described in cattle, and of neurophysins I, II, and III in several other mammalian species including humans, rabbits, and rats. Production of the neurophysins is genetically linked with synthesis of the peptide hormones. The proteins are specifically (but not covalently) bound to the peptides, and are released along with the hormones into the systemic circulation. It has been reported that neurophysins combine with ADH to form crystalline complexes, and with oxytocin to form amorphous complexes.

Molecular weights ranging from 10,000 to 30,000 have been found; at least some of the differences are related to formation of peptide chain dimers and oligomers.

There are indications that one neurophysin is closely associated with ADH and another with oxytocin. Deficiency of one specific protein (determined from studies utilizing specific antibodies) has been linked with deficiency of ADH in the Brattleboro strain of rats. The proteins and hormones seem to be synthesized together. Amino acid analogs which interfere with neurophysin synthesis also interfere with hormone production, although the related amino acids do not appear in the hormone molecule.

According to one concept, both the neurophysin and the hormone are components of a single, biologically inactive precursor molecule which is later lysed to yield active components. According to another, the two are synthesized separately within

the same cell and later associate, but their formation is in some way linked.

Functions of neurophysins have not been unequivocally established. In addition to the possibility that they are components of a common precursor, it has been proposed that neurophysins (1) combine with the hormone and thereby protect it from intracellular degradation and from leakage out of the secretory granule, (2) act as a hormone carrier in the blood plasma, (3) complement or synergize with actions of the hormone, (4) participate in binding of the hormone to target organ receptors, (5) exert specific actions of their own described below, or (6) perform functions which have not yet been detected.

There is little evidence to support any of the preceding; and there are reasons for questioning most of them. If the proteins combined with the hormone to protect them against leakage from the granule, it would be expected that they would also affect the osmotic pressure within the granule exerted by the small peptide molecules; attempts to demonstrate this have yielded negative data, and there is no support for the concept that the peptides are present as free molecules within the granule. When protein-free peptide hormones are injected, they travel to their target organs and exert specific actions; addition of neurophysins does not seem to enhance activity, prolong the action, or affect the binding to target organs. Neurophysins have been reported to exert actions such as enhancement of lipolysis and stimulation of the heart, and to exert MSH-like and plasma calcium-regulating activity. But fairly large amounts are needed to elicit such actions, they are not readily demonstrated in many species known to regularly secrete neurophysins, and there is nothing unique about the actions which cannot be performed by more potent hormones directly implicated in the processes. There is no evidence for interaction of neurophysins with hormones regulating such functions as lipolysis or plasma calcium maintenance.

Release of ADH from the neural lobe in response to osmotic stimuli, and actions of the hormone on the kidney which lead to water conservation were described in Section III. The question of whether certain supraoptic cells function as both os-

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moreceptors and neurosecretory cells has not been definitively answered (Section III). Stimuli arising from pressoreceptors seem to clearly affect supraoptic cells via synaptic connections. Cholinergic agents can promote ADH release, and cholinesterase has been identified within the supraoptic nuclei. The vesicles of the axons are believed to be predominantly cholinergic. Catecholamines and evidence for adrenergic receptors have not been found directly associated with the neurosecretory cells, but catecholamines have been identified in nerve fibers penetrating the nuclei from the outside. It is possible that tuberoinfundibular neurons participate in regulation of functions of magnocellular components.

Termination of supraoptico-hypophysial tract fibers on the pars intermedia was noted, and reasons for associating the pars intermedia with regulation of water and electrolyte metabolism were presented in Sections III and in Chapter 23. It has been proposed that ADH (vasopressin) can act on pars intermedia cells in much the same way that "typical" neurotransmitters act on their target structures. If this is true, then one more distinction between nervous and endocrine function becomes blurred.

It was also noted in earlier sections that ADH is released in response to a variety of "stress" stimuli, that ADH in fairly high concentration can exert important influences on the ACTH-adrenocortical system, and that substances related to ADH may participate in release of ACTH in some of the lower vertebrates. Glucocorticoids seem to confer protection of mammals against toxic effects of excess ADH. Morphological changes in axons of the tracts have been observed in response to adrenalectomy or to administration of adrenocortical hormones.

Cells of the supraoptic nucleus respond to injection of estrogens, and exhibit morphological signs of increased activity after castration. The functional significance of these findings is unclear.

THE PARAVENTRICULAR NUCLEI^{8, 7, 8, 10, 11}

The paraventricular nuclei lie close to the lining of the third ventricle and may form broad vertical bands extending toward the hypothalamic sulcus. They are

dorsal, caudal, and medial to the supraoptic nuclei. Although the paraventricular nuclei are smaller and less clearly defined in many species, they contain similar cells. In addition, a second, smaller cell type (not found in the supraoptic groups) has been described. Axons join with those of the supraoptic cells to form the supraoptico-hypophysial nerve tracts. The secretory granules contain oxytocin and neuropeptides (and possibly some ADH).

Functions of oxytocin have been described in sections on the uterus and mammary glands (Section V). But many pharmacological agents and physiological stimuli affecting ADH release exert similar (if quantitatively different) influences on oxytocin secretion. Thirst may elicit release of both hormones. Hemorrhage affects mostly ADH secretion directly, but oxytocin secretion may follow a different time-course of release in some animals. While stimulation of the female reproductive tract and mammary glands seems to elicit mostly oxytocin secretion, increased amounts of ADH have been detected in several animal types.

Paraventricular cells take up and are affected by estrogens of the circulating blood. The "small" cells have been implicated in synthesis and release of FSH-RF (but not of LH-RF); however, no specific hypothalamic hormone having such activity has been isolated from the cells.

Indirect evidence links the paraventricular nuclei with release of other hypothalamic hormones. Disturbances of growth and thyroid function as well as disruption of gonadal functions have been observed following destruction of the paraventricular nuclei. The nature of the defect resulting from such lesioning has not been determined.

Fishes do not have paraventricular nuclei. Peptides chemically and biologically related to neurohypophyseal hormones of higher vertebrates are produced in the preoptic nuclei. Fishes have, in addition, a unique structure at the caudal end of the spinal cord, the *urophysis*. Cells of this structure show some morphological similarities to hypophysial neurosecretory cells of other vertebrates. Some limited information on possible functions of octapeptide hormones in fishes was presented in Section III.

THE "HYPOPHYSIOTROPHIC AREA"^{7, 8, 10}

It has been noted several times in the text that the structural integrity and secretory functions of the adenohypophysis are subject to continuous regulation by hypothalamic hormones which reach the pituitary gland via the hypothalamo-hypophysial portal system. Removal of the pituitary gland from its continuous contact with the hypothalamic hormones, to a distant site within the same organism, leads to atrophy of thyrotrophs, corticotrophs, gonadotrophs, and somatotrophs, and this is associated with decreased secretion of TSH, ACTH, FSH, LH, and STH (although PRL secretion is usually increased).

Similar disturbances of adenohypophyseal function result from extensive bilateral destruction of the median eminence region, or from interruption of the hypothalamo-hypophysial portal circulation.

If the pituitary gland is implanted within certain parts of the hypothalamus, normal cytology and secretory functions are maintained. The term *hypophysiotrophic area* (HTA) has been used to designate such sites. Presumably, the pituitary cells establish communication with hypothalamic cells that secrete the release factors. (Implantation into other parts of the brain, including hypothalamic sites outside the HTA, does not maintain function.)

The hypophysiotrophic area has been most intensively studied in the rat. It extends forward from the arcuate nuclei to the optic chiasma and upward to the paraventricular nuclei, and backward from the arcuate nuclei to the premammillary region. The median eminence and parts of the ventromedial and premammillary nuclei are included.

Electrical stimulation of the hypophysiotrophic area in intact animals has been observed to promote increased secretion of TSH, ACTH, FSH, LH, and STH, and to decrease secretion of PRL. Median eminence extracts have potent release factor activity, and some of the release factors described above have been isolated from such extracts. Since the median eminence itself contains few cells which look as if they might engage directly in active hormone synthesis, it is believed that release factors found in that area arise mostly in other parts of the hypophysiotrophic re-

gion, and that the median eminence is a major site for transfer of the hormones to the pituitary gland.

Attempts have been made to subdivide the HTA into specific regions, e.g., thyrotrophic, gonadotrophic, corticotrophic, etc. Usually, it is not possible to clearly separate the functions. It has not been established that production of each of the releasing hormones is confined to a small region, or that localizations found in one species can apply to another. Lesions destroying hormone-secreting cells at one site are likely to affect axons passing through to other parts of the hypothalamus; and implantation or stimulation studies are exceedingly difficult to localize.

THE MEDIAN EMINENCE^{7, 8}

The median eminence is sometimes said to constitute the "final common pathway" between the nervous and endocrine systems. On the basis of structure and function, it has been divided into three zones.

The Ependymal Zone or Inner Layer

The large cuboidal ependymal cells which line the third ventricle send microvilli and bulbous projections into the ventricle, and extend long processes outward as well. Some of the latter reach to the perivascular spaces of the portal vessels while others form intimate associations with neuronal elements of the median eminence and with cells of the arcuate nucleus. They have been implicated in uptake from and secretion of materials into the cerebrospinal fluid and, therefore, with provision of a means of communication between the blood capillaries and the ventricles of the brain.

The Fibrous Zone or Middle Layer

Axons of the hypothalamo-hypophysial nerve tracts pass through this region on the way from the magnocellular nuclei to the neural lobe; some terminate within this region.

The Palisade Zone or External Layer

Fibers of the tuberoinfundibular tract terminate within the palisade zone; this accounts for the high content of hypothe-

lamic hormones derived from parvicellular regions. The hormones are transferred to capillaries of the hypothalamo-hypophysial portal system. The palisade zone also contains catecholaminergic fibers of the tuberoinfundibular system and is rich in dopamine and noradrenaline.

THE ARCUATE NUCLEI^{3, 7, 10}

The arcuate (or infundibular) nuclei form a continuous mass of grey matter around the floor of the third ventricle. They lie just below the ventromedial cells, and extend from about the middle of the hypothalamus backward to the premamillary nuclei.

They contain large, clear cells which are smaller than those of the magnocellular regions. Two types have been described by some authors, but others have observed three which exhibit changes in appearance and relative numbers associated with development, maturation, and reproductive functions. The highly developed Golgi complex with numerous sacs and vacuoles, the appearance of the endoplasmic reticulum, and the polysomes and vesicles filled with electron-dense matter seen in some of the cells, are all consistent with a high level of synthetic and secretory activity.

The neurons give rise to mostly fine, unmyelinated fibers of the tuberoinfundibular system (which also receives input from the ventromedial nuclei). Much of the hormone content of the median eminence is believed to originate within the arcuate nuclei. Afferent fibers of what is sometimes called the *preopticotuberal system* course through the anterior hypothalamus and terminate in the arcuate nucleus. (Other afferents of that system go to the ventromedial regions.)

Fibers of the corticohypothalamic tracts pass through the suprachiasmatic nuclei before reaching the arcuate nuclei. Since the suprachiasmatic region has been implicated in regulation of rhythmic patterns for secretion of several of the pituitary hormones, this probably has functional significance.

As noted above, the arcuate nuclei are included within the hypophysiotropic area. They are also part of the more restricted *gonadotrophic area* (so named because structure and function of the gonadotrophs

seem to be well preserved when pituitary implants are placed there). The region is evidently directly involved in maintaining tonic or "steady-state" secretion of FSH and LH. Male animals with lesions separating the arcuate nuclei from their afferent connections maintain normal spermatogenesis and functions of accessory reproductive structures, and testosterone concentrations in the blood are kept within the normal range. Females with similar lesions secrete some FSH and LH and can develop ripe ovarian follicles. But they do not ovulate because afferent neurons from more anterior parts of the hypothalamus are needed to provide the stimuli for LH surges.

Cells of this region seem to participate directly in gonadal hormone negative feedback mechanisms. It has been stated that estrogens implanted into this region inhibit secretion of both FSH-RF and LH-RF, but that implants of progestagens can selectively inhibit LRF alone. Estrogen influences on the arcuate nuclei may also be directly involved in precipitation of the onset of puberty.

Unfortunately, observations of this kind do little to help resolve the question of the existence of a single or multiple gonadotrophin release factors. Action of estrogens could be interpreted as inhibiting release of a single FSH/LH-RF, and it is possible that the progestagens gain access to the portal system and influence pituitary responses to the hypothalamic hormone. Alternatively, it can be suggested that estrogens affect release of both FSH-RF and LH-RF, while progestagens selectively influence just one hypothalamic hormone. Electrical stimulation of the arcuate region leads to release of both FSH and LH.

Stimulation of the preoptic-anterior hypothalamic areas increases LRF secretion sufficiently to induce ovulation (in suitably primed animals). Destruction of this region (or interruption of the preoptico-tuberal fibers leading to the arcuate nucleus) has no influence on tonic secretion of gonadotrophins, but the LH surge required for ovulation does not occur in otherwise intact animals. Administration of nembutal (which acts on neuronal pathways but has little direct action on neurosecretory functions) also blocks the LH surge if adminis-

tered at appropriate times, and electrical stimulation of the preoptic area of nembutalized animals can then induce the ovulatory surge of LH. For these reasons, regulation of LRF changes associated with ovarian cycles has been ascribed to the preoptic areas. Effects of estrogens and of environmental stimuli on estrous cycles also seem to be mediated via the preoptico-tuberal system.

Strong stimulation of the central nervous system can promote secretion of LRF, and both cholinergic and adrenergic mechanisms are involved. Dopamine administered into the third ventricle also provides powerful stimulation.

Induction of premature ovulation in experimental animals following injection of catecholamines and following exposure of animals to a variety of other (naturally occurring and artificial) stimuli supports the concept that differences between spontaneous and induced ovulators (Chapter 22) are quantitative rather than qualitative.

Some of the influences of environmental lighting on the reproductive system are mediated via nerves from the anterior hypothalamus acting on the arcuate nuclei cells. (Practical application of stimulatory influences has been made, *e.g.*, by egg farmers.)

The arcuate nuclei receive inhibitory as well as stimulatory impulses. Lesions of the ventromedial nuclei lead to increased LH secretion if arcuate cells remain intact. Precocious puberty sometimes results from interruption of inhibitory pathways.

THE VENTROMEDIAL NUCLEI^{7, 10}

Two main cell types have been described. This region of the hypothalamus has been directly implicated in detection of blood glucose changes, participation in regulation of food intake, and in control of growth hormone secretion. Both stimulatory (GRF, SRF) and inhibitory (SRIF, somatostatin) hormones have been localized to this region. (See also Section V).

Neuronal pathways connect the ventromedial with adjacent lateral hypothalamic regions. Communication depends at least in part on extension of dendrites of each component into the neighboring parts of the other. Damage to cell bodies and nerve

fibers can lead to extensive disruption of metabolic functions. Animals with lesions of ventromedial and neighboring areas often develop hyperphagia, obesity, "finnickiness," and what has been termed "sham rage." (But see also Section V).

The ventromedial nuclei have also been directly implicated in regulation of FSH and TSH secretion, possibly through synthesis of FSH-RF and TRF. Anatomical demarcation from the dorsomedial nuclei is often indistinct; but stimulation or lesioning of the more dorsal portions does not elicit the same responses.

THE ANTERIOR HYPOTHALAMUS^{1, 7, 8, 10}

While direct secretion of hypothalamic hormones has not been ruled out, this region and the adjacent preoptic areas seem to function primarily in regulation of activity of other parts of the hypothalamus. (Fibers to the arcuate region implicated in regulation of LH surges were mentioned above; the anterior hypothalamus may also function in the prolactin surges associated with estrous cycles and pseudopregnancy in rats.)

Some anterior hypothalamic neurons seem to be directly sensitive to changes of the temperature (and especially to elevations) of the blood supplied to that region, and to participate in protection against hyperthermia. Cells of the same location have been implicated in hypothalamic coordination of parasympathetic nervous system functions.

The term "anterior hypothalamic nuclei" is applied by some authors to indistinctly defined cell clusters located dorsolateral to the suprachiasmatic nuclei; because of the poor definition of boundaries, many prefer to designate such cell groups as the "anterior hypothalamic area."

The *suprachiasmatic nuclei* are situated just above the optic chiasma, but close to the third ventricle. They are made up of densely packed clusters which are especially prominent in rats but have been clearly recognized in several other mammals (including humans). Cells of this region are relatively small; they have recently been implicated in generation of a number of circadian rhythms, and evidence has been accumulating for direct

neuronal communication with the retina which provides for synchronization of the rhythms with changes in environmental lighting. (See also Chapter 25). This region is especially rich in serotonin.

A *retrochiasmatic area* containing cells with structural features in common with those of the supraoptic nuclei has been described in rats and some other mammals.

The magnocellular nuclei and the cell clusters of the preoptic regions are often classified as belonging to the anterior hypothalamus.

A single (unpaired) grouping, the "medial preoptic nucleus," is located just dorsal to the third ventricle; paired "preoptic periventricular nuclei" contain cells which are difficult to distinguish from the ependyma lining other parts of the third ventricle. The "lateral preoptic nuclei" are closely associated with the medial forebrain bundle.

THE POSTERIOR HYPOTHALAMIC NUCLEI^{7, 8, 10}

The *posterior hypothalamic nuclei* are situated close to the third ventricle. Many of the smaller, more numerous, densely packed neurons contain a yellow pigment. Larger scattered cells resemble those of the magnocellular nuclei. The dorsal border of the posterior nuclei in some species is marked by the hypothalamic sulcus, but the caudal parts merge with the premammillary nuclei.

As noted above, the posterior nuclei have functional linkages with the periventricular fiber system connecting to other hypothalamic nuclei, ventricular and dorsal parts of the thalamus, and to the midbrain. This region has been most closely associated with hypothalamic regulation of the sympathetic nervous system. Damage to this area impairs ability to adjust to cold environmental temperatures, and has also been said to induce lethargy, sleepiness, and disruption of some visceral functions.

THE MAMMILLARY NUCLEI^{7, 10}

Important efferent pathways arise in this region, and afferent pathways from the midbrain and other parts of the brain ter-

minate here. The nuclei and fibers seem to be important for regulation of some visceral functions and for mediation of certain forms of behavior including those related to sexual activity. Unlike the situation described for more anterior regions of the hypothalamus, the mammillary regions may show extensive degeneration if connections to other parts of the brain are severed.

The *medial mammillary nuclei* fill most of the volume of the mammillary bodies in humans and some other species, and provide the origins of the mammillothalamic tracts. Clear morphological subdivision into medial and lateral portions can be made in humans and several other mammals. The mammillothalamic tract is most closely associated with the medial parts, while fibers of the fornix enter the more lateral aspect.

The neighboring *lateral mammillary nuclei* are not believed to be functionally associated with the thalamus. *Intermediate mammillary nuclei* (intercalated nuclei) between the medial and lateral mammillary nuclei have been described in some species. Small *supramammillary nuclei* located more dorsally are also recognizable.

The *tuberomammillary nuclei* located within the lateral hypothalamus, and the *premammillary nuclei* (ventral to the posterior nuclei) are not considered to be part of the mammillary complex.

THE LATERAL HYPOTHALAMUS

The lateral hypothalamus extends from the preoptic to the mammillary regions. While nerve fiber groups are most prominent in this region, distinct cell clusters, the *lateral hypothalamic nuclei*, and the *nuclei tubercles* are fairly prominent. The MFB (which fills much of the lateral hypothalamus) and connections between the lateral and medial hypothalamic components were described above.

THE 'THYROTROPHIC AREA'^{8, 7, 8, 10}

Electrical stimulation of the arcuate nuclei leads to release of TSH as well as of gonadotrophins. But TRF seems to be synthesized over a much broader region. The "thyrotrophic area" has been described as extending into the suprachiasmatic and

retrochiasmatic portions and into the ventromedial nuclei. It may include the entire HTA.

TRF has been identified in several extra-hypothalamic parts of the brain, and it has been proposed that this tripeptide performs functions other than stimulation of adenohypophysial thyrotrophs. Potent influences on PRL secretion in humans and some other mammals were mentioned in Section VI. While no stimulation of other types of adenohypophysial cells have been found with doses in excess of those affecting thyrotrophs and lactotrophs, there are observations are consistent with influences on mood and behavior.¹⁸

A role of TRF in regulation of thyroid hormone function seems to be well established. As noted above, thyrotrophs exhibit morphological changes and depression of secretory functions when removed from the hypothalamo-hypophysial portal circulation, while hypothalamic stimulation of the intact animal or administration of TRF clearly leads to increased TSH secretion. Moreover, there is evidence for central nervous system regulation of the thyroid hormone system which requires an intact hypothalamus.

TRF secretion is diminished after most strong, stressful stimuli, e.g., surgical trauma, burns, anoxia, food deprivation, and extremely cold or hot environmental temperatures. It is also diminished after administration of central nervous system depressants and noxious agents such as formalin. Experimental evidence has been presented for an inverse relationship between TRF and CRF release; but there are conditions under which it is possible to augment release of both simultaneously, or to stimulate secretion of one without affecting the other.

Moderate increases in intensity or duration of environmental lighting tend to enhance thyroid hormone secretion in day-light-active animals exposed to alternating light and dark periods. Changes in the opposite direction have been described in nocturnal animals such as rats and mice. The effects of light require the presence of an intact hypothalamus.

In humans, depression of thyroid function has been observed to be associated with severe stress and anxiety; but some

individuals consistently put out larger amounts of thyroid hormones during milder anxiety states. The clinical literature contains numerous case histories describing onset of thyrotoxicosis in patients during a "letdown" period following removal of a situation which has induced prolonged and severe anxiety. (For example, it has been seen in women heavily burdened with the care of an invalid relative following either recovery or death of the invalid.) Many physicians do not believe that psychological factors play an important role in etiology of hyperthyroidism; but aggravation of existing conditions is well recognized.

Thyroid hormones clearly participate in thermoregulatory mechanisms. The role of TRF in maintenance of body temperature is considered below.

On the other hand, the TSH-thyroid hormone system possesses a high degree of autonomy. Pituitary glands transplanted to sites removed from direct hypothalamic influences maintain some function, and thyrotrophs are clearly subject to direct regulation by thyroid gland hormones. T3 and T4 antagonize TRF influences on thyrotrophs and reduce TSH secretion. When plasma levels of thyroid hormones are depressed (by surgical excision of the thyroid gland or administration of thiouracils and similar agents), some compensatory elevation of TSH secretion is achieved in animals with hypothalamic lesions. And some compensatory hypertrophy of remaining thyroid gland tissue can be seen after partial thyroidectomy. It has also been proposed that catecholamines directly stimulate thyroid gland cells.¹ In all cases, regulation of thyroid function is less effective in the absence of normal TRF influences.

The question of the importance of plasma levels of thyroid hormones for regulation of TRF cells has not received adequate answers. Studies in which implants of thyroid hormones into the hypothalamus have led to suppression of TRF release have been criticized on the basis of the possibility that some thyroid hormone leaked into the portal vessels and ventricular system or that the experimental procedures affected neurons which regulate the TRF-secreting cells. Moreover, local-

ized concentrations of the hormones were probably much higher than those found in the undisturbed control animal. There is some evidence for *long-range* influences of thyroid hormones which can be abolished by destruction of anterior (but not posterior) parts of the hypothalamus.

THE "CORTICOTROPHIC AREA"^{2, 6, 7, 8, 10}

The median eminence component of the HTA seems to be especially rich in CRF activity. But determination of anatomical sites for CRF synthesis is difficult for many reasons, some of which were noted in Section III. The chemical structure of CRF has not yet been established, and some investigators believe that two or more related peptides are involved. Moreover, corticotrophs can be strongly influenced by agents produced in the hypothalamus (e.g., vasopressin) which seem to be quite distinct from "true" CRF. In addition, evidence has been presented for release of a nonhypothalamic or "tissue" CRF during prolonged severe stress; and a direct stimulatory influence of catecholamines on adrenocortical cells has been suggested.

Hypothalamic control of the ACTH-adrenocortical system seems to be far more important than TRF regulation of the TSH-thyroid hormone complex. This seems to make good "physiological sense", since rapid rises in ACTH and glucocorticoid output and maintenance of elevated steroid hormone levels constitute essential components of the response to conditions of stress. Effects of such discharge are evident within moments, and they contribute substantially to survival. By contrast, there is little evidence that thyroid hormones exert rapid actions, or that prompt elevations of thyroid hormone levels are essential for physiological adjustments.

The "corticotrophic area" of the hypothalamus seems to control "tonic" secretion of ACTH, and to thereby maintain the functional integrity of the corticotrophs of the adenohypophysis. If this region is separated from other parts of the brain, sufficient ACTH is secreted to prevent atrophy of the zona fasciculata cells of the adrenal cortex; and glucocorticoid hormone levels are adequate for support of metabolic needs of unstressed animals. But hypothalamic

"deafferentation" impairs or abolishes responses to a variety of stress stimuli and disrupts the usual secretory patterns. Neurons outside the corticotrophic area exert both stimulatory and inhibitory influences. While there is some evidence for direct inhibitory influences of steroid hormones on adenohypophysial cells, the quantitative importance seems to be far less than is the case for thyroid hormone influences on thyrotrophs. Effects of glucocorticoids on corticotrophs can be antagonized by administration of hypothalamic preparations containing CRF activity.

Under normal conditions, glucocorticoid secretion is cyclic. Circadian patterns have received the most attention; glucocorticoid concentrations in the blood plasma tend to rise to peak levels in the early morning in daylight-active animals, and in the late afternoon in nocturnal ones. Shorter (around 3-hr) and much longer rhythms have been observed. Differences between peak and trough levels tend to be greater in females than in males. Some physiological implications of adrenocortical rhythms were mentioned in Chapter 8.

Although adrenocortical cells maintained in tissue culture, and adenohypophysial cells separated from hypothalamic and glucocorticoid influences, seem to exhibit intrinsic rhythmicity of function, circadian rhythms of adrenocortical function in the intact organism depend upon neuronal input to the "corticotrophic area." It has been reported that small bilateral lesions of the anterior hypothalamus can abolish the rhythms without impairing "basal" secretion or some of the responses to stress. The rhythms are entrained by environmental stimuli, especially light, and are in phase with sleep-waking patterns; but they may persist when experimental changes are made in either.

The suprachiasmatic nuclei have been directly implicated in maintenance of glucocorticoid secretion patterns. Serotonin rhythms in the hippocampal and amygdaloid regions are in phase with adrenocortical rhythms and are believed to exert controlling influences. Studies utilizing pharmacological agents point to additional cholinergic influences.

Partial deafferentation of the hypothalamus can lead to chronic elevation of gluco-

corticoid concentrations in the blood plasma. Tonic inhibitory influences from posterior hypothalamic and cerebral cortical regions have been proposed.

Neurons in many parts of the brain are known to pick up glucocorticoids from the blood plasma, and to be affected by their presence. Some may be directly involved in regulation of adrenocortical responses to changing needs. But ACTH is known to affect brain functions which seem to be independent of elevation of glucocorticoid levels; for example, ACTH (and the chemically related MSHs) affect the rate at which conditioned reflexes can be established and extinguished. It seems likely that both ACTH and CRF are involved in as yet undisclosed functions.

THE HYPOTHALAMUS AND REGULATION OF BODY TEMPERATURE^{1, 4}

Neurons within the posterior hypothalamus which are not directly responsive to temperature changes, receive information from receptors of the skin and other regions affected by exposure to cold environments, and from numerous internal structures when the body temperature is actually lowered. Ascending spinothalamic tracts for transmission of the information have been identified. Objectively observable responses to stimulation of the posterior neurons include peripheral vasoconstriction, piloerection, increased tone of skeletal muscle, elevated metabolic rate associated with accelerated lipolysis, and also shivering if chilling is severe. It is usually possible to demonstrate increased secretion of catecholamines, and often of adrenocortical hormones.

More anteriorly located hypothalamic neurons are directly sensitive to changes in the temperature of the blood supplying that region. Responses to a rise in temperature include peripheral vasodilation, sweating, panting, and release of antidiuretic hormones.

It is clear enough that destruction of the posterior hypothalamus impairs responses to cold environmental temperatures, while damage to the anterior regions can lead to hyperthermia. But the concept that the posterior hypothalamus contains

a "cold center" or that the anterior hypothalamus contains a "heat center" is not fully consistent with available data.

Responses to environmental temperature changes are complex and they involve peripheral as well as central mechanisms. Receptor pathways enter the thalamus before reaching the hypothalamus; they also ascend to the cerebral cortex and travel elsewhere within the organism. Conscious awareness usually leads to changes in behavior, and the latter play a substantial role in protection against displacement of body temperature settings. Sweat glands respond directly to local stimuli; and animals with spinal cord sections that interrupt efferent pathways from the hypothalamus are capable of shivering when exposed to cold air or water.

Sensations of heat and cold are almost always associated with subjective reactions of pleasure or discomfort; such reactions can indirectly affect the hypothalamus.

The sensitivities of hypothalamic and peripheral receptors, and responses to their activation, vary with the conditions of the organism. Animals exhibit diurnal fluctuations of body temperature and are affected differently by stimuli presented at one time of the day as compared with another. Sudden changes in temperature invoke different responses than changes of equal magnitude presented gradually or in cycles. Animals previously exposed to the changes are different from "naive" animals; and those acclimated to special environments are dissimilar to siblings raised under more usual conditions. Additional variables include age, sex, nutritional history, endocrine status, and extent of development of the skeletal musculature. Delayed responses to chronic exposure can differ markedly from acute reactions. Reduction of air temperatures by a few degrees arouses qualitatively different responses than sudden, severe chilling. And extremely cold temperatures may actually impair mobilization of mechanisms used to cope with more moderate disturbances.

Hypothalamic responses to changes in environmental or internal temperatures are invariably associated with hypothalamic influences on processes not directly related to heat gain or loss and regions not

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directly related to "thermoregulatory centers" contribute to over-all response. For example, hypothalamic regulation of food intake has been most commonly associated with the ventromedial nuclei; but animals exposed to cold quite consistently increase their voluntary intake. Under many experimental conditions, animals are unable to cope with cold environments if food is restricted to the quantity taken regularly in a heated laboratory.

As noted in the introductory section of this chapter, the hypothalamus is an important site for reception of information concerning the nature of the internal and external environments, for processing of the information, and for transmission of signals. It sets in motion a number of mechanisms which aid the organism in coping with changing situations, and it coordinates many different kinds of activities. But one hypothalamic function is intimately related with another; and all hypothalamic functions interact with those of many other parts of the body.

Recently acquired information has led to drastic revision of earlier concepts of hypothalamic roles. An excellent example comes from studies of thyroid gland responses to cold exposure.

Thyroxine exerts important controlling influences on metabolic rate. Thyroidectomized animals succumb readily to reductions of environmental temperatures that are well tolerated by intact controls; and they are unable to effectively acclimate to cold temperatures, even when the fall is gradual. Thyroidectomized animals can be fully protected by administration of thyroid hormones. Some early studies have described morphological changes in thyroid glands of cold-exposed animals consistent with an augmented rate of thyroid hormone secretion. It has also been shown that some changes in the thyroid gland may not take place if there is extensive damage to the hypothalamus. All of the preceding seem to fit into a unified picture: As a result of cold exposure, the hypothalamus increases TRF output; this in turn leads (via TSH) to augmented thyroid hormone secretion and consequent elevation of metabolic rate. By thus increasing heat production, animals are able to cope with cold environmental temperatures.

But available facts do not support the concepts. TRF secretion may not be at all affected by a *small* reduction in environmental temperature (even when the stimulus invokes limited cardiovascular and metabolic adjustments). With a *moderate* fall in temperature, *transient* elevation of TRF is seen in certain mammals (*e.g.*, rats) but may not be seen at all in others (*e.g.*, humans). The TRF does not remain elevated if animals are maintained under conditions which lead to acclimation (acclimatization). *Severe* cold tends to *reduce* rather than elevate TRF secretion.

Studies on several different mammals have revealed that elevations of metabolic rate in response to cold are attributable to influences of *catecholamines* on lipolysis and other metabolic processes (Section III) and to increased activity of skeletal muscles. Thyroxine concentrations in the blood plasma are not elevated. (But there are indications that TSH concentrations tend to rise; and some studies have shown an increase in the plasma T₃:T₄ ratio.)

Evidence for increased secretion of thyroxine without elevation of plasma thyroxine concentrations seems to be strong. It has been shown that rats and some other animals substantially increase the rate at which thyroxine is secreted into the bile and eliminated with the feces. The enhanced biliary secretion results directly from food intake responses to the cold environment; it can be reproducibly altered by changing the composition of the diet. (When fecal loss of thyroxine is reduced by dietary manipulation, thyroxine secretion is also reduced.) The elevated TSH secretion rate is attributed to diminished negative feedback influences of circulating thyroid hormones. Increased thyroid hormone secretion then compensates for increased rate of fecal removal.

Hypothalamic mechanisms directly favor increased heat production and conservation, largely through activation of the sympathetic nervous system and secretion of norepinephrine. Calorigenic and "permissive" actions of thyroid hormones (Section V) are essential for support of the processes. But evidently "normally" available quantities of thyroid hormones can suffice. Although TRF is synthesized in the hypothalamus, and its release can be

acutely stimulated in response to cold exposure, it is not justified to conclude that TRF release contributes substantially to adaptation to the cold.

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VIII

THE PINEAL AND THYMUS GLANDS

25. The Pineal and Thymus Glands

ARE PINEAL AND THYMUS GLAND FUNCTIONS CLOSELY INTERRELATED?

Neuroendocrinologists have recently unraveled some of the mysteries of the pineal gland, while immunologists have been defining previously unsuspected functions of the thymus gland. Research in the two fields has proceeded in divergent directions, and has been conducted in dissimilar laboratories.

The situation was once very different. It was not at all uncommon in the past to find research papers on thymus and pineal glands emanating from the same laboratory, or for studies on the two structures to be pursued simultaneously, sequentially or even alternatingly. Reports on one of the organs usually contained references to the other.^{29, 54, 63}

The older literature contains numerous suggestions that both the thymus and the pineal attain maximum size and function early in life, and that they regress during childhood or at puberty. Both have been implicated in regulation of growth, maturation, and reproduction, and especially in protection against premature onset of puberty. Both have been observed to enlarge following castration, to be reduced in size during pregnancy and at other times associated with augmented secretion of gonadal hormones, and to be affected by estrogen administration. Both the thymus and the pineal have been reported to participate in control of sodium, potassium, and water balance, and to exert actions antagonistic to those of the parathyroid hormone. Summer-winter differences in functions of each have been described; and the two glands have been said to be affected by changes in environmental lighting, environmental

temperature, and modifications of the dietary intake. And both have been implicated in mediation of seasonal changes in thyroid gland functions. Evidence has been presented for interaction with adrenocortical functions; and both the thymus and pineal have been said to be affected by and to participate in response to stress. In addition, both the thymus and pineal glands have been associated with responses to and protection against development of malignant tumors.

There are remarkable similarities in the history of research in the two areas. At one time endocrinologists assumed without question that both the thymus and the pineal secrete important hormones; and studies were performed which paralleled the kinds used to investigate other endocrine organs. This was followed by a period in which highly respected investigators (whose work in different areas could be readily confirmed by independent laboratories), encountered difficulties repeating even their own experiments. Explanations were eagerly sought for inability to reproduce studies described in the literature.

Speculations on the causes of the difficulties went in several directions. Some investigators scrutinized structures of the two organs, and came to the conclusion that the cytological characteristics are not consistent with "true" endocrine function. Others proposed that both the thymus and pineal secrete multiple substances which exert antagonistic actions; and that any one extract is likely to contain both stimulatory and inhibitory principles. The concept was extended, and suggestions were made that the balance of components could change with age or prevailing conditions. Still others emphasized the technical

difficulties involved in removal of the organs (hemorrhage, infection and damage to neighboring structures) and the poor health of the few survivors. They wondered if only animals with preexisting abnormalities or those with ectopic tissues could withstand the extirpation sequelae. But it was also observed that an occasional survivor in which no aberrant tissue could be found, remained in apparently excellent health. Unfortunately, much of the very early work was done before research grants were available to support the projects. Investigators performing studies at their own expense tried to make do with limited numbers of experimental animals; and few included sham operated controls in their studies. Additional misinterpretations arose from failure to appreciate that animals were often exposed to inadequate diets and sub-optimal housing conditions.

It was suspected quite early that special kinds of experiments would have to be devised to study thymus and pineal functions, and that timing could be of critical importance. There were indications that age, sex, season of the year and dietary history all influenced the consequences of either thymectomy or pinealecotomy. But even when studies were devised in which effects of extirpation could be demonstrated and reproduced, it usually proved impossible to reverse the effects by administration of tissue extracts or homogenates, or by implanting glands from other animals.

Considerable excitement was generated by reports that thymectomy of successive generations of rats could lead to progressive impairment of growth and maturation, while injection of successive generations of rats with thymus gland extracts led to emergence of offspring with high birth weights and precocious development.¹³

Eighth generation rats treated with thymus extract were reported to have birth weights averaging 6.5 g, to have teeth and open ears at birth, to start hair growth after 1 day, and to open their eyes after 1.5 days; males had descended testes by days 2-3, while females cast their first litters by day 43. By contrast, un.injected controls were found to weigh 4.6 g at birth, with ears opening on days 2.5-3.5, eyes opening on days 14-17, teeth erupting at 8-10 days, hair growth starting at 12-16 days, and first litters cast on day 102.

Although effects of pinealecstasy were unimpressive, injection of successive generations of

rats with pineal extracts was reported to retard growth but to advance puberty.

The hope was raised that studies of this kind might shed some light on processes regulating both normal and malignant growth. However, attempts by independent laboratories to reproduce the findings were mostly unsuccessful.¹⁴ Questions were soon raised concerning the theoretical possibility of transmitting acquired characteristics to offspring, and wonder was expressed that young animals of later generations reported to be very large and vigorous at the time of birth (because of treatment of parents, grandparents, great-grandparents, etc. with thymus extracts) never attained a greater size or weight at maturity than did untreated controls of the same strain.

The validity of the work was soon doubted to the point where financial support for pursuit of further research in this area became virtually unobtainable. And indeed, there were good reasons for questioning the reported findings.

In time, the frustrations associated with attempts to define functions of the thymus and pineal glands, growing suspicions that the structures do not, in fact, produce hormones, and the excitement and greater rewards for work in other areas of endocrinology, led to a sharp decline in interest in thymus and pineal physiology. For a few years, the two structures were lumped together in textbooks under chapter headings such as "Structures of Unknown Endocrine Function". Later, even such references were deleted.

Interest in the pineal gland was rekindled after identification of *melatonin* (at first believed to be produced exclusively in that organ), the demonstration of circadian periodicity of enzymatic activity in the pineal, and elucidation of the nature and importance of pineal innervation (described below). Links to periodic functions of the reproductive system were soon established, and the pineal has now regained respectability as a member of the endocrine system. It is worth noting that a structure originally believed to deteriorate during childhood or adolescence is now thought to exert important influences on functions which do not emerge until after the onset of puberty.

Interest in the thymus gland was rea-

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wakened by demonstration of the key role played by that structure in development and function of the immune system.^{9, 14, 15, 16} Although it soon became apparent that devastating effects of thymectomy on immune systems of otherwise untreated animals can be demonstrated only when the surgery is performed very early in life (within the first few days after birth in rats and mice), it is widely recognized that the thymus gland grows rapidly after that time, and that it develops exquisite sensitivity to hormones secreted after puberty. There has been little recent interest in the United States in possible endocrine functions of the thymus gland of adult animals, although reports from European and Japanese laboratories have been published in American journals. An occasional paper has appeared linking the thymus to reproductive system or electrolyte balance physiology. A few investigators have also considered possible interrelationships with the pineal gland.^{15, 33a, 40}

Some of the established information on the two structures is presented below. This is followed by speculation on ways that thymus and pineal glands may interact.

STRUCTURE AND INNERVATION OF THE PINEAL GLAND

The Mammalian Pineal^{11, 16, 21a, 22}

The "typical" mammalian pineal gland is a small, compact, knoblike structure located just above and behind the roof of the third ventricle and attached to the latter by a small stalk. It commonly assumes a position close to the superior colliculi of the midbrain. The cavity of the ventricle may extend into the stalk for a variable distance as the *pineal recess*; but the gland does not usually contain a lumen.

The pineal starts as a bilateral thickening of the neuroectoderm of the third ventricle roof between the habenular and posterior commissures. Fusion into a single midline structure occurs early. In a few of the mammals, the gland is represented by little more than a thickening of the diencephalic roof.

The fully formed gland is covered by meninges, and there is disagreement about whether these effectively bar communication between the pineal and the third

ventricle (via the pineal recess or the more posteriorly located suprapineal recess). Conflicting interpretations have been attached to observations that materials injected into the ventricles gain access to the gland interior. The fenestrated endothelium of the rich capillary blood supply permits free entry of substances injected into the bloodstream. According to one hypothesis, transition can be traced from pineal glands of "lower" vertebrates which secrete predominantly into the cerebrospinal fluid, through intermediate types, to the mammalian gland which secretes exclusively into the blood capillaries.

The *pinealocytes* (pineocytes, parenchymal cells) are widely believed to be derived from neural ectoderm. They are not neurons, but resemble neurosensory cells. They also have characteristics consistent with special secretory functions; and a mesodermal origin has been proposed by some. Mitochondria are abundant, the Golgi complex is well developed, and there is a variable component of smooth and rough endoplasmic reticulum; and lipid droplets, vesicles, and lysosomes are consistently seen in the cytoplasm. Separate functions have been linked with the "dark" and "light" pinealocytes of the rabbit⁴⁵ and the rat,⁷⁴ but it has also been proposed that the two morphological types are functional variants of a single kind of cell in at least some species. Long cytoplasmic processes containing microtubules extend between and around neighboring cells; most terminate in the vicinity of the perivascular spaces.

Glia cells resembling those occurring elsewhere in the nervous system, *pigment cells* and *fibroblasts* are seen regularly. Some authors report finding mast cells, but others believe that components so designated differ both morphologically and functionally from "typical" mast cells found elsewhere.

A *ground substance* resembling that of bone often contains *calcareous deposits*. The latter (also known as concretions, corpora arenacea, acervuli, and psammoma bodies) are more prevalent in glands of older individuals. Pineal glands of human adults consistently show calcification. An older concept that the pineal becomes non-functional after puberty has been abandoned because of recent evidence that en-

zymatic activity is maintained; some findings point to a *positive* correlation between hormone synthesis and calcification.

The functional innervation in at least some of the mammals consists predominantly of *adrenergic postganglionic fibers* from the cervical sympathetic ganglia (and possibly to a lesser extent from the carotid plexuses). Axons enter the pineal via the nervus or nervi conarii (paired in some forms but not in others) often in association with the blood vessels; some terminate near the pinealocytes and others in the perivascular spaces.

Cholinergic fibers from the superior cervical ganglia have been identified in rats; in other mammals, preganglionic parasympathetic fibers may enter from the superficial petrosal nerves. It is possible that functional innervation is different in nocturnal (as compared with daylight-active) animals. One hypothesis (based on studies in the rabbit) states that cholinergic fibers control the release of serotonin from "light" pinealocytes, while adrenergic fibers influence utilization of the serotonin by the dark cells.

In most mammals, neurons which enter the pineal from neighboring brain regions seem to simply loop through the gland without making synaptic connections. In primates there is some evidence for direct epithalamic innervation.

Pineal Glands of

Nonmammalian Vertebrates^{10, 18, 21a, 22, 76}

In many (but not all) of the poikilotherms, the pineal is a *saccular* structure containing functioning *photoreceptor cells*, neurons, and direct synaptic connections with other parts of the brain. In some, a distinct "pineal proper" can be distinguished from a second structure known variously as the *parapineal*, *frontal organ*, or *Stirnorgan*. The latter is typically located extracranially, anterior and superior to the pineal proper, and connected to diencephalic structures by a long stalk. It may contain a retina-like component, lens, and cornea, as in the well developed "third eye" (parietal eye) of some of the lizards. A related structure is seen in frogs. But tailed amphibians have a single, saccular structure.

In some of the poikilotherms the parapineal is located on one side and the pineal proper on

the other. In lampreys and some of the fishes the pineal proper is larger, and may be located anterior or superior to the parapineal. In certain snakes the pineal is a single, compact structure similar to that of mammals. Crocodiles do not seem to have even rudimentary pineal glands.

Photoreceptor cells may be present in either or both pineal and parapineal components. Glands exhibiting electrical responses to light stimulation also seem to perform secretory functions. Many believe that mammalian pinealocytes (which are not directly light-sensitive) evolved from photoreceptor cells.

Pineal glands of at least some of the birds seem to have retained limited photosensitivity, and to be directly affected by light entering through the skull. Cells resembling rudimentary photosensors have been described for certain species.

HORMONES OF THE PINEAL GLANDS^{16, 21a, 22, 74}

Pineal Components Which May Function as Hormones

Melatonin (5-methoxy-N-acetyltryptamine, MLT) and enzymes catalyzing its formation from plasma-derived tryptophan have been identified in large numbers of vertebrate pineal glands. MLT has also been found in blood plasma, body tissues, and urine of many mammalian species; in at least some, the plasma concentrations fall to undetectable levels following pinealectomy.

The pineal gland is the major (and possibly sole) source of plasma MLT in humans and some other mammals; and diurnal variations in plasma concentrations have been directly related to rhythmic patterns of enzyme activity within the gland. Small quantities of MLT are synthesized by mammalian retina and Harderian gland, but these may not contribute to plasma content of the indole-amine. Somewhat larger amounts are formed in the eyes and brains of poikilothermic vertebrates, and there is also evidence for synthesis in frog skin.

Injected labeled MLT spreads rapidly throughout the body, crosses the "blood-brain barrier" and enters many cell types. Highest concentrations accumulate in the pineal, but considerable quantities are taken up by sympathetic nerves, iris, pituitary and adrenal glands, the testes, and the ovaries. MLT is degraded by hepatic en-

zymes, and the metabolites have been identified in urine. MLT injected into cerebral ventricles is rapidly concentrated in the pineal, hypothalamus, and midbrain.

Serotonin (5-OH-tryptamine, 5-HT) is an intermediate and it, too may function as a pineal hormone. However, 5-HT is synthesized by many cells outside the pineal, and its accumulation in most parts of the body depends on such synthesis. Relatively high concentrations of 5-HT are found in the brainstem, gastrointestinal tract, and platelets, and also in mast cells of some species.

Other substances thought to be secreted by the pineal include *5-OH-tryptophol* (5-H-ol), *5-Methoxytryptophol* (5-M-ol), and some known small peptides. Additional pineal peptides, lipids and carbohydrate-containing moieties which may be released have not been chemically characterized. The pineal also contains *histamine* but it may not be exported in substantial quantities.

Opinion is divided on whether components of the pineal gland identifiable in plasma should be characterized as "true" hormones. Objections to the designation have been made on the basis of one or more of the following: (1) Synthesis of the described molecules has been shown to take place (at least to a limited extent) in structures outside the pineal gland. (2) Synthesis and release are under nervous system control and there are reasons for suggesting that functions resemble those of neurotransmitters. (3) With the exception of demonstrated influences on pigmentation, most actions appear to be indirect; there are few studies in which administration of a pineal component has been shown to directly reverse effects of pinealecstasy.

Biosynthesis of Serotonin and Melatonin

The biosynthetic pathway for conversion of plasma-derived tryptophan to serotonin and melatonin, the associated enzymes and cofactors are shown in Figure 25-1. Some of the tryptophan taken up by pineal gland cells goes into other pathways (Figure 25-2).

The rate-limiting step for MLT (and 5-HT) synthesis is the conversion of tryptophan to 5-OH-tryptophan (5-HTP), a reaction catalyzed by the enzyme *tryptophan hydroxylase*.

Activity of tryptophan hydroxylase is high in the pineal compared with other tissues tested. (It is a mixed function oxidase which catalyzes transfer of molecular oxygen to several amino acids including phenylalanine.)

5-HTP synthesis can be markedly enhanced by acute or chronic dietary administration of large quantities of tryptophan, and can be decreased by feeding a diet excessively rich in phenylalanine. Under physiological conditions, plasma tryptophan concentrations are linked to food intake patterns. But circadian rhythms of serotonin synthesis are not readily disrupted by short periods of food deprivation.

5-HTP is rapidly converted to 5-HT. The enzyme (5-HTP-decarboxylase, L-aromatic acid decarboxylase) also catalyzes removal of a CO₂ moiety from tyrosine, DOPA, and histidine.

The pineal contains an additional, more specific histidine decarboxylase. The decarboxylase reaction does not seem to be a regulatory site for indoleamine rhythms, although influences of sympathetic denervation and of environmental lighting have been described.

The serotonin (5-HT) content of the pineal tends to rise sharply during the daylight hours, and may attain a peak concentration 6-9 times greater than the low point seen at night. (There are species variations in 5-HT concentration, and also changes related to the season of the year.)

Serotonin content falls during hours of darkness, because the amine is then rapidly converted to *N-acetylserotonin* (Ac-HT). The reaction is catalyzed by *serotonin-N-acetyltransferase*. Concentrations of Ac-HT are highest when those of 5-HT are lowest. Fifty to seventy-fold increases in enzyme activity and 10-30-fold increases in Ac-HT associated with the dark phase have been found in rats.

Conversion of Ac-HT to MLT is catalyzed by the enzyme *hydroxyindole-O-methyltransferase* (HIOMT).

The Role of Norepinephrine in Pineal Hormone Synthesis

Norepinephrine enhances (1) the rate of tryptophan uptake by pinealocytes, (2) activity and synthesis of tryptophan hydroxylase and therefore formation of 5-HTP, (3) activity and synthesis of *N-acetyltransferase* and therefore rate of con-

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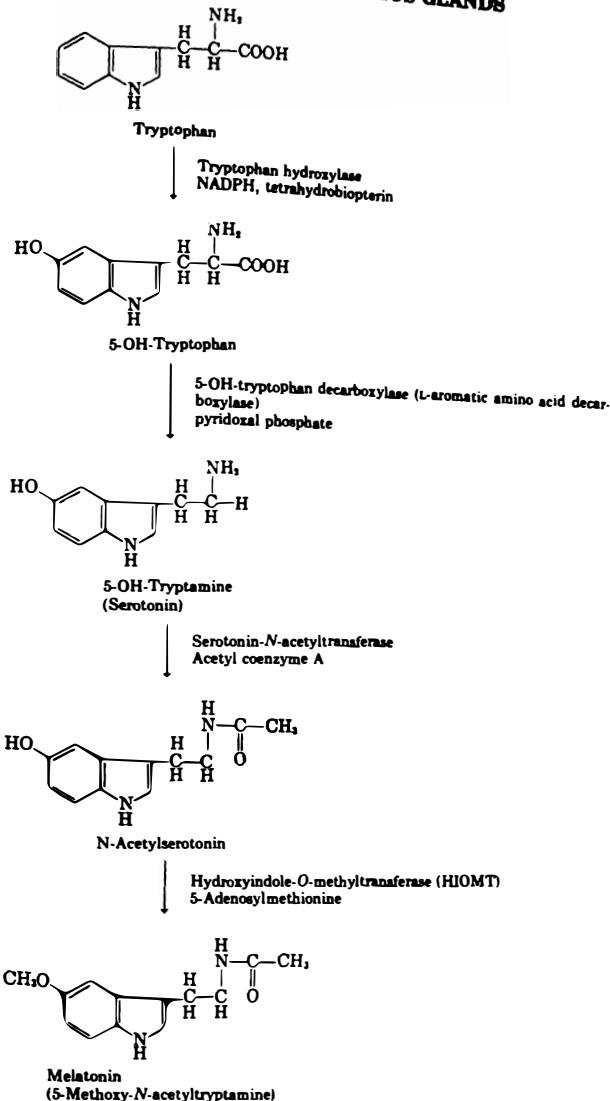


FIG. 25-1. Major pathway for biosynthesis of melatonin

version of 5-HT to Ac-HT. Effects on conversion of 5-HT to Ac-HT are quantitatively of great importance for maintenance of pineal gland rhythms.

The influences of norepinephrine on the two enzymes (but not on tryptophan uptake) involve activation of pineal adenylate cyclase and formation of new proteins, and a cAMP-dependent protein kinase has been identified. The

actions can be mimicked by administration of d-cAMP. A β -adrenergic receptor has been implicated, since norepinephrine influences can be blocked by administration of β -blocking agents (propanolol) while α -blockers (phenoxybenzamine) are ineffective. Effects of the catecholamine can be abolished with inhibitors of protein (but not of RNA) synthesis. Tryptophan hydroxylase activity can be inhibited with pharmacological agents such as parachloro-

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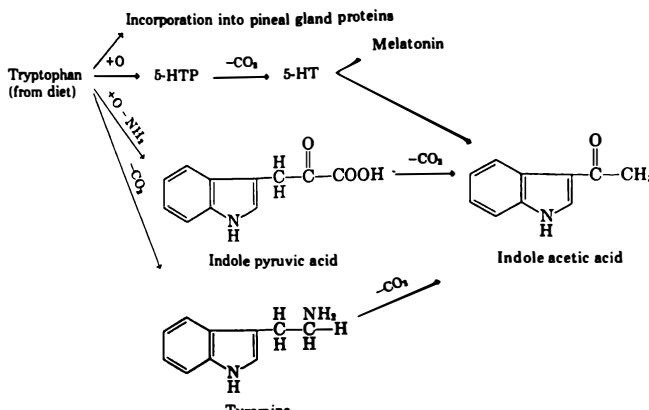


FIG. 25-2. Pathways for tryptophan metabolism in the pineal gland.

phenylalanine (pCPA) and α -propyldopaceta-mide.

The norepinephrine content of the pineal gland is greatest during darkness. When intact rodents are exposed to alternating light and dark periods, norepinephrine secretion, tryptophan uptake, tryptophan hydroxylase activity, *N*-acetyltransferase activity, ACHT, HIOMT activity, and MLT content of the pineal all show a pattern of diurnal variation with highest values found at night (while pineal content of 5-HT is low). Exposure of animals to continuous darkness leads to loss of norepinephrine, tryptophan hydroxylase, 5-HTP decarboxylase, Ac-HT, HIOMT, and MLT rhythms; but the *N*-acetyltransferase diurnal pattern is maintained. (The latter, however, is no longer entrained to environmental lighting; therefore the hour at which one animal exhibits peaks may be different from that seen in another animal.) Destruction of the innervation to the rat pineal gland leads to reduction of norepinephrine content and loss of all known pineal rhythms. It seems, therefore, that the presence of adequate amounts of norepinephrine is required for persistence of *N*-acetyltransferase rhythms, but that maintenance of some other pineal patterns depends upon continuation of norepinephrine rhythms. Blinding leads to much the same effects as exposure to continuous darkness.

When animals are maintained under conditions of constant light, norepineph-

rine secretion is inhibited and pineal rhythms are abolished. Therefore, effects of continuous light are in many ways similar to pineal gland denervation.

The pineal gland tends to get smaller after denervation or exposure to continuous light. This is consistent with evidence that norepinephrine plays a role in cell growth and cell division.

Because of its limited distribution (mostly within pineal glands of mammals, with very small amounts in Harderian glands and retinas), and because of the linking of circadian patterns of activity to light-dark cycles, it was once believed that the HIOMT enzyme exerted the major influence over MLT rhythms. But it is now known that changes in activity of *N*-acetyltransferase are of much greater magnitude, and that they are more closely linked with rates of MLT synthesis. In many studies investigators failed to find circadian variations in HIOMT activity; and there are conditions (e.g., strenuous locomotor activity) under which MLT concentration changes do not follow patterns of HIOMT activity.

The HIOMT rhythm is usually seen only in animals exposed to alternating periods of light and darkness. Activity of the enzyme and the MLT content of the pineal increase when nocturnal animals are maintained in the dark, while opposing influences of darkness have been found in primates and in some of the birds.

Thus, while *N*-acetyltransferase activity increases during the time in which noctur-

nal animals are moving about, the rise coincides with periods of rest in diurnal animals. By contrast, HIOMT rhythms may be more closely linked with locomotor function and the associated pattern of body temperature elevation.⁷⁴ Long-range stimulation of HIOMT activity in diurnal animals exposed to increased day length seems to be more reproducible than circadian responses to day-night alternations.

The formation of MLT seems to be controlled to some extent by mass-action influences of Ac-HT at times when *N*-acetyltransferase activity is high. But there are indications from pharmacological studies employing oxotremorine (which is muscarinic and increases brain acetylcholine content) and atropine (which blocks cholinergic responses and decreases brain acetylcholine) that cholinergic mechanisms also play a role in regulation of HIOMT activity. Either the relative dominance of cholinergic:adrenergic innervation, or the functions subserved by cholinergic nerves, may be very different in daylight-active as compared with nocturnal animals.

While blinding and pineal denervation abolish differences in HIOMT activity of pineal glands of adult rats kept in alternating light and darkness, the stimulatory influences of light on several bird species are not blocked in this way. Since light effects can be blocked by covering the head region in the vicinity of the gland, it is thought that light directly penetrates the skull to affect the pineal. Blinded infant rats are also sensitive to environmental light; it is believed that light in these animals is sensed by the Harderian glands. (Adult rats do not exhibit light sensitivity after blinding.)

In addition to promoting melatonin formation, HIOMT catalyzes transfer of methyl groups to other products of serotonin metabolism; it thereby contributes to synthesis of additional pineal gland components, especially 5-methoxytryptophol.

Some tryptophan is utilized for synthesis of pineal gland peptides and proteins, and substantial amounts are converted (via indole pyruvic acid) to *indole acetic acid* (IAA). Very small amounts may be decarboxylated to *tyramine*. Little of the latter is found in pineal glands, probably because only small quantities are formed and there is rapid conversion to IAA.

Two different monoamine oxidase (MAO) enzymes have been identified, one associated with pineal innervation and the second within pineal parenchymal cells. They can be distinguished because Catron (α -methylphenylethylhydrazine) specifically blocks only the parenchymal enzyme. No circadian variations in activity of the enzymes have been found. Adrenergic nerve endings pick up serotonin; and neuron content of the latter seems to vary inversely with that of norepinephrine. Sympathetic nerve MAO affects mostly 5-HT picked up by the nerves and contributes little to over-all 5-HT metabolism. Parenchymal cell MAO promotes conversion of comparatively larger amounts of 5-HT to metabolites, especially IAA. Actions seem to assume greatest importance during daylight hours in rats when acetyltransferase activity is at a minimum.

The metabolic fate of injected (labeled) melatonin has been traced, and it is likely that amine released by pineal gland into the bloodstream is handled in the same way. After hepatic hydroxylation at the 6 position (Fig. 25-3), most of the amine is conjugated with sulfate and excreted in the urine. Ten to fifteen per cent may be conjugated with glucuronic acid; and very small amounts of free 6-OH-

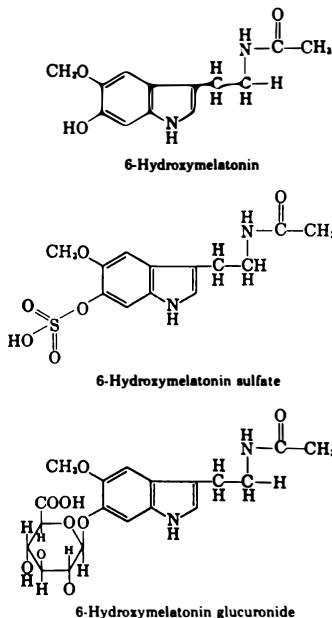


FIG. 25-3. Urinary metabolites recovered after administration of melatonin.

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melatonin are also excreted. The very rapid rate of hepatic hydroxylation probably accounts for differences seen after intravenous as compared with intracerebral injection or pellet implantation of MLT.

5-Methoxytryptophol and 5-Hydroxytryptophol^{22, 61, 74}

MAO catalyzes oxidation of some of the serotonin to an unstable intermediate, 5-OH-indole-3-acetaldehyde. Much of the latter is further oxidized to 5-OH-indole-3-acetic acid (HIAA). (The reaction takes place in serotonin-containing tissues outside the pineal gland, and is the major pathway for metabolism of whatever pineal 5-HT does not get converted to melatonin.) HIOMT catalyzes methylation of HIAA to 5-methoxyindole-3-acetic acid (MIAA). The reactions are shown in Figure 25-4. No hormone functions have been attributed to HIAA in animals. (A role in regulation of plant growth and phototropism is well-defined.)

Some of the 5-OH-indole-3-acetaldehyde is reduced to 5-OH-tryptophol. The latter can be methylated to form 5-methoxytryptophol.

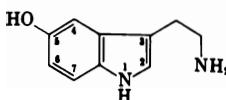
Some of the aldehyde intermediate may be directly methylated before oxidation to the acid or reduction to the alcohol (Fig. 25-4). Both the methoxy acid (MIAA) and 5-OH-tryptophol

have been regarded as possible pineal hormones.

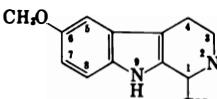
Related reactions within the pineal glands include methylation of serotonin to yield 5-methoxytryptamine, and oxidation of 5-

"Adrenoglomerulotropin"^{10, 22}

It was proposed some time ago that a hormone secreted by the pineal gland directly stimulates aldosterone production by zona glomerulosa cells of the adrenal cortex. The structure of adrenoglomerulotropin is compared with that of 5-HT in Fig. 25-5. It has been stated by different



5-OH-Tryptamine
(Serotonin)



1-CH₃-6-methoxy-1,2,3,4-tetrahydro-2-carboline
(Methoxytetrahydroharman)
(Adrenoglomerulotropin)

FIG. 25-5. Comparison of proposed structure of adrenoglomerulotropin with that of serotonin, illustrating numbering systems.

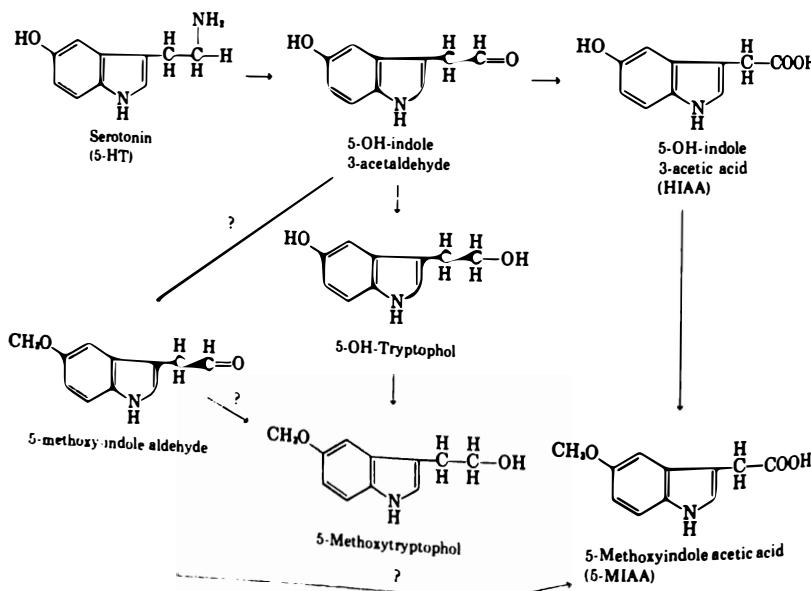


FIG. 25-4 Synthesis of HIAA, 5-methoxytryptophol, and 5-MIAA in pineal.

authors that (1) there is no convincing evidence that the pineal produces an adrenoglomerulotropin, (2) proposed structure is incorrect, and (3) while the described molecule can be found in pineal extracts, it is formed during the extraction procedure and does not represent a true physiological entity. (See also Section II, and discussion of pineal influences on electrolyte metabolism below.)

Pineal Peptides and Proteins^{18, 19, 22, 74}

Several small pineal peptides have recently been shown to influence functions of the reproductive system. The group includes arginine vasotocin (previously identified only in submammalian vertebrate neurohypophysis), lysine vasotocin, and a number of small molecules which clearly differ chemically but which have not yet been fully characterized. The pineal also contains substantial quantities of TRF, small amounts of gonadotrophin releasing factors, and probably some oxytocin.⁷² Tryptophan-rich proteins have recently been implicated in pineal regulation of hypothalamic functions.⁴⁶ The protein content and aminopeptidase activity of the gland are consistent with a rapid rate of peptide synthesis.

Histamine²²

Pineal histamine content is high, and a specific histidine decarboxylase (distinct from the L-aminoacid decarboxylase mentioned above) has been identified. Some authors believe that histamine is formed by pineal mast cells, but others deny that "typical" mast cells can be found in the gland.

Dopamine⁴⁶

Pinealocytes contain substantial quantities of dopamine. No direct relationship to sympathetic innervation has been established.

Pineal Gland Lipids and Carbohydrates^{18, 22}

No lipid unique to the pineal has been identified, and no lipid has thus far been implicated in endocrine functions. However, it has been reported that stress, hormones, and other factors markedly influence the lipid content of the pineal gland.

Radioactive phosphorus is avidly taken up by pineal cells; most is incorporated into as yet incompletely characterized lipids and carbohydrate-containing molecules.

NERVOUS PATHWAYS MEDIATING INFLUENCES OF ENVIRONMENTAL LIGHTING^{18, 19, 21a, 22}

There is clear evidence in many *poikilothermic* vertebrates that photoreceptor cells of the pineal convey information on environmental lighting directly to the other parts of the brain via pineal nerves. In *birds*, photoreception through the skull or via the Harderian glands may supplement information relayed from the retina.

Light can directly penetrate the skulls of *immature mammals*; light admitted in this way or through photoreceptor components of Harderian glands may regulate physiological functions. In *adult mammals*, photoelectric cells placed within brain tissue are affected by changes in environmental lighting; but it has not been demonstrated that structures other than retinal components of the eyes contribute to physiological mediation of light influences on pineal glands. Response of adult mammals maintained in continuous darkness are similar to those of animals subjected to orbital enucleation or retinal destruction. While receptors for both visual perception and pineal regulation are located in the retina, separate neuronal pathways to effectors are utilized.

Visual perception requires the *primary optic tracts* which lead from the optic chiasma to the lateral geniculate bodies of the thalamus and make functional connections with the occipital cortex. Additional fibers of the primary optic tracts terminate at the superior colliculi and tectal nuclei. The smaller, *superior accessory optic tracts* terminate in the midbrain tegmentum. Destruction of primary tract fibers not directly involved in visual perception (or of superior accessory tracts) leads to disturbances in responses to light, e.g., pupillary constriction and postural changes. But such destruction has no influence on pineal gland rhythms; and the rhythms are maintained when animals are blinded by severing the primary optic tracts.

Light travels to the pineal gland via the *inferior accessory optic tracts* which leave

the main pathway at the chiasma, cross, and travel through the *medial forebrain bundle* to the mesencephalic tegmentum. After passage through the tegmentospinal system to the upper thoracic part of the spinal cord, preganglionic autonomic fibers terminate in the *superior cervical ganglion* on each side. Postganglionic sympathetic fibers originating in the ganglia terminate within the pineal gland. Pathways to the pineal can be interrupted without disturbance of vision by severing the inferior accessory optic tracts, damaging the medial forebrain bundle, or interrupting post ganglionic pathways to the pineal. Both visual and pineal pathways are disrupted by damage to the retina or to the optic nerves.

An additional retinohypothalamic pathway has been recognized for some time in nonmammalian vertebrates, and special techniques have recently been employed to demonstrate the existence of such pathways in many mammalian species including rodents, carnivores and primates. Termination has been traced to the suprachiasmatic nuclei (Chapter 24).

As noted above, pineal *N*-acetyltransferase activity retains a circadian pattern in animals kept in continuous darkness; but the rhythm is abolished after denervation of the pineal gland. When animals are blinded by orbital enucleation or section of both optic nerves but innervation to the pineal is left intact, the circadian rhythm persists but it is "free-running"; i.e., it is no longer "entrained" to environmental lighting. Peaks and troughs of activity may occur at hours very different from those seen in animals with functioning optic nerves. On the other hand, destruction of both primary and superior accessory optic tracts does not interfere with entrainment of the rhythms, provided the retinae and optic nerves are left undisturbed. Since HIOMT rhythms are lost after severing the primary optic tracts, it is apparent that rhythms of the two enzymes are controlled differently.

The *N*-acetyltransferase rhythm is abolished by destruction of the suprachiasmatic nuclei, or by making a "postchiasmatic cut" between the nuclei and their caudal associations. A "prechiasmatic cut" which severs the retinohypothalamic connections leaves the rhythm intact but free-running. Such information has led to

development of the concept that *generation* of the rhythm resides in the hypothalamic cells, while entrainment requires input from the retina to the hypothalamus.

It is interesting to note in this connection that a similar relationship between the suprachiasmatic nuclei and their associated pathways seems to be involved in generation and maintenance of glucocorticoid rhythms, and that the appearance of the glucocorticoid pattern in young rats coincides with the time period in which a functional retinohypothalamic pathway can first be detected. Mechanisms for generation of the rhythms remain to be elucidated.

While effects of rearing animals in darkness can be largely explained on the basis of removal of photoperiod stimuli, exposing animals to continuous light seems to inflict damage to the retina, and may induce total blindness in albino rats. Circadian pineal rhythms are completely lost. Female rats kept in continuous light also tend to go into a condition of constant estrus (Section V), in which ovarian follicles ripen but ovulation and formation of corpora lutea are blocked.

PINEAL GLANDS AND PIGMENTATION

A useful discussion of physiological mechanisms for control of pigmentation must contain precise definition of the nature of the function examined, the kinds of cells involved in the response, the species (and often the strain) of animals, the age of the animals, previous treatment and other relevant factors.

General Nature of Pigmentation and of Pigment Cells^{2, 17}

Most "colored" animals have more than one kind of pigment cell, and the appearance at any given time depends not only on the morphological and physiological condition of such cells, but also on the arrangement of pigmented and associated non-pigmented structures, and on the direction of the light. The structures affect the apparent color through combinations of absorption, reflection and diffraction of light, and by means of differential light scattering and interference phenomena.

Pigment cell functions of the various species are affected by such things as neuronal stimulation and inhibition (involving more than one type of neurotransmitter), hormones (including melanocyte stimulating hormones, catecholamines, gonadotrophins, prolactin, thyroxine, ACTH, melatonin, androgens and estrogens), and direct influences of environmental light, humidity and temperature, as well as the ionic makeup of fluids surrounding the cells.

A single hormone may affect only one of the cell types within a specific organism, it may simultaneously stimulate (or inhibit) more than one type, or it may exert opposing influences on different cell types. Cells with similar morphology may exhibit widely divergent *sensitivities* to the same agent within the same organism at any given time, and the *same cell* may exhibit different sensitivities at different times. (An interesting observation is that melanophores within the spotted areas of a frog respond differently from those located nearby in the clearer region of the intact animal, but the contrast may not be discernible if the excised skin is studied.) A single cell can respond to more than one kind of stimulus. A hormone may exert seemingly opposite actions on morphologically similar cells of two closely related species. Genetic differences in hormone sensitivity have frequently been encountered within the same (often closely inbred) strain. Animals exhibit *developmental* and *physiological* alterations in responsiveness to the various types of stimulation, and especially striking differences may be encountered when larvae are compared with adults, when animals go through changes in reproductive functions, at different times of the day, and during different seasons.

Hormones can affect growth, mitosis, and differentiation of precursor (blast) cells; synthesis and degradation of the pigments; movements of pigments within the cells or from the cells to surrounding structures; modifications of structures into which pigments are deposited; responsiveness of pigment cells to other kinds of stimuli; and also shedding and molting of colored parts of the organism. Mechanisms for regulation of color change may be interrelated with mechanisms for control of quite different functions, e.g., body temperature or certain forms of behavior. The

importance of pigment changes for attraction of members of the opposite sex, or for signaling readiness to mate, has been established in several species.

Even the use of the term *pigment cell* may generate confusion. It is defined by some investigators as one in which a "colored" substance is synthesized and stored. There is a difference of opinion concerning inclusion of structures containing white materials which reflect light and impart iridescence. Some authors apply the term *pigment cell* to colorless structures physiologically related to those that actually produce the pigments.¹⁷ (Thus, one hears of "amelanotic melanocytes which are related to "true" melanocytes but lack pigment accumulations and differ from melanoblasts.) Still others include any "colored" cells which have the capacity to concentrate pigments derived from neighboring cells or from dietary sources. Melanophages are classified as *pigment cells* by some, and as *macrophages* by others.

Most of the research on pigmentation has been conducted on poikilothermic vertebrates and on invertebrates. Some of the derived information can be cautiously applied to the understanding of mechanisms in homeotherms.

The term *chromatophore* is widely used to designate any pigment cell. But some reserve the term for those in which rapid *physiological* changes in appearance can be brought about without concomitant alteration of total pigment content. (The changes usually result from intracellular migration of granules or pigment-containing organelles within a cell having fixed boundaries; but the possibility that some cell types engage in true expansion and contraction has not been ruled out.)

The term *chromatocyte* is usually applied to cells containing pigment in less movable form. But some authors use it for any *pigment cell* and others for all *vertebrate* ones.

Pigment cells have been classified on the basis of color. The term *erythrophore* is obviously suitable for those that appear red; but some cells included in the group appear orange or yellow. *Xanthophores* are often yellow; but they can impart green coloration to animals which might otherwise appear blue, and cells appearing orange-

colored have also been classified as xanthophores.

Leucophores or *iridophores* contain opaque materials usually arranged geometrically in the form of platelets which reflect light and thereby impart a whitish, silvery or iridescent appearance. But since light may also be scattered, such cells can contribute to pinkish, bluish, or golden coloration. The term *xantholeucophore* designates an association of two different cell types.

Melanophores provide the greatest problems for classification on the basis of color, since while most appear black or brown, some contain red or yellow pigments. Moreover, most animals have more than one type of pigment cell, each type can contain pigment mixtures, and the appearance is affected by anatomical arrangement of the pigment cells and by associations with other structures which contribute to light absorption, reflection, diffraction, and scattering.

Another system of classification is based on the chemical makeup of the pigment. According to this system, *melanophores* (or *melanocytes*) are pigment cells containing melanins. "True" or eumelanins appear

brown or black, while *phaeomelanins* can be yellow or red. (But some zoologists use the term melanophore to describe very dark cells of invertebrates which actually contain ommochromes derived from tryptophan.)

Melanins are high molecular weight heteropolymers of tyrosine metabolites associated with proteins. The major component derived from tyrosine seems to be indole-5,6-quinone (Figure 25-6), but additional tyrosine oxidation products have been identified. Phaeomelanins also contain cysteine derivatives; both 5-cysteinyl DOPA and a 2-cysteinyl isomer are present in substantial quantities. The enzyme tyrosinase catalyzes synthesis of both DOPA and Dopaquinone.

Some established steps in the biosynthetic pathway are shown in Figure 25-6. Pigment-protein complexes are deposited on a preformed matrix of protein fibers derived from the Golgi complex. The resulting organelle is called a *premelanosome* during the formative stages, and a *melanosome* once melanization has been completed. The terms melanosome and melain granule are sometimes used interchangeably.

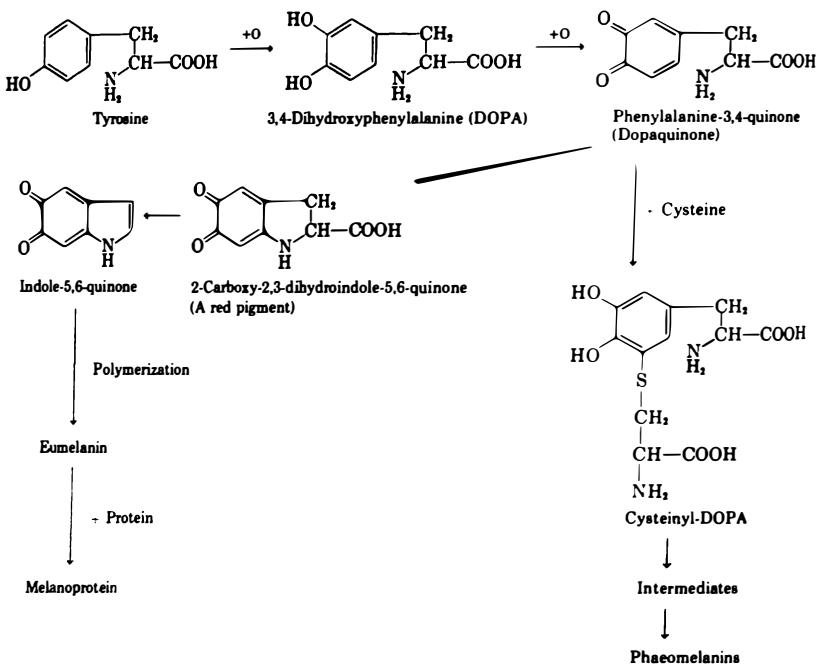


FIG 25-6. Some steps in the biosynthetic pathway for eumelanins and phaeomelanins.

Distinctions are often made between *dermal* and *epidermal* melanophores (or melanocytes) since they differ in location, structure, function, and hormone sensitivity. The dermal types are most commonly involved in rapid "physiological" color changes, while epidermal melanophores transfer pigment to other cells and function predominantly in "morphological" changes. However, melanocytes are also found in nerves, in the eye, the areolae, the visceral organs, and in association with blood vessels. All chromatophore precursors are derived from the embryonic neural crest.

The principal pigments of iridophores are *purines*. Guanine is the most abundant, but hypoxanthine, uric acid, adenine and isoguanine have also been found (Fig. 25-7). Purines are usually deposited in crystalline form. While the term *guanophore* appears in the literature, the terms iridophore and leucophore are more commonly used.

Lipophores contain lipid-soluble pigments. A distinction can be made between *pteridophores* and cells containing carotenoids. But many pteridophores contain carotenoids, and the latter accumulate even before pteridine deposition is obvious. Carotenoids are derived from plant materials; chromatophores possess the ability to select and modify the precursor molecules. (Animal breeders often feed selected carotenoids to birds and fish to promote development of attractive coloration.) *Riboflavin* may also be present (Fig. 25-8). Lipophores are classified as *xanthophores* or *erythrophores*.

The term "physiological" is widely applied to color changes resulting from reversible movements of pigment "granules" or pigment-containing organelles within a single cell, while "morphological" color changes involve more slowly developed and longer lasting alterations of the quantity of pigment present or of movements of colored materials into cells which do not directly synthesize pigments.

Physiological changes have been most extensively studied in melanophores; but these are often accompanied by changes in iridophores.

Most poikilothermic vertebrates acquire at some stage in their life cycle, the ability to adapt rapidly to changes in environmental lighting and background coloration. Many possess sensors for detection of *albedo*, or ratio of light falling directly into the eye to light reaching them through background reflection.

Animals placed against a dark background may exhibit outward migration of melanosomes from a perinuclear position towards the periphery of the cell. The change in appearance has been likened to that seen when one blows on a spoonful of powdered charcoal initially located in the center of a white tablecloth. The analogy is useful in some ways, but melanosome movement is more ordered, and is often accompanied by reciprocal inward migration of reflecting platelets of iridophores. Pigmented organelles seem to follow defined pathways, and microtubules have been implicated in regulation of the movement. In the maximally aggregated state, melanosomes have a somewhat globular or

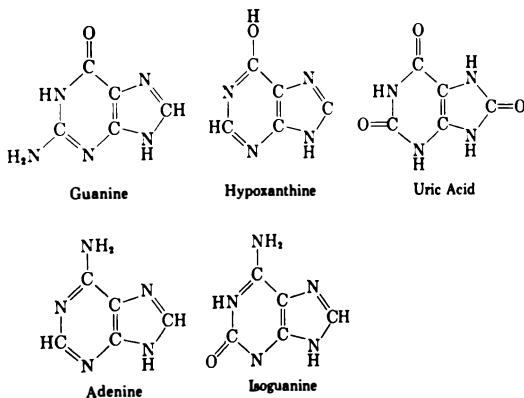


FIG. 25-7. Purine pigments of iridophores

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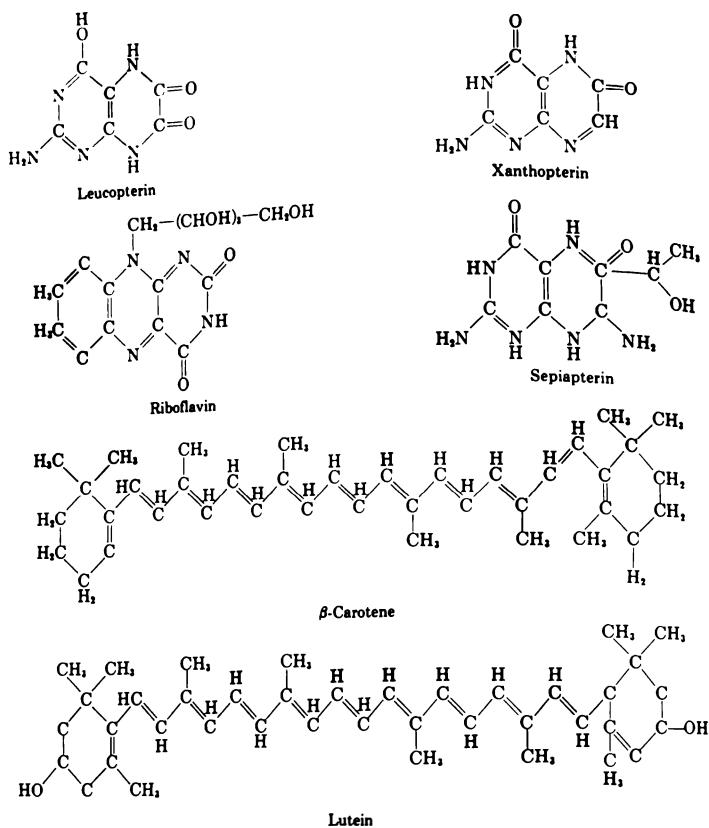


FIG. 25-8. Some pigment components of lipophores.

punctate appearance; when dispersed they appear star-like or *stellate*, and the maximally dispersed melanophore is said to be *reticulate*. The term *melanophore index* (MI) refers to semiquantitative descriptions of the magnitude of response:

- Stage 1 Punctate (maximal aggregation)
- Stage 2 Punctate-stellate
- Stage 3 Stellate
- Stage 4 Stellate-reticulate
- Stage 5 Reticulate (maximal dispersion)

The MI has been useful for estimation of hormone activity, although some subjectivity is involved in the designations. Photometric procedures for use with isolated amphibian and reptilian skins have been developed which are sensitive, rapid, and objective; but the photometric measurements do not separate out associated changes in iridophores.

Increasing the hydrostatic pressure leads to dispersion of melanosomes of some species, while elevation of environmental temperature and exposure to agents which stabilize the gelated state, induce changes in the opposite direction. Such observations are consistent with the belief that dispersion-aggregation responses are directly related to cytoplasmic solation-gelation phenomena. It has also been found that hormones and other stimuli which elevate cAMP concentrations within the melanophore cytoplasm tend to promote melanosome dispersion. The demonstration that it is absolutely essential to have sodium ions in the medium bathing melanophores if melanophore stimulating hormones are to promote dispersion, that the amount of dispersion may be directly related to the quantity of sodium entering the cell, and that

other monovalent cations cannot be substituted, is interesting from several points of view. In addition to providing insight into mechanisms for migration of pigment granules, it lends support to the concept that melanocyte stimulating hormones regulate physiological processes unrelated to pigmentation but directly related to sodium movements. (See also Chapters 13 and 23.)

Dermal melanophores are large and flat. The movement of melanosomes into elongated processes which extend over iridophores obscures the underlying white pigment. In some species the iridophores also contract in response to the same stimuli which affect melanosome dispersion. When animals are placed on a white background, aggregation of melanosomes exposes the iridophores; the effect is enhanced by dispersion of the reflecting platelets.

Morphological color changes are best seen after chronic stimulation. Prolonged exposure to dim light and dark backgrounds leads to synthesis of additional melanin which is transferred to epidermal structures; deposition of iridophore purines may be simultaneously diminished. Prolonged exposure to light-colored backgrounds can lead to reduction of melanin synthesis and increased purine deposition.

It has been proposed that conditions promoting migration of melanosomes lead secondarily to changes in synthesis; the hypothesis has not been adequately tested.

Regulation of Pigmentation: Role of Peripheral Nerves²

Rapidly moving fishes are more likely than most other vertebrates to encounter sudden differences in background color in the presence of predators. It is obvious that prompt changes in protective coloration can favor survival. Innervation of chromatophores has been demonstrated in many species, and the cells also respond to administration of neurotransmitters. Nervous control of chromatophores has also been found in chameleons. There is little evidence for chromatophore innervation in most other vertebrates; but it has been observed that patterns of localized pigmentation disturbances in humans tend to follow the nerve pathways.

Stimulation of sympathetic nerves and administration of catecholamines usually

lead to melanosome aggregation. α -Adrenergic receptors have been identified in some species.

Acetylcholine can promote melanosome aggregation *in vitro* when tested on frog skin; but there is little evidence for direct cholinergic innervation of chromatophores. Lightening responses following administration of acetylcholine have been attributed to stimulation of sympathetic ganglion cells, or to influences on MSH release.

Some *innervated* chromatophores are clearly responsive to hormones; the latter may enhance rapid responses and also promote long-range morphological changes. In certain fishes the responses to hormones can be demonstrated only after denervation. In other varieties, at least some of the pigment cells lack hormone receptors. Tail fin chromatophores of amphibian larvae seem to respond directly to light stimulation.

Melanocyte Stimulating Hormones^{2, 12}

The hormones most directly associated with promotion of pigment synthesis and dispersion of melanosomes are known as *melanocyte* or *melanophore stimulating hormones* (MSHs). Since synthesis in many species has been localized to the pars intermedia or the neurointermediate lobe of the pituitary gland, they are also known as *intermedins*. Neither designation is completely appropriate, since the hormones affect chromatophores other than those synthesizing melanin, while cells producing them have been localized to the pars distalis or neurohypophysis of some species (Chapter 23).

The structures of mammalian α - and β -MSHs were described in Chapter 23. Chromatophore stimulating peptides have been isolated and characterized for many vertebrate species. All contain the heptapeptide sequence shown below, and there is good reason to believe that those not yet fully analyzed are chemically related.



The heptapeptide is a powerful stimulant of chromatophores. The smallest molecule known to exert similar action is the tetrapeptide His-Phe-Arg-Tyr contained within the heptapeptide. Elongation of the chain at both COOH- and NH₂-terminals (up to a total of 13 amino acids) enhances potency. α -MSH found in sev-

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eral mammals is a tridecapeptide; its structure may be identical in all species. It is somewhat more potent than the slightly longer β -MSH.

The described amino acids sequences are contained within all known ACTH molecules; and all ACTHs have limited MSH-like activity (but not all MSHs have ACTH potency). In some of the goldfish, pigment cells respond to ACTH but not to MSH. ACTH has been clearly implicated in contributing to the hyperpigmentation of Addison's disease.

Hypophysial lipotrophins and hypothalamic peptides with CRF activity have also been observed to stimulate melanophores.

Both dermal and epidermal melanophores of poikilothermic vertebrates respond to MSHs. Solutions containing as little as 0.01 pmol/ml promote dispersion, and similar effects are obtained after administration of whole pituitary extract, extracts prepared from pars intermedia or neurointermediate lobes, and blood plasma taken from darkened animals of the same species. Iridophores of some amphibians contract in response to the hormone, and responses of xanthophores have been observed in a few species.

Removal of MSH-producing cells (by hypophysectomy) leads to reduction in melanocyte numbers and to melanosome aggregation in most amphibians, in cyclostomes and at least in some of the elasmobranch fishes, and in reptiles. Reciprocal changes in iridophores have been seen in amphibians.

It is obvious that warm-blooded animals whose integument is covered with fur, hair, or feathers have little use for a hormone which promotes movement of pigment organelles within the underlying dermis. But MSH has been clearly implicated in stimulation of *synthesis* and *transfer* of melanin in integumental structures of mammals. It evidently plays an important role in mediation of seasonal changes in the color of the pelage of some; for example, appearance of the brown coat of weasels seems to be directly dependent upon the presence of the hormone. Humans suffering from MSH-secreting tumors develop hyperpigmentation of the skin and darkening of the hair; reversal is sometimes achieved by removal of the tumor. When MSH is injected into human subjects, skin darkening

becomes apparent within 24 hr. It has been proposed that the hormone promotes rapid distribution of pigments in addition to slower stimulation of new melanin synthesis. (The human hypophysis does not include a distinct pars intermedia; but melanocytes are found within the gland, and a pars intermedia has been identified in human embryos. The human hypophysis seems to contain additional, as yet undefined molecules with considerable melanotropic potency.)

Since birds never develop a pars intermedia, it is perhaps not surprising that few respond to MSHs from other vertebrates. (MSH is effective in red chickens and certain other species in which the hormone has been detected.) Feather color in the weaver finch is unresponsive to MSH, but is known to be directly influenced by interstitial cell-stimulating hormone, an effect which can be elicited in the absence of the testes. A curious observation is that, whereas bill coloration in the finches requires the presence of androgens, bill coloration in the wydah is directly dependent upon the pituitary gonadotrophin.

There is considerable evidence that other hormones can influence pigmentation in certain animals. In metamorphosing amphibians, thyroxine directly increases the number of chromatophores and may play a role in pattern formation. Prolactin seems to directly affect some pigment responses in fishes, and to synergize in other cases with MSH.

It has been reported that some fishes lighten after administration of MSH. Interpretation of such data requires use of appropriate controls since the stress related to handling and injection can affect chromatophore innervation or release of catecholamines and other agents implicated in paling responses.

Estrogens seem to directly enhance melanization in mammals, and localized effects of administration of the hormone have been observed. Estrogens may also synergize with the MSHs, or affect their release. Increased pigmentation of the areolae during normal pregnancy and the localized pigmentation of the face which occurs in some women during pregnancy or while taking oral contraceptives, have been attributed to high estrogen concentrations.

Regulation of MSH Release^{2, 12, 19}

There are unsettled questions concerning whether darkening and paling responses result from increase and decrease, respectively, of amounts of MSH present, or whether a second "lightening" hormone is needed in animals which darken in response to the intermedins. It seems likely that several kinds of control mechanisms operate in the various animal types.

Hypothalamic inhibitory control over MSH secretion can be exerted via neurotransmitters, hormones, or both. Hormones implicated in both inhibition and stimulation of MSH release were described in Chapter 23.

Transplantation of the pituitary gland to a site removed from hypothalamic influence, or damage to certain parts of the hypothalamus, is often followed by mitosis of pigment cells, increased synthesis of melanin, and (in some vertebrates) dispersion of melanosomes. Weasels with pituitary transplants tend to grow dark fur at times of the year when others produce white or light coats.

Adrenergic neurons terminating in the vicinity of the pars intermedia have been demonstrated with fluorescent staining techniques; and administration of dopamine or norepinephrine can reduce MSH secretion by pars intermedia transplants isolated from the hypothalamus.

The observed terminations of hypothalamo-hypophysial tract axons in the pars intermedia region are consistent with the concept of magnocellular control over pigmentation. But it has been noted (Chapter 23) that the pars intermedia tends to be large in animals capable of excreting highly concentrated urine; and a role of MSH in regulation of sodium metabolism has been described (Chapter 13). This raises the possibility that some pigmentation changes arise as "side-effects" of hormonal regulation of sodium metabolism; it is also conceivable that physiological changes in melanocyte function contribute in some way to electrolyte balance. (Melanosome dispersion is directly linked with sodium uptake.)

There are reasons to believe that innervation to the pars intermedia is exclusively inhibitory in some species; and that MSH secretion varies inversely with activity of

the neurons. But in others, stimulation of the hypothalamus sometimes leads to increased MSH secretion; this could result from either activation of pathways promoting MSH release, or through inhibition of inhibitory neurons.

On the basis of quantitative studies in the frog, it has been proposed that those animals possess light-insensitive adrenergic neurons that exert tonic inhibitory control and additional light-sensitive neurons that inhibit activity of the former. Under conditions of low illumination, the light-sensitive components become maximally active and thereby indirectly effect MSH release. Other animal types may have neurons which directly activate the pars intermedia cells.

The Pineal Gland and Pigmentation^{2, 11, 22, 29}

Melatonin in very low concentrations is a potent agent for stimulation of melanosome aggregation in dermal melanophores of amphibian larvae and of some very young (embryonic and larval) fishes. It is effective even in the presence of MSH, and seems to act directly at the target organ level by competing with MSH for receptor sites.

Certain kinds of observations raise the possibility that melatonin plays an important role in regulation of pigmentation in these animals. The indole-amine has been identified in the pineal glands of several amphibian species, and it is effective when administered to tadpoles *in vivo*. Melatonin synthesis is evidently increased during periods of darkness, and it is known that amphibian larvae blanch when deprived of light. Moreover, the blanching response can be abolished by damaging diencephalic regions in which pineal structures are located. Blanching is difficult to demonstrate in untreated hypophysectomized larvae, but hypophysectomized larvae treated with MSH exhibit the reaction. (Therefore, the pineal influence does not depend on inhibition of MSH release.) Paling following dark exposure is also seen in eyeless larvae, and there are good indications that the photoreceptors are part of or directly associated with the pineal.

However, it is difficult to define the role of the pineal gland (or of melatonin) in

such animals. Young (embryonic and larval) forms do not exhibit responses to changes in background coloration when light is present, and older animals which have the capacity to change their appearance under such conditions, exhibit little or no reaction to the administration of melatonin. (Dermal melanophores undergo developmental loss of sensitivity to the indole-amine; and the minimal responses of such cells in the adult tend to be obscured by the presence of overlying epidermal melanophores which are totally unaffected by melatonin.) Moreover, pinealectomized adult frogs may still pale when placed on a light background.

One can only speculate on whether blanching of very young animals in darkness has functional significance. It is possible that predators with sensory mechanisms very different from ours have greater difficulty finding pale than dark larvae. The light color could also provide an advantage to larvae swimming about and suddenly entering a region of light background.

But perhaps the blanching response has no direct meaning as such, and merely represents a *developmental stage* (which confers no advantage but also no disadvantage), as the young animals prepare for very different adult pineal gland functions. Another possibility worth considering is that less energy is utilized by melanophores in the aggregated state. Energy conservation can be important to a rapidly growing embryo or larva with minimal reserves, especially if it becomes necessary to seek out food sources in the lighter regions where plants grow.

Presently available evidence points to pineal gland regulation of pigmentation in some mammals but not in others.⁴⁴ Pineal gland implants and melatonin administration induce formation of light colored fur in weasels during times of the year when they would otherwise grow a dark coat. No such influences on color coat have been found in rats; but it has been observed that melatonin increases, while pinealectomy decreases in MSH content of the pituitary gland. Pineal influences in the weasel may also be mediated indirectly via the pituitary, since melatonin is ineffective in weasels in which the pituitary has been transplanted to a site distant from the hypothal-

amus. Melatonin may promote release of a hypothalamic MIF.

Some success has been described for attempts to treat melanization in dogs by administration of melatonin. Melanized human subjects have not responded. It has been pointed out in this connection, that the condition in humans seems to involve primarily the epidermal pigment cells, while only dermal pigment cells are responsive to MSH at least in some species. However, it seems more likely that differences in responses of dogs as compared with humans are related to species variation in secretion of a hypothalamic inhibitor of MSH release following exposure to melatonin.

THE PINEAL GLAND AND REPRODUCTIVE PROCESSES^{21a, 22, 42, 61, 71, 74}

Young persons with tumors of the pineal gland often suffer from either delayed or precocious onset of puberty.^{11, 12} While mechanical pressure exerted by tumors on neighboring structures can cause difficulties, there is good reason to believe that most disruptions of reproductive patterns are related to pineal gland secretions. Hypertrophy of the parenchyma is often associated with inhibition (and cell destruction with stimulation) of reproductive hormone secretion. For unknown reasons, the incidence of pineal tumors in combination with unusual timing of puberty is far greater in human males than in human females.

Several kinds of observations point indirectly to pineal gland regulation of the human reproductive system. For certain ones, the association is highly speculative. More direct evidence for similar influences in experimental animals is cited below.

Melatonin and the enzymes catalyzing its synthesis have been identified in human pineal glands. A substance effective in experimental animals has been obtained from human urine and named gonadotropin-inhibiting substance (GIS).¹² It seems to be similar to an agent found in the urine of cows, rabbits and rats, and it has been reported that GIS disappears from the urine of rats after pinealectomy. Pineal gland extracts have been administered to human patients for clinical purposes, and have been observed to exert inhibitory influences on the reproductive system.

Effects of environmental lighting and of blinding on reproduction have been cited as indications of pineal gland influences. The onset of puberty is said to be accelerated in blind girls. But other aspects of reproduction seem to be inhibited; for example, the fertility of married blind women is said to be extremely low. In Northern Finland (which has very long winter nights and very long summer days), the incidence of conception is lowest in January and highest during the spring and summer months. While factors other than the pineal must surely contribute, the data on multiple births (associated with increased output of gonadotrophin in the 75% of cases attributable to multiple ova) are far more impressive. Such births peak in the summer and are rare in January. Menstrual cycles have been reported to be stabilized in women experiencing great irregularity, following deliberate nighttime exposure to light during the mid-cycle. There have also been suggestions that the earlier onset of puberty in human females today as compared with some decades ago, and in human females living in urban areas as compared with rural areas can be related to increased use of artificial illumination during the winter months. Peak times for onset of the first menstruation seem to occur during late spring and early summer.

Much of the experimental investigation of pineal gland influences on the reproductive system has utilized small rodents which are readily available, and whose anatomical make-up permits easy performance of pinealectomy. It has become increasingly apparent that species variation place sharp limitations on the transfer of information to the understanding of pineal physiology of other animal types. Special problems arise from studies conducted on animals with very marked seasonal fluctuations in reproductive functions, animals that hibernate, and those that are by nature nocturnal. There are serious questions concerning the physiological significance of studies on nocturnal forms such as rats and mice reared under the usual laboratory conditions, in which animals are exposed to long periods of light and handled during daylight hours when they would otherwise be least active.

Data derived from several different

mammalian species indicate that removal of the pineal gland can lead to onset of precocious puberty in both males and females, to accelerated growth of testes and accessory reproductive structures, to enlargement of some of the latter in adult animals, and to evidence of increased ovarian activity. The findings have emanated from several independent laboratories. Occasional emergence of contradictory data has been interpreted by many to mean that some as yet undefined factors must be controlled before the pineal role can be fully elucidated.

The golden hamster raised under natural conditions exhibits regression of the reproductive system before it goes into winter hibernation, and recrudescence shortly before it emerges in the spring. The natural cycle is conducive to perpetuation of the species, since it provides for mating and birth of the young during periods of the year most favorable for survival.

When exposed to progressively longer nights as winter approaches, the male undergoes testicular atrophy, interruption of spermatogenesis, and reduction in size of the accessory reproductive structures. The LH content of the blood is lowered, and the weight and bioassayable content of FSH and LH of the pituitary gland decline. In female hamsters, related changes include reduction of ovarian weight and interruption of ovarian cycles.

There is an associated increase in the size and weight of the pineal gland, increased volume of pinealocyte nuclei, and heightened activity of the HIOMT enzyme. All of the described changes can be delayed or attenuated by exposure of the animals to artificially long days.

Blinding leads to reproductive system atrophy even under intense and prolonged environmental illumination. Influences of natural or artificial light deprivation on the pituitary gland and reproductive structures can be prevented by performing a pinealectomy prior to onset of declining function. Some reversal of dark-mediated inhibition can be achieved by removing the gland at a later time. Denervation of the pineal has similar effects.

On the basis of such observations, it has been proposed that synchronization of the hamster reproductive system with environmental cues is mediated via the pineal

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gland; when information from retinal receptors is relayed, the pineal responds by releasing antigenadotropic principles. Since pituitary gonadotrophin content is reduced, peripheral structures remain responsive to exogenous hormones, and certain other studies seem to rule out the pituitary gland, it is widely believed that pineal influences are exerted on the hypothalamus.

It is likely that regulatory mechanisms in the hamster are more complicated than is suggested by the hypothesis. Animals eventually become refractory to prolonged exposure to darkness, and they resume reproductive growth and function. (And hibernating animals prepare for copulation while still confined to darkened burrows.) A proposal consistent with such observations is that the sensitivity of the hypothalamus to pineal influences undergoes seasonal variation and may be lowest in the springtime. An alternate explanation is that prolonged secretion of pineal hormones leads secondarily to synthesis of anti-pineal substances, or that the pineal gland itself changes the kinds and quantities of hormones released.

Attempts to reverse the early effects of light deprivation by injection of MLT have been uniformly unsuccessful in hamsters, although the methoxy-indoleamine has been shown to exert antigenadotropic activity in other species (see below). There have been reports that arginine vasotocin and some as yet uncharacterized pineal peptides can induce inhibition. This could mean that antigenadotropic principles of the hamster pineal differ from those of some other mammals. But it was recently observed that repeated implantation of pellets containing either MLT or 5-methoxytryptophol in beeswax effectively prevented involution of reproductive organs, depletion of pituitary LH stores, and lowering of plasma LH concentrations which would otherwise occur in golden hamsters subjected to conditions of 23 hr of darkness each day. (The pellets did not interfere with regrowth of reproductive structures and resumption of function when animals were returned to conditions of long daylight.) Several hypotheses have been proposed, including: (1) MLT and related pineal derivatives of some species inhibit synthesis or release of other pineal gland hormones which depress gonadotro-

phin release. (2) Methoxyindoleamines of some species render neuroendocrine mechanisms for gonadotrophin release insensitive to pineal peptides or other pineal hormones. (3) MLT enhances synthesis or release of progonadotropic principles. (4) Methoxyindoleamines exert (direct or indirect) stimulatory influences on the gonads.^{21c}

Ferrets are also seasonal breeders. Under natural conditions in temperate zones, they come into estrus once a year—in the springtime as the days become longer. The onset of estrus can be accelerated by exposure of the animals to artificially long days during the fall, or by providing short periods of light during the evening hours. Ferrets will then mate and give birth at times unusual for the species. A 14-hr day was found to maximally accelerate the cycle. In a study performed in England, ferrets exposed to light went into estrus in early January while controls were delayed until March or April.

Early onset of estrus cannot be induced by artificial illumination in ferrets which have been blinded (by removal of the eyes or destruction of the retinae), or in animals which have been subjected to pineal gland denervation or pinealectomy. Effects of blinding or pinealectomy cannot be duplicated by severing the main optic tracts, if the retina and pineal are left intact.

Neither the pineal gland nor the eyes are needed for onset of estrus. Blinded and pinealectomized ferrets still go into estrus at fairly regular intervals. The findings are consistent with pineal gland mediation of effects of artificial illumination on the timing of estrous cycles.^{21a}

Repeated injection of either MLT or of 5-methoxytryptophol (MTol) did not restore the ability of pinealectomized animals to respond to increased environmental illumination. MLT was also without influence on timing of estrus in intact ferrets kept under natural lighting conditions. However, MLT did delay onset of estrus in intact animals exposed to long days. The findings are consistent with the concepts that the pineal secretes both progonadotropic and antigenadotropic principles, and that MLT is antigenadotropic in these animals. Evidently, addition of MLT to animals with fully functioning pineal glands is ineffective; but when light reduces the amount of MLT released,

injection of the methoxyindoleamine can delay onset of estrus.

Male short-tailed weasels undergo seasonal atrophy of the reproductive system during winter months, with recrudescence in the springtime. Repeated implantation of MLT in beeswax during summer months was shown to promote atrophy of the testes during summer months, and to block recrudescence of winter-atrophied structures in the springtime. It is not clear whether apparent differences in sensitivity of these animals as compared with the ferrets to administration of MLT is related to differences in species, sex or method of MLT presentation.

Increased environmental illumination can accelerate onset of estrus in the horse, mink, raccoon, hedgehog, cat, rabbit, and vole.⁴² But not all animals with seasonal breeding patterns respond in this way. Sheep and goats usually mate in the fall; estrus in these species can be accelerated by exposure of animals to artificially long nights during the summer months. In different mammals, environmental factors other than lighting (temperature, rainfall, food availability, etc.) may be of greatest importance for adjustment of the timing of reproductive cycles.

Investigators who breed their own rats have long been aware of seasonal fluctuations in the average time required for conception to occur, in numbers of young per litter, in birth weights of the newborn, and in average weights at the time of weaning. The differences are especially marked if mothers are exposed to the small vicissitudes of ambient lighting and temperature which are difficult to avoid in laboratories in which protection against entry of sunlight and against draughts is incomplete. Cyclical changes are encountered even when light and temperature seem to be completely uniform. But seasonal variations in reproduction are subtle in rats; and some births occur at all times of the year. The reproductive system does not undergo obvious regression. Therefore, the rat is not a suitable candidate for studies of the kinds described in hamsters. However, there is abundant evidence for a regulatory role of the pineal gland. Responses of mice are similar and often of greater magnitude.

Unlike the hamster, the rat readily responds to inhibitory influences of exoge-

nously administered MLT, MTol, and other pineal derivatives. Puberty can be delayed by administration of either of the methoxy-indole amines to immature animals. A less pronounced delay can be effected by rearing young animals with intact pineal glands in darkness, or by blinding the animals at an early age.

Underfeeding, premature weaning, and deprivation of olfactory stimuli sensitize animals to pineal gland and light-deprivation influences on the reproductive system. While moderately underfed young rats mature late, animals fed the same amount but also pinealectomized enter puberty at the usual time. Rats that are just blinded or just reared in the dark exhibit only minor reproductive system responses; but the effects of light deprivation are much exaggerated if the olfactory bulbs are excised. Removal of olfactory bulbs alone does not have great influence on reproductive functions of rats exposed to alternating light and darkness.

Increasing the daylength has only minor influences on established ovarian function, if animals are exposed to at least brief intervals of darkness during every 24-hr period. This is not surprising, considering that rats spontaneously go into estrus every 4 or 5 days. Under some conditions, light can abbreviate 5-day cycles to 4; but shortening beyond this has not been achieved.

Changes in appearance of the pineal gland have been directly linked with phases of the estrous cycles; and castration of young animals can lead to pineal gland hypertrophy. MLT injection into the cerebral ventricles on the morning of proestrus blocks the LH surge and ovulation in mice. Rats repeatedly injected with MLT may go into a condition in which ovarian follicles ripen but few if any corpora lutea are formed.

Influences of exogenous MLT on compensatory hypertrophy of one ovary when the other is removed are inconsistent; but both MTol and arginine vasotocin can block or impair the response.

Compensatory ovarian hypertrophy depends heavily upon increased secretion of FSH as well as LH, while LH is primarily involved in ovulation and formation of corpora lutea. For these reasons and others cited below, it has been proposed that melatonin affects mostly LH secretion in

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rats and mice, while 5-methoxytryptophol exerts inhibitory influences on FSH secretion. The concept is supported by many but not all experimental findings. A direct inhibitory influence of MLT on responses to LRF by rat anterior pituitary glands in culture has been demonstrated.⁷⁹

Stereotaxic implantation of melatonin or 5-OH-tryptophol into the median eminence region of the hypothalamus or the reticular formation of the midbrain, and injection of these agents into the cerebral ventricles, all lead to reduction of both pituitary gland and plasma LH concentrations, while implantation into the cerebral cortex or pituitary gland and injection into the hypothalamo-hypophysial portal system are ineffective. Similar studies utilizing either 5-methoxytryptophol or serotonin have led to reduction of FSH. (But there have also been indications that melatonin can under some conditions affect FSH release.) It has been proposed that pineal principles exert their influences through effects on serotonergic neurons of the hypothalamus. Since neonatal androgenization affects preoptic mechanisms regulating LH release (Section V), and the treatment also sensitizes animals to pineal gland influences, the preoptic region of the hypothalamus has been proposed as a target site for melatonin and related agents. But it has also been shown that the pineal influences the content of a fluorescent protein present in cells of the arcuate and ventromedial nuclei which have been implicated in inhibition of gonadotrophin release factor secretion.⁴⁵

In vitro inhibitory influences of melatonin on steroid hormone secretion by the testis, and responses of ovaries of hypophysectomized gonadotrophin-treated rats to melatonin administration raise the possibility of additional actions on the gonads.

The pineal may also affect PRL secretion in rats and hamsters through influences on PIF. Rats exposed to "short days" have large pituitary stores and low plasma concentrations of PRL, while hamsters raised under such conditions have low pituitary PRL content. In hamsters it has been found that implantation of pineal compounds can elevate pituitary PRL but not to the level seen in animals exposed to more prolonged lighting. Therefore, light may affect hamster PRL secretion by pi-

neal-independent mechanisms as well. Direct penetration of light into the hypothalamus has been proposed.

It is evident from the preceding that the pineal gland exerts controls over the reproductive system, but that many questions regarding their nature have yet to be answered.

PINEAL GLAND INFLUENCES ON WATER AND ELECTROLYTE METABOLISM^{21a, 22, 41, 46}

It has been reported that pinealectomy leads to significant lowering of plasma sodium and potassium concentrations,⁴⁶ potassium diuresis,^{21a} and sodium retention.²² The effects are seen only under specified conditions, and seem to be associated with changes in rates of secretion of hormones more directly implicated in the regulation of electrolyte and water metabolism. Neither pinealectomy nor the injection of pineal gland derivatives seems to seriously compromise the ability to maintain circulating blood volume, or to abolish appropriate responses to situations such as salt deprivation or the administration of agents which markedly affect blood pressure.

The pineal gland contains small peptides of the kind synthesized by the magnocellular nuclei of the hypothalamus. There is no evidence that such peptides are released in quantities sufficient to directly regulate renal or hemodynamic mechanisms. On the other hand, pinealectomy affects the supraoptic nuclei, and the effects are reversible with melatonin and pineal extracts.⁴⁵

It is possible that influences on salt and water metabolism assume importance at particular ages or developmental stages, during certain times of the day, or during certain seasons of the year, and that other regulators assume dominance during periods of relative pineal quiescence. It is also possible that influences on reproductive or other functions involve secondary changes in electrolyte metabolism, and that the pineal releases additional humoral agents which help to maintain proper balance. If either of the preceding is true, then removal of the pineal gland would not be expected to lead to profound alterations of body chemistry.

Very large increases in rates of aldosterone secretion have been observed in rats at specified intervals following removal of the pineal gland.^{21a} It has also been reported that pineal extracts can induce sodium retention in intact but not in adrenalectomized rats.²² Additional indications that the pineal may affect aldosterone secretion have been presented in Chapter 11 and earlier in this chapter.

The pineal has been directly implicated in adaptation to cold environments^{21a, 81} and it seems to play a role in release of ADH in mammals. A thermoregulatory role in birds has also been described.^{77, 78} It also seems to be involved in mediation of responses to a variety of stress stimuli associated with activation of the ACTH-adrenocortical system. But many observations are consistent with pineal gland inhibition of ACTH secretion. Some seem to be exaggerated when animals are exposed to darkness; but not all influences of environmental lighting on the ACTH-adrenocortical system can be related to the pineal gland.

Under specified conditions, it has been observed that pinealectomy leads to elevation of plasma corticosterone concentrations, depletion of adrenal ascorbic acid and cholesterol content, and sometimes enlargement of the adrenal gland. Blinding and anosmia lead to impairment of compensatory hypertrophy of one adrenal gland when the other is removed, and the hypertrophy response is restored by pinealectomy. Most of the information was obtained from rat studies, but blinding of hamsters can also impair the compensatory adrenal hypertrophy.

Similarly, it was observed that intraventricular injection of pineal indoleamines can lead to lowering of plasma corticosterone levels, and to reduced ACTH responses to administration of histamine. (Since ACTH responses of reserpine-treated animals were not found to be affected by administration of pineal gland derivatives, it has been proposed that influences are exerted on serotonergic inhibitory neurons.^{21a})

The pineal gland of at least some rodents appears to be more active during times of darkness, while ACTH secretion peaks during the late afternoon. The pineal may participate in regulation of adrenocortical

rhythms. Pinealectomized animals maintain their adrenocortical secretory rhythms and they retain ability to shift the timing in accordance with alterations of photoperiods. There are indications that pinealec-tomy reduces the time lag for readjustment.⁷⁴

Some pineal influences on electrolyte metabolism may be accomplished via modification of thyroid hormone secretion. Thyroid gland enlargement has been described in pinealectomized rodents. This is consistent with pineal inhibition of TSH secretion. But the pineal may also more directly affect the thyroid gland, since it has been reported that thyroids of pinealectomized-hypophysectomized rats show less severe atrophy than those of just hypophysectomized animals. Studies in which measurements were made of thyroid uptake of radioactive iodide, thyroid hormone secretion rates, plasma concentrations of thyroid hormones, or of pituitary TSH content have been variously interpreted as indicating that pinealectomy leads either to increased TSH synthesis or no change. TRF has been identified in pineal glands,⁷² but the significance of the finding has not been established.

Still other kinds of data point to pineal gland influences on the secretion of renin, MSH, prolactin, and STH, all of which have been implicated in regulation of electrolyte metabolism. Some authors have observed that thymus glands are enlarged following pinealectomy¹¹; a possible role of the thymus in electrolyte metabolism is described in Section III and also later in this chapter.

Behavioral Influences

The pineal gland has been clearly implicated in regulation of locomotor activity patterns in birds^{77, 78} and in rats.⁷⁴ There are also indications that it exerts influences on reproductive and maternal behavior.⁷⁴ Beneficial effects of administration of melatonin-free pineal extracts to schizophrenic patients have been described.⁷⁴

THE THYMUS GLAND: STRUCTURE AND EMBRYOLOGICAL ORIGINS^{1, 6, 10, 14, 21b, 23a}

The developmental anatomy of the mammalian thymus gland may provide

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clues to the functions of this still enigmatic member of the endocrine system.

Epithelial components of the thymus take origin from the entoderm (and possibly to a minor extent from the ectoderm) of the embryonic pharyngeal pouches. The latter are hollow at first, but soon growth of solid cords of epithelial cells leads to obliteration of the lumen; and epithelial cells become closely associated with the surrounding mesenchyme. A similar developmental pattern has been described for "more typical" endocrine glands.

Thymic rudiments have been recognized in fishes (which do not develop parathyroid glands) as well as in amphibians, reptiles, birds, and mammals. A "protothymus" has been described in lampreys. Hagfishes have a "primitive" spleen and lymphocyte-like cells. No typical thymus organ is visible, but these animals show some ability to reject homografts and may have an as yet undiscovered thymus-like structure or collections of cells performing thymus type functions.¹

Thymus glands of mammals arise in close proximity to the origins of the parathyroid and thyroid glands and the ultimobranchial bodies.¹⁰ Humans and some others form thyroid-thymus ligaments. It is common for cells of the four types to mingle during their migration to sites occupied in the more mature animal. The results of such mingling can often be demonstrated histologically in adult glands.

Functional associations between the four structures may be established even when anatomical mingling cannot be shown. Calcium metabolism is controlled primarily by parathyroid and ultimobranchial derivatives (Section IV), but is influenced by both the thyroid and the thymus. Thyroid and parathyroid hormones affect thymus gland mitoses, and the thymus may influence thyroid functions.

Parathyroid tissues begins to develop from the dorsal part of the third pouch on each side by the time the human embryo is 5 weeks old. Thymus cells become visible in the ventral regions of the same pouches by the 6th week; some make direct contact with parathyroid rudiments. Additional parathyroid tissue arises from parts of the fourth pouch next to ultimobranchial cells of the fifth. Some thymus cells also appear in the fourth pouch. Thyroid cells undergo early proliferation in the floor of the pha-

rynx and they migrate by the 6th week to a site close to the developing pharyngeal pouch structures. According to some observers, additional thyroid cells originate from the fourth pouch.

As the thymus primordia migrate caudally and medially (and come into close association with the developing thyroid), parathyroid tissue is carried along; but complete separation of thymus and parathyroid cells is effected later in many species.

The main portion of the thymus rudiment on each side fuses superficially with its counterpart, while the extreme caudal part narrows, elongates, and fragments. Most of the fragments deteriorate; but some may form small, independent masses of thymus tissue, while others can be incorporated into the developing thyroid. Clumps of thymus cells may separate from the loosely organized mass during migration. It has been estimated that about 20% of humans have bits of such tissue in widely separated regions such as the jaw, the base of the skull, and deeper regions of the mediastinum. Analogous development in laboratory animals can contribute measurably to the difficulties of performing a complete surgical thymectomy. (Partial thymectomy has proven ineffective in many studies.)

Epithelial components of the thymus rudiments are closely associated with mesenchymal cells, and such association is evidently essential for normal differentiation.

Epithelial fragments will develop in tissue culture if placed in contact with mesenchyme from distant portions of the embryo, and fetal thymus transplants containing only epithelial cells will differentiate in the presence of mature connective tissue of older hosts. While no additional humoral factors seem to be required at early stages, spleen and other tissue may provide stimulation later.

Lymphocyte precursors ("stem") cells originating in the yolk sac migrate first to the liver and later to the mesenchyme of the pharyngeal pouches. (Earlier suggestions that epithelial components can give rise to lymphocytes were based on appearance of such cells in cultures of embryonic thymus epithelium⁶; it is now believed that the epithelial tissue contained undifferentiated and unrecognized stem cells which

had migrated in prior to removal of the samples for culture.

Lymphoblasts can usually be identified within the thymus before they appear in lymph nodes, spleen, or blood. They are dependent upon the thymus for early development. Early removal of thymus rudiments leads inevitably to severe reduction of the lymphocyte population of the blood and thoracic duct lymph, and failure to establish lymphocyte colonization of "thymus dependent" cortical regions of lymph nodes and perivascular portions of the spleen. Cultures of embryonic spleen and bone marrow do not develop a lymphocyte population if tissues are removed before the thymus gland has exerted its influence. A syndrome has been recognized in human infants with congenital absence of the thymus gland. Such infants exhibit (among other things) severe reduction of blood and tissue lymphocyte populations.

As the thymus continues its development, lymphocyte numbers increase dramatically because of a combination of inward migration of mesenchymal stem cells and profuse proliferation of resident cells. It soon becomes possible to distinguish populations of large, medium and small lymphocytes, with the last predominating.

Epithelial cells send out processes to join with those of neighboring cells, and a continuous network is formed. (The term *reticulum* has been applied to the epithelial lattice; but it may generate confusion, since the term *reticular cell* designates mesenchyme-derived phagocytic cells which appear later.)

The network becomes filled with lymphocytic elements, while mesenchymal cells surround the developing structure. There is both histological and functional evidence for later establishment of a continuous epithelial barrier between lymphocytes and the blood vessels that arise in the thymus interior. (Thymus lymphocytes at a later stage respond to intrathymic but not to intravenous injection of antigens.)

With further development, the periphery (cortex) becomes heavily populated with small lymphocytes, while the more vascular interior (medulla) contains small numbers of mostly large lymphocytes. The divisions are obvious under low magnification, since the cortex contains 2-3 times as

many lymphoid cells, while epithelial cells of the medulla contain more cytoplasm and exhibit staining reactions different from those of the cortex. But usually there is no clear line of demarcation. Later, medullary lymphocytes show evidence of living longer, and exhibit greater resistance than those of the cortex to radiation injury and to hormonal influences.⁵

The histological appearance of the medullary epithelial cells is consistent with synthesis and secretion of humoral substances which find their way into the circulation; and radioautographic studies on thymus glands of very young mice support the concept. The remarkably high rate of mitosis of cortical lymphocytes in direct contact with epithelial components is also consistent with *localized* (intrathymic) release of humoral regulators by cortical epithelial cells.^{21b}

Concentric arrangements of epithelial cells (Hassall's corpuscles or bodies, thymic corpuscles) form in the medulla of most species. They vary in size and number. The simplest ones consist of one or two flattened cells surrounding a more rounded one, while others are large and multicellular, with arrangements that resemble the concentric layers of an onion. Cells within the interior of the corpuscles degenerate and become hyalinized, cystic, or calcified. Keratinization of outer layers has been attributed to mechanical pressure and deprivation of capillary blood supply.

The corpuscles usually first appear soon after the thymus has become obviously colonized with lymphocytes (around the age of 3 months in human fetuses), and their numbers increase rapidly for a time. Some authors regard them as sites of localized degeneration, but others believe they are regions of especially high activity. Increased rate of formation has been described in young animals when the latter are exposed to stress or given thyroid hormones, and also after radiation of the thymus. The thymic medulla contains an acid mucopolysaccharide which stains strongly with PAS. Glucocorticoids (released during stress) retard release of the material from the thymus, thereby favoring its accumulation in cystlike formations. The corpuscles seem to be formed soon after discharge of the PAS-staining material. Accumulation of a substance with

similar chemical properties has been observed in the plasma of intact rats during the thymus-regeneration phase which follows administration and then withdrawal of glucocorticoids. (No rise in plasma content of this substance has been found in glucocorticoid-treated thymectomized rats.) Hassall's corpuscles have not been found in healthy rats, while they are almost always seen in mice and most other laboratory species, and also in humans.

Myoid cells with striations similar to those of skeletal muscle are present in the medulla,⁷⁰ usually close to the Hassall's corpuscles.⁶

Connective tissue derived from mesenchyme forms a complete capsule in the fully formed gland, and separates right and left lobes (which may function independently). The connective tissue penetrates the cortex to the level of the medulla, dividing the gland into lobules. Small and large animals show differences in number rather than in size of lobules. Efferent lymphatic vessels coursing through the interlobular connective tissue of the cortex drain into mediastinal and tracheobronchial lymph nodes. There is no afferent lymphatic supply. "Germinal centers" resembling those of lymph nodes have been found in thymus glands of animals exposed to certain infectious agents. Some authors believe they do not form in normal thympuses, while others report regularly finding a few in thymus glands of young animals.

The inferior thyroid and internal mammary arteries send branches to the thymic cortex. Venules within the medulla empty into thyroid and innominate veins and into the superior vena cava.

The thymus of most mammals is located largely or entirely within the mediastinum. In rats surgical removal requires cutting of the sternum to the level of the second or third rib and exposure of the thoracic cavity to atmospheric pressure. In infant mice, the thymus can be removed more easily by aspiration.

The thymus assumes a cervical position in some mammals, e.g., guinea pigs. Australian marsupials such as the quokka have both cervical and thoracic glands.

The spleen of most mammals becomes lymphoid much later than the thymus, and

true lymph nodes appear after the spleen has differentiated. In humans the thymus is lymphoid by the end of the 3rd prenatal month and the spleen by the 5th, while lymph node differentiation may not be completed before the end of the 1st postnatal year.

INVOLUTION OF THE THYMUS GLAND^{6, 8, 12, 14, 22c}

Age Involution^{22a, b}

Extensive human autopsy data indicate that the thymus gland attains its maximal *organ weight: body weight* ratio during late fetal or very early postnatal life, that it is large during early childhood, and that a process of "age involution" begins around the age of 4 years. Growth of the gland does however continue during childhood, so that maximum *absolute* weight is seen around the time of puberty. From then onward, absolute size declines, lymphocyte numbers fall off even more rapidly, and active elements appear to be partially replaced by adipose tissue which accumulates in the interlobular septae and gradually invades the cortex. In aging humans, the ratio of cortical to medullary tissue becomes sharply reduced; but medullary components also undergo some atrophy.

Increased numbers of lipid-rich "foamy cells" appear in the cortex, while epithelial cysts line by ciliated or mucous type cells develop in the medulla. Mast cells (usually seen only in interlobular locations of young glands) may also accumulate within the medulla.

Since many of the common laboratory animals are born after relatively short gestation periods, it is not surprising that reported timetables for growth and involution are different from those of the human, and rapid thymic growth during the first few post-natal weeks has been repeatedly described.

Large numbers of early studies of thymic influences on growth and maturation were based on the concept that the gland functions importantly only early in life when it is relatively large. It has been proposed that the young thymus gland produces both growth-promoting and growth-inhibiting factors, and that something (including a substance named *infertine*) protects

against premature onset of puberty. (The belief that infertine is a thymic hormone was later questioned by many, including those making the original suggestion.)

Hormonal, "Accidental," and Nutritional Involution³

At all ages, the thymus gland responds rapidly to several hormones. Within 24 hr after administration of glucocorticoids, it shrinks to a small fraction of preinjection size. (Reductions by up to 90% can occur in young glands.) A similar effect is achieved with administration of ACTH or by exposure of animals to any of a wide variety of stress stimuli, provided the adrenal cortex is present.

Actions of glucocorticoids on the thymus gland were described in Chapter 7. Lymphocyte depletion results from destruction of existing cells and also through inhibition of mitosis. (An early suggestion that glucocorticoids promote outward migration of lymphocytes has been discounted.)

The thymus is exquisitely sensitive to X-irradiation. It also involutes rapidly when animals are exposed to a wide variety (but not all types) of infectious agents. Glucocorticoids play a role in involution related to infection.

Since young thymus glands contain relatively larger numbers of lymphocytic components, changes are more obvious. Responses to glucocorticoids are so predictable, they form the basis for bioassay methods.

Hormonal influences are specific.^{3, 14, 59a} Involution follows administration of estrogens and androgens as well as of glucocorticoids. But deoxycorticosterone (a mineralocorticoid) and progesterone may promote thymus gland enlargement. Histological studies indicate that responses to estrogens differ qualitatively from responses to glucocorticoids.

Hormonal influences are also additive. Greater involution is seen after administration of submaximal dosages of a combination of glucocorticoids with sex steroids than when the same dose of either is given alone. Withdrawal of naturally occurring hormones leads to thymus gland enlargement; heavier glands are found in animals subjected to combined adrenalectomy-gonadectomy than in those suffering removal of only one source of steroid hor-

mone. And testosterone can promote thymic involution in a gland that has enlarged following adrenalectomy.

Thymus glands also shrink when nutrition is suboptimal (in animals receiving inadequate quantities of balanced diets, or those offered diets which satisfy caloric needs but are deficient in essential components). Acute changes in thymus glands following food deprivation of previously well nourished animals are similar to those seen after glucocorticoid administration. Glucocorticoid hypersecretion seems to be involved in responses to food deprivation; and the presence of pycnotic nuclei and signs of necrosis indicate cell destruction. Chronic undernutrition leads to delayed impairment of new cell formation; influences of inanition on adenohypophysial hormone functions, and especially on secretion of STH and TSH, play a role.

All of the described conditions (hormones, nutrition, aging, stress) affect cortical lymphocytes to a far greater extent than medullary epithelial components. But while hormonal and stress effects are rapidly reversible in young animals (and thymic weight can be fully restored within 2 days after withdrawal of the condition), age changes tend to be progressive. Transplantation of the thymus gland of an aging animal to a young recipient does not lead to "rejuvenation" of the old thymus, although the latter retains the ability to respond to host hormones. Glands of infant animals can grow in older hosts whose own thymus is undergoing involution.⁶

Thymus restoration after chronic underfeeding may be less complete than after acute effects of hormone administration. The nutrition-deprived thymus may become more responsive to aging influences. But hormones do not affect timing of age involution.

Lymphoid tissues outside the thymus gland respond in similar fashion to hormones affecting the thymus; but they appear to be far less sensitive. Some reduction of blood lymphocyte levels has also been found after exposure of adrenalectomized animals to very mild stresses. This might indicate that very small changes can be effected in the thymus which are not mediated via the glucocorticoids; alternatively the effect could be unrelated to thymic physiology, since it may involve redistribution of lymphocytes rather than reduction of their numbers.

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How Extensive, Universal, and Significant is the Phenomenon of Age Involution?

Those who fully subscribe to the concept that the *function* of the thymus declines rapidly after puberty usually agree that the *absolute* weight of the gland varies over a wide range in normal individuals of the same age, and that the weight rarely if ever declines in middle age and beyond to less than half that seen around the time of birth (although gland:body weight ratio falls off significantly and adipose tissue content is increased). The thymus gland in old age continues to be a defined, encapsulated structure containing substantial numbers of metabolically active cells.

In some laboratory species for which extensive data are available, thymus glands of animals well past their "prime" are often three times as heavy as those of weanling animals. (The reduction of gland:body weight ratio is partly attributable to the large gain in nonthymus body weight; the ratio may be higher in healthy, lean, reproductively active young adults than at the time of onset of puberty.)

According to some observers, concepts of the magnitude of age involution may be distorted because of the manner in which data are collected. The effects of stress, infection and undernutrition were mentioned. It has been pointed out that conclusions about age involution in the human are largely based on comparisons of autopsy material of older persons with those from children and adolescents. Older persons are usually examined following prolonged periods of chronic illness, in which the disease itself, poor nutrition, and drugs have led to "accidental" thymus gland involution. On the other hand, most very young people come to autopsy because of unexplained death of a previously apparently healthy individual. Causes of death in the young include acute poisoning, accidents, and rapidly fulminating infections. Some childhood diseases lead to thymic enlargement. Limited data on sudden, accidental death in older persons point to a lesser of (but still real) "age involution".

Distortions may also arise from selection of certain animal species. The mouse is an excellent subject for many kinds of studies on the thymus gland; and aging mice usually have very small thymuses. On the

other hand, healthy, well-fed rats of at least some strains retain considerable masses of apparently metabolically active thymus tissue almost to the time of natural death, provided that they are protected against stress. Therefore those who work exclusively with mice may draw different conclusions from those working exclusively with rats.

Relationships Between Endocrine Gland Weight and Hormone Function

General Considerations. The weight of an endocrine gland is often a poor indicator of its functional state. Thyroid glands stimulated by TSH, and adrenal glands stimulated by ACTH, are large by comparison with structures not subjected to such influences. But glands that store their products can enlarge when hormone *release* is inhibited. Gland weight can also be increased by the presence of large numbers of nonfunctioning cells (as in some kinds of endocrine tumors). Thyroid glands undergo marked hypertrophy when iodine deficiency or administration of goitrogens impairs ability to synthesize thyroid hormones.

It is possible that some weight changes in the thymus cannot be directly related to function. Cell death rate is accelerated at times when cell proliferation is stimulated. In well nourished young animals, larger thymus weights may be related to storage of reserves, or to influences of STH on nonhormonal components.

Can Small Thymus Glands Be More Active Than Larger Ones? The possibility that a smaller thymus gland may be more active than a larger one is suggested by the finding that the glands of anencephalic fetuses are enlarged, and that normal size can be restored by administration of hormones believed to be secreted by the pituitary glands of healthy fetuses. (The presumption is that the normal condition is the more active, but proof of this is lacking.)

The very rapid involution in response to stress and to steroid hormones is also consistent with the concept that the "involved" gland is active. It has been proposed that destruction of lymphoid components leads to release into the bloodstream of nutritional or regulatory molecules

which are especially useful to peripheral organs in times of stress or augmented activity of the reproductive system (see also Chapter 7). Another suggestion is that destruction of lymphoid components removes an otherwise present inhibitor of thymus epithelial cell function. (The concept of the presence of a tonic internal "brake" on hormonal function has been applied to other endocrine glands; e.g., some believe that ascorbic acid serves this purpose in "resting" cells of the adrenal cortex.)

There are reasons to believe that the small thymus glands of older (nonsenile) individuals are highly active, rapidly responsive structures, even after some adipose tissue has accumulated. Such glands consistently enlarge following adrenalectomy or gonadectomy, and exhibit additive responses to combination of the procedures. Thymus glands of adult rats also enlarge following administration of PRL (and in the presence of a PRL-secreting tumor) and will usually show a similar response to chronic administration of STH, parathyroid hormone and doses of thiouracils sufficient for stimulation of TSH secretion without complete suppression of thyroid hormone synthesis. (Thymus gland enlargement is a common finding in human adults suffering from hyperthyroidism.)

Thymus glands of older adrenalectomized, gonadectomized, or doubly operated animals may be smaller than those of similarly treated younger animals, but they are substantially larger than those of unoperated controls of the same age. "Age involuted" thymus glands undergo further reduction in size in response to stress or to administration of androgens, estrogens, or glucocorticoids. Such observations are consistent with lifelong maintenance of active function.

Hormonal secretion is widely believed to be accomplished by epithelial components, and the latter predominate in glands subjected to involution of all types. Many effects of thymectomy can be reversed by implantation of thymus fragments containing only epithelial cells, or by implantation of glands enclosed within diffusion chambers with pores large enough to permit egress of molecules but not of cells. (Only epithelial cells and fibroblasts

remain viable in such implants.) It has been reported that cortex-depleted glands of older cattle contain larger quantities of an active principle (thymosin, see below) than do the lymphocyte-rich glands of young calves. Medullary epithelial cells of patients with myasthenia gravis are believed to synthesize and secrete another thymus derivative, *thymin* (*thymopoietin*).^{6, 10} When doses of ACTH or of glucocorticoids sufficient to promote thymic involution are given to such patients, disease symptoms may be temporarily exacerbated; this has been attributed to accelerated release of *thymin*. In an animal study, it was reported that the thymic hormone *content* is elevated in postcastration hypertrophy, but that the gland *releases* the hormone to lymph nodes and spleen more slowly.¹¹ Since lymphocytic elements can account for up to 90% of the volume of young thymus glands, marked reduction in size in response to stress is not necessarily indicative of epithelial component depletion.

The possibility that animals that have attained a certain age or state of maturity require some thymus-derived hormone which younger animals need only during times of stress, is worth considering. The need for the hormone could also change with onset of active function of the reproductive system.

Another thought is that lymphocytic functions lead to a buildup of long-lived medullary cells, and that the need for such activity declines when sufficient numbers of cells have accumulated. A very reduced cortical volume may suffice for maintaining the activities in the adult, whereas a large volume is required for the build-up during youth. A further, and perhaps conflicting concept is that some of the disorders associated with aging result from diminished availability of cortex-derived humoral factors. There have been suggestions that older persons might benefit from administration of thymic extracts. As a general rule, attempts to combat aging processes through administration of hormones have been notoriously unsuccessful since either the aging process is unrelated to hormonal imbalance, or else older tissues tend to become unresponsive to administered preparations. But the fact remains that older individuals show re-

duced tendency to reject foreign cells and greater susceptibility to tumor development. Agents which destroy thymus gland cells retard rejection of organ transplants.

Reasons for Considering That There Is No Relationship Between Thymus Weight and Thymus Gland Function. Unlike "typical" endocrine glands, the thymus does not seem to be regulated by negative feedback inhibition.⁶⁻¹⁴ Animals that have been partially thymectomized seem to function normally, and it has been stated that thymic remnants do not undergo compensatory hypertrophy. Transplantation of large numbers of thymus glands to mice does not seem to lead to involution of the host thymus, even when all transplants develop a blood supply and remain viable. Some increase in numbers of circulating lymphocytes has been described; but this does not seem to adversely affect the animals.

Thymus functions may be internally regulated, and it is possible that each lobule operates as an independent unit. The regulation could involve a balancing of the quantities of humors with opposing biological actions, and also stimulation of release of extrathymic physiological antagonists.

It has been independently reported from different laboratories that large doses of thymic extracts can exert inhibitory influences on the very functions which are stimulated by smaller doses.¹⁵ Two kinds of explanations have been advanced: (1) biphasic actions of a single hormone, and (2) the existence in extracts of minute quantities of very potent inhibitors whose presence cannot be detected until a certain threshold dose has been administered.

One laboratory has described the presence within the thymus gland of *thymosin* which stimulates the lymphatic system in specific ways and of *thymostatin* which opposes its actions. A potent *antithymosin* has also been obtained from blood sera of thymosin-treated animals. Another laboratory has reported on electrophoretic separation of a powerful inhibitor from preparations of a lymphocyte-stimulating hormone (LSH) derived from thymus glands. All of the preceding seem to be peptides or proteins.¹³

Thymosterin, which seems to be a steroid that differs chemically from adrenocortical and gonadal hormones, has also been

prepared from thymus tissue and is said to inhibit certain thymic functions. While *promine* and *retine* are no longer believed to be thymus hormones, they can be obtained from thymus tissue and shown to exert opposing influences on tumor growth. Additional preparations have been described which elevate and others which lower serum calcium concentrations and exert opposing actions on bone; and still other extracts have been reported to either increase or decrease blood glucose concentrations or to stimulate or depress certain reproductive processes.¹³

INFLUENCES OF THE THYMUS ON THE LYMPHOPOIETIC SYSTEM^{1, 20, 74}

Chemotaxis

As noted above, lymphocyte precursors (stem cells) migrate at a very early stage from the embryonic yolk sac, pass through the liver, and then enter the mesenchyme of the thymic primordia. The existence within the mesenchyme of a factor which attracts the stem cells is consistent with observations that lymphocytes are identifiable in the thymus tissue of most species before they can be detected in spleen or bone marrow, with later continuous entry of lymphocytes from bone marrow and other sources, and with host colonization of thymic grafts. Chemotactic influences of thymus gland derivatives, including a preparation of purified glycoprotein (homoeostatic thymus hormone, HTH) have been described.

Only those stem cells that will become lymphoblasts seem to accumulate in embryonic thymus. It is possible that stem cells undergo early changes in the liver, and that only those committed to the lymphoblast line acquire the ability to respond to the attraction afforded by the thymic rudiment. An alternate concept is that all hemopoietic stem cells are attracted to the thymus, but only those that will become lymphoblasts are retained; or that any stem cell which happens to come under thymic influence tends to develop into a lymphocyte precursor.

Stem Cell Differentiation^{5, 9, 13, 14}

Early destruction of thymic rudiments permanently impairs development of the

lymphocytic system. An inductive influence of thymic epithelial cells is consistent with experimental findings.

It is widely believed that stem cells are "pluripotential" at some stage; *i.e.*, they are capable of giving rise to erythroid and myeloid as well as to lymphoid elements. Since healthy thymus glands do not ordinarily produce erythrocytes or granulocytes, it has been proposed that the thymus synthesizes one or more substances which inhibit differentiation of erythroid and myeloid cells. It has been reported that erythroid and myeloid cells proliferate more rapidly in spleen tissue if the thymus is removed. There are also some incompletely understood associations between certain forms of thymoma and either hypoplastic anemia or granulocytopenia.⁶

Some of the stem cells will eventually develop into T-cells (see below), and at some stage become *prethymic cells*. The site and timing of the commitment to such development has not yet been established; the process could occur in embryonic liver. Since "committed" cells have been found in athymic nude mice, it has been proposed that the stages leading to commitment occur outside the thymus gland.^{7*} However, there are those who believe that athymic mice possess functioning thymus tissue for a limited but sufficient period during early development to provide the conditions necessary for commitment.

While the thymus is the earliest site for differentiation of lymphocytes in the embryo, it is not the exclusive site. Some stem cells seem to go directly to other tissues, and undergo development into B-type cells (see below) even in thymectomized animals. It has been reported that lymphocytes can be detected in the lymph nodes of cats before they are seen in the developing thymus.¹

Lymphocyte Proliferation^{6, 8, 13, 14}

Within the Thymus. The thymus gland provides an environment highly conducive to lymphocyte proliferation. A remarkably high rate of mitotic activity (estimated to be 5–10 times greater than that for lymphocytes within lymph nodes) persists throughout most of the life span of the individual.

Epithelial cells of the cortex may either

produce or accumulate a locally acting stimulant, since lymphocytes in contact with such cells multiply profusely. The stimulant may actually arise in the medulla; but mitotic rates within the medulla are low.

Outside the Thymus. The thymus gland seems to export an agent which promotes lymphocyte proliferation in lymph nodes, bone marrow, and spleen. A number of preparations from thymus glands have been found to exert such actions. Medullary extracts are highly potent, and there is reason to believe that a hormone is produced by medullary epithelial or "reticular" cells.

Several peptides have been described which differ from each other in such properties as heat stability, carbohydrate and lipid content, number of SH groups present, and apparent molecular weight. The preparations most intensively studied include lymphocyte-stimulating factor (LSF) and *thymosin*. The latter has been shown to rapidly increase protein, RNA, and DNA synthesis in lymph node lymphocytes; its effects can be antagonized by administration of antithymosin sera.

Reports from different laboratories suggest that the active agent is a sulfated mucopolysaccharide, a glycoprotein, or a component of thymus gland lipid fractions.^{13, 14}

Thymus gland extracts, and glands enclosed within diffusion chambers can partially restore the depleted lymphocyte population seen after thymectomy. Extracts of other tissues (including those from lymphatic tissue outside the thymus) are ineffective. (But it has pointed out that lymph node cells of animals past the neonatal stage can be stimulated to respond to antigens and nonhormonal agents, and that the possibility of such stimulation must be considered when interpreting evidence for lymphocyte proliferation.) It is not known whether thymic influences are accomplished by one or perhaps several different humoral factors.

Lymphocyte Migration^{6, 8, 13, 14}

There is good evidence that something like 95% of thymic lymphocytes of laboratory rodents past the perinatal stage sur-

vive no longer than 3-4 days. Most cells die within the gland; but others migrate out via diapedesis, enter the circulating blood, and travel to "colonization sites" of the lymph nodes and spleen. The numbers emigrating are sufficient to contribute substantially to the total body lymphocyte pool. (Thymectomy of adult animals leads gradually to a lymphocyte depletion which cannot be corrected by administration of thymic extracts that reverse other thymectomy defects.)

The supply of lymphocytes from the thymus is proportionately greater in fetal and perinatal animals. Neonatal thymectomy of some species can lead to up to 90% reduction of blood lymphocyte counts, up to 97% reduction of thoracic duct lymphocyte populations, and to failure to colonize "thymus-dependent" paracortical and extrafollicular regions of lymph nodes and periarteriolar and perifollicular regions of the spleen.

In animals in which bone marrow function has become established, chromosome marker studies indicate that the bone marrow supplies stem cells to the thymus, and that such cells and their progeny leave the thymus to take up residence in other lymphatic tissues. Bone marrow cells also travel to and settle in thymic grafts.

Glycoproteins on the surfaces of lymphocytes seem to play a role in determination of the site to which the cells migrate. Differences have been found between those which tend to go to the spleen as compared with those that enter the lymph nodes.

It has been proposed that the thymus gland possesses mechanisms for recognition and destruction of lymphocytes which have the potential for damaging normal body tissues, and that healthy thymus glands engage in "immune surveillance" through production of enormous numbers of mutant cells and selection of those suitable for survival and export. Products released by destroyed lymphocytes may participate in local regulation of thymus gland functions.

T Cells^{18, 20, 22, 76}

Immunologists recognize the existence of at least two major populations of lymphocytes which have similar appearance under the light microscope, but which differ in functions, surface properties and condi-

tions required for maturation.

Thymus-dependent or *T cells* require the presence of the thymus gland for development of "immunocompetence" (the ability to respond appropriately to antigenic stimulation). They are most directly involved in *cellular immunity*, i.e., immune responses mediated by sensitized cells but not by antibodies which circulate in the blood plasma. (However, subpopulations of T cells are also recognized and at least some types affect B cell functions, see below).

Animals deficient in T cells have impaired ability to cope with viral, fungal, protozoal, and certain of the bacterial infections, they do not display the usual delayed hypersensitivity responses (e.g., tuberculin reactions which first become visible 24 hr after administration of test substances to individuals previously exposed to the microorganisms), and they may be unable to reject grafts of foreign tissue (including skin grafts from other species). They are also more susceptible to certain oncogenic (tumor-producing) viruses and to carcinogenic chemicals.

T cells seem to participate in protection against growth of certain kinds of tumors. Mechanisms for recognition and rejection of spontaneously arising mutant cells may be similar to those needed for rejection of grafts of alien tissues.

Effects of T cell deficiency cannot be corrected by administration of blood plasma or serum from normal animals of the same strain, or by injection of γ -globulins. Some transient restoration has been achieved through presentation of very large numbers of thoracic duct lymphocytes, still larger numbers of blood lymphocytes or enormous quantities of thymocytes. The short-term effectiveness is consistent with the belief that T cells must be continually renewed.

Undifferentiated but committed *pre-thymic cells* (prothymocytes) seem to come under the influence of a thymic inducer or hormone which some believe is present within the cortex of the gland. The cells become transformed into *immature thymus cells* that are immunologically incompetent. The process involves changes in cell surface properties so that specific antigens can be recognized. (Surface antigens of mouse thymus cells have been designated Thy-1 or θ , TL, and Ly, and

sets of genes coding for their formation have been described. Related antigens are found on the surfaces of immature T cells of other species including humans.) It is not known whether the antigens are synthesized at this time, or whether changes in cell properties lead to their appearance at the cell surface.

Athymic nude mice do not have immature thymus cells; but they have lymphocytes which can undergo the transformation *in vitro* if exposed to cAMP, thymosin, thymopoietin, endotoxins, and certain other agents. Evidently cell division is not involved. While cAMP (and possibly also PGE₁) has been implicated in the process, the thymic influence seems to be specific for prethymic cells. The other agents affect lymphocytes of different types as well. The thymus may play a role in generation of cAMP. c-GMP also affects the process, while excessive amounts of cAMP are inhibitory.

Immature T cells have relatively large quantities of θ -antigen at the surface, and cells of most strains also have the TL antigen. (TL negative strains have been developed.) The cells are immunoincompetent and sensitive to the destructive effects of cortisone.

Mature, competent, cortisone-resistant cells have been identified in the medulla of the thymus gland. A factor in the medulla may promote conversion of immature to mature types. The process involves surface changes, including disappearance of the TL marker, and reduction in available theta antigen. Mature and immature cells can also be separated by differential centrifugation.

It is possible that some prethymic cells bypass the cortisone-sensitive stage and become directly transformed into immunocompetent or mature thymic cells. There are indications that prethymic cells are not all alike; certain ones seem to be destined to become future "helper" cells while others become "suppressor" cells.

After leaving the gland, postthymic cells evidently undergo at least one more change during which they develop sensitivity to certain antigens. A circulating thymic humoral factor has been implicated. Its relationship to factors promoting conversion of precursor cells to mature thymus cells is not yet clear. Thymosin may act at both sites, while there are

indications that thymopoietin acts predominantly within the gland.

Following appropriate immunological stimulation, responsive postthymic cells are transformed into large, proliferating *blast cells*. (Blast cell formation can be experimentally induced by administration of a number of plant-derived "mitogens" such as phytohemmaglutinin (PHA) and concanavalin A (Con A); differences in actions have been noted.) Diverse cell types seem to be involved in mediation of cellular immunity and related phenomena. They have been named according to the type of actions observed: killer, memory, helper, suppressor, and effector. At least some attach to target cells by means of small cytoplasmic processes or *uropods*, and it is suspected that they inject materials through such processes.

Certain actions of T cells have been directly linked with release of *lymphokines*. The presence of such agents can be demonstrated 4–6 hr after antigenic stimulation. Production can be impaired or delayed by administration of puromycin.

The lymphokines are probably not released into the bloodstream under physiological conditions, but their presence in culture media is seen after cells are exposed to mitogens. Described factors include a lymphotoxin (LT) which is capable of killing a variety of cells, a *macrophage chemotactin* which stimulates migration of phagocytic cells to sites of target cells, a *macrophage activation factor* that enhances macrophage motility and phagocytosis, and a migration inhibitory factor (MIF) that immobilizes macrophages *in vitro*. It is believed that activated T cells can stimulate other competent T cells via release of a *mitogenic factor* after attracting them with a *lymphocyte chemotactin*. In addition, there has been discussion of the existence of a *transfer factor* obtained from human lymphocytes which can induce sensitivity to tuberculin, diphtheria toxoid, and certain other antigens when injected into recipients. Lymphokines do not behave as antigens. Although they seem to be proteins, they are not chemically related to antibodies.

Animals with T cell deficiency can have near normal titers of *circulating* antibodies and adequate numbers of plasma cells. (The number of plasma cells and reticular elements may actually increase in lymph

node areas normally occupied by T cells.) Responses to certain bacterial antigens (e.g., tetanus toxoid or *Pneumococcus III* capsular polysaccharide) can be complete; but antibody formation in the presence of some other antigens (sheep erythrocytes, bovine serum albumin) is defective, and the titer of at least one immunoglobulin is reduced.

Clinical conditions are known in which development of the thymus gland is incomplete, and there is defective T cell function.¹ When development of the third branchial pouch is abnormal, the condition may be complicated by parathyroid hormone deficiency.

There are unresolved questions concerning whether T cells must actually *reside* for a time within the thymus gland, or whether they can differentiate elsewhere under the influence of a humoral factor secreted by the thymus. When thymectomized animals are given grafts of lymphocyte-containing thymus tissue from animals of the same strain, complete restoration of cellular immunity is achieved. The restoration may also be complete when thymus epithelial tissue of the same species (but of different strain) is retained by the host and is populated by host lymphocytes. However, only partial restoration has been observed after administration of any known cell-free extract. Thymus glands enclosed in Millipore chambers which permit release of humoral agents but not of cells, are effective for only limited periods of time, and it has been proposed that they affect only preexisting postthymic cells. In particular, thymus gland deprivation impairs the ability to respond to certain antigens; and if extrathymic lymphatic tissue is depleted at the time of implantation of a chamber-enclosed gland, lymphocyte numbers do not return to normal levels.

Most observers accept the concept that T cells require the inductive influence of the thymus itself plus one or more additional humoral factors released by the gland. A competence-inducing factor (CIF) present in lymph nodes and spleens of intact, but not of thymectomized animals has been described.

Animals thymectomized at weaning or older age are usually normal in appearance and they display cellular immunity reactions. It is believed that such animals re-

tain a population of long-lived T cells, but that they are unable to form new ones. Evidence for this arises from development of T cell deficiency several months after thymectomy in rats, and inability of the animals to cope with destruction of pre-existing cells (by X-irradiation or administration of ALS or cyclophosphamide).

It is not yet known whether thymic humoral factors promoting lymphocyte mitosis are identical with those promoting maturation of T cells. Some purified preparations said to contain a single active principle seem to provide for both functions.

B Cells^{18, 20}

Thymus-independent or *B cells* are required for *humoral immunity*, i.e., for functions dependent upon ability to release antibodies into the circulating blood. When appropriately stimulated by specific antigens, competent B cells become transformed into *plasma cells* which synthesize and secrete large quantities of specific gamma globulins into the bloodstream. The blood plasma of animals so stimulated can be used to passively immunize other animals against the same antigens.

Antibodies protect against bacterial and viral invaders in several ways. They can combine with antigens on their surfaces, and bring about clumping (agglutination) of microorganisms, can immobilize them by combining with flagellae, and can reduce penetrating power of some of the viruses. Antibody combination with some of the bacterial toxins leads to "neutralization" of the toxins. Changes in surface properties of microorganisms can render the latter more susceptible to engulfment by phagocytic cells. Interactions between antibodies and some of the antigens lead to "fixation" of plasma components known collectively as "complement." Accumulation of complement on surface membranes of micro-organisms can lead to cell lysis.

While most B cell functions are beneficial to the organism, attachment of antibodies or of antigen-antibody combinations to previously healthy tissue can lead to cellular injury, inflammation, and immediate hypersensitivity reactions. Anaphylactic reactions, vascular injury (Arthus reaction), hay fever, and some forms of glo-

merulonephritis are among the conditions arising from such phenomena. Some involve release of mediators such as histamine, kinins, and slowly reacting substance (SRA).

Animals with B cell deficiency have reduced plasma concentrations of gamma globulins, and impaired resistance to most bacterial infections and to viral reinfections. But they may retain full ability to reject foreign tissues. A mouse-specific B lymphocyte antigen (MSBLA) has been identified on surfaces of mouse B (but not T) lymphocytes; and similar antigens are known to be present on B cells of other animals.

Clinical conditions are recognized, in which B cell functions are inadequate, and there is a deficiency of plasma cells and of plasma immune globulins, but in which T cell functions are retained.^{1, 8, 9} (There are other clinical conditions associated with problems of stem cell differentiation, in which both B and T cell functions are seriously impaired.)

Because of differences in surface charge, B cells can be separated from T cells by electrophoresis. They can also be selectively destroyed by agents that do not affect T cells.

In chickens and other birds, maturation of B cells requires the presence of a small, sac-like structure at the posterior end of the gastrointestinal tract known as the *bursa of Fabricius*. The latter is a lymphoepithelial organ attached to the cloaca by a short stalk. It develops from the hind-gut epithelium at about the time that the thymus gland becomes obviously lymphoid; but the bursa does not accumulate lymphocytes until later. (In chick embryos the thymus is recognized as an epithelial organ around the 9th day after fertilization, and it becomes lymphoid by the 12th day; epithelial components of the bursa are visible by the 12th day, but lymphocyte colonization takes place during days 15-18, while the spleen becomes lymphoid close to the time of hatching at 21 days.) The bursa involutes after attainment of sexual maturity. Birds do not develop true lymph nodes.¹

Humoral immunity can be impaired in birds by performing a surgical bursectomy, or by exposure of the bursal region to X-rays. In bird embryos, it is also possible to destroy the bura without seriously dam-

aging the thymus gland, by injection of a large dose of testosterone. Cellular immunity is retained in bursectomized birds.

It is difficult to surgically remove the thymus of chickens, because many lobes are scattered over a wide area; but the thymus can be destroyed by radiation. After thymus gland destruction, T cell (but not B cell) function is impaired. Complete disruption of development and function of the immune system can be brought about by destruction of both bura and thymus.

The equivalent of the bura in other vertebrates is not known. Cartilaginous fishes have a rectal gland, and turtles have an anal sac, which may perform analogous functions.

It has been proposed that some combination of lymphoid structures outside the thymus collectively performs bursal functions in mammals.¹ The candidates include the Peyer's patches of the intestine, the tonsils, appendix, and spleen. Theoretical objections have been raised to each, because they develop relatively late and they differ from both the bura and thymus in that they contain cells directly responsive to antigens reaching them via the blood circulation. Moreover, they do not involute appreciably during aging or after administration of low doses of hormones. In rabbits, greater impairment of the immune system has been observed if appendectomy is combined with thymectomy than after just thymectomy; and still greater deficiency results from additional destruction of the tonsils and Peyer's patches.

Another possibility is that the embryonic liver of mammals exerts influences leading to B cell differentiation before the cells reach extrathymic lymphoid tissues. Since the bura equivalent remains undefined, it is not possible to perform the equivalent of bursectomy in mammals. But congenital abnormalities have been noted in patients in which there is a deficiency of B cells, plasma cells, γ -globulin synthesis and certain antibody reactions, while T cell function is maintained. The observations speak against (but do not rule out) the possibility that mammalian B cells require the influence of a thymic factor separable from those promoting T cell maturation.

The bura and its mammalian equivalent may function through release of hu-

moral factors. Restoration of B cell function through implantation of bursa fragments in diffusion chambers has been described in birds; but (unlike the situation for the thymus), some other organs also seem to partially restore B cell function. No humoral factor has been identified, and the possibility that B cells reside for a time within a bursal equivalent has not been ruled out.

Interactions Between T-cell and B-cell Systems^{10, 78}

The T and B cell systems are mutually dependent. Some of the T lymphocytes are said to be "helper cells." They exert as yet undefined influences on B cells which are essential for B cell responses to certain antigens. They could function by presenting antibody to the B cells, or through interaction with macrophages. "Helper cells" have been implicated in responses to administration of sheep erythrocytes and of bovine serum albumin. It is suspected that these are the ones which require residence in the thymus.

Other T lymphocytes, the "suppressor cells" exert inhibitory influences and seem to provide protection against disorders which could arise from precipitation of antigen-antibody complexes in previously healthy tissues.

Tolerance to specific antigens can be induced by chronically exposing young animals to low antigen concentrations, or by "overwhelming" older ones with very high dosages. Tolerance can be developed in thymectomized animals, but the dosages and time periods required are different from those of intact controls. Some forms of tolerance are gradually lost; the thymus seems to be involved, since the loss is much slower after thymectomy.

Experimental induction of "autoimmune diseases" is accomplished by presentation of unusual antigens; effects of such presentation are greatly enhanced by simultaneous administration of nonspecific stimulants of the immune system (e.g., Freund's adjuvant, which consists of mineral oil, an emulsifying agent and killed mycobacteria). The antigens can be naturally occurring but normally sequestered molecules (such as thyroglobulin or some of the muscle proteins), artificially modified substances derived from the animal, or materials derived from other organisms

which are similar to (but not identical with) the animal's own antigens and therefore exhibit cross-reactivity.

Certain clinical disorders may arise in similar ways. Inflammation of the thyroid gland can lead to release of (otherwise sequestered) thyroid gland proteins, and such release can lead secondarily to autoimmune thyroiditis in which antithyroid antibodies can be recovered from the serum. Antigens from certain intestinal bacteria cross-react with proteins normally present in the colon; they have been implicated in the etiology of some forms of ulcerative colitis. Although humoral antibody production is clearly involved, the thymus gland may play a protective role.

T cells may also directly promote cytotoxicity, release of tissue antigens, and subsequent stimulation of humoral antibody formation. Thymectomy has not proven effective for alleviation of disorders believed to have originated in this way. (It could be argued that "prophylactic" thymectomy might have avoided the onset; but it is unlikely that thymus glands will be removed for such purposes.)

There is speculation on mechanisms for production of so many different kinds of B cells, each capable of responding to only one specific antigen and of providing for release of one specific antibody type. Some observers believe that the embryonic thymus produces a staggering number of different antigens which affect future B cells, and that the antigens "select" specific "precommitted" B cells for proliferation. There are also questions about whether B cells can produce humoral antibody without first undergoing transformation to plasma cells, whether there are additional B type "helper cells", and whether some macrophage functions which participate in immune responses remain to be discovered. Cells differing from both T and B types have been found in lymph nodes and spleen, and have been implicated in the process of presentation of antibodies to the B cells.

WASTING DISEASE^{6, 8, 13, 28}

Neonatally thymectomized rats and mice often succumb to a characteristic "wasting disease" in which growth is seriously impaired. The animals pass through a moribund period during which they appear dirty and unkempt, have difficulty

walking, and suffer from diarrhea. The condition has been attributed to overwhelming infection by microorganisms, since it is not seen in animals raised under germ-free conditions, and can be at least partially alleviated by administration of suitable antibiotics. Evidently, intact young animals are able to cope with the same kinds of microorganisms. There are reasons to believe that disruption of the endocrine system contributes substantially to the wasting syndrome.²⁵ Loss of a trophic function of the thymus has been proposed. (Enrichment of the diet has not proven helpful.)

Thymectomized animals can be protected against development of the wasting disease if they are given transplants of thymus glands from other animals, and the grafts also restore the ability to reject foreign tissue. Some of the thymic extracts also afford protection. It has been reported that thymectomized animals that do not develop wasting disease exhibit abnormalities of the endocrine system, and that thymus extracts which protect against wasting disease can also reverse the endocrine changes.

The term "runtling" has also been applied. But most authors reserve that term for autoimmune disorders involving graft vs. host reactions, in which growth is seriously impaired after administration of isologous cells to young animals with intact thymus glands.²⁶

STATUS THYMOLYMPHATICUS²⁷

At one time it was believed that sudden ("crib") death in infants that had appeared to be healthy, could be attributed to suffocation from pressure of an abnormally large thymus gland against respiratory structures. The concept was based on reports that thymus glands of infants dying of "suffocation" were especially large. In some cases broad thymus shadows were found in siblings, and it was recommended that the glands be irradiated to reduce their size.

Systematic studies subsequently revealed that thymus weight and size found at autopsy of infants dying suddenly were usually within the normal range for infants of that age. (In a few exceptional cases, thymic hyperplasia associated with hemorrhagic destruction of the adrenal cortex was found.) Doubt was cast on the proba-

bility that even excessively enlarged thymus glands could exert sufficient mechanical pressure to prevent movements of respiratory gases. (Adrenalectomized animals with thymuses twice normal size do not die from suffocation.)

The concept that sudden or crib death results from "status thymolymphaticus" has been abandoned. The "sudden infant death syndrome," (SIDS) is now variously attributed to adrenocortical failure, fulminating viral pneumonia which is not readily detected by routine procedures, autoimmune disorders which interfere with respiratory reflexes, congenital defects of the autonomic nervous system, or to as yet undisclosed conditions which require investigation. Recent autopsy findings point to chronic oxygen deficiency preceding the time of death. It is not known whether evidence for augmented production of erythrocytes bears any relationship to suspected influences of the thymus gland on the bone marrow and spleen (p. 421).

The incidences of leukemia and of breast cancer have been found to be greater in individuals subjected to "prophylactic" irradiation of the thymus during infancy than in matched control populations. The findings support the concept that the thymus gland protects against proliferation of mutant cells. Further support derives from observations that patients treated with steroid hormones, antilymphocytic sera, and other agents given to block thymus functions for the purpose of extending the viability and preventing the rejection of organ transplants, have exhibited especial susceptibility to tumor development.

MYASTHENIA GRAVIS^{28, 70, 75}

Myasthenia gravis is a severe disorder in which neuromuscular transmission is impaired, and there is extreme weakness of striated muscles including those involved in respiration and swallowing. The incidence of true thymoma in patients suffering from the disease is estimated at 10-15%; and it has been reported that at least 70% of thymus glands within the normal weight range contain unusual collections of medullary epithelial cells, germinal centers and other evidence of inflammation.

Thymectomy has proven effective for alleviation of the symptoms in some pa-

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tients, and is especially likely to be beneficial in persons having the disease for a short time, and in young females.

Attempts to demonstrate direct *in vitro* "curare-like" effects of thymus gland extracts on myoneural junction preparations were unsuccessful. (A few early claims were discounted when it was recognized that the extracts were rich in potassium, and that actions on the myoneural junction could not be demonstrated if extracts were first subjected to dialysis.) More recently, it was reported that chronic treatment of whole animals with thymic extracts can induce delayed disturbances in neuromuscular transmission.

The suspicion that myasthenia gravis is a disorder of the immune system was aroused not only by the high incidence of thymic pathology, but also because of the presence in the sera of many patients of antibodies against myoid cells of the thymus. (The cells share an antigen with that of the I bands of striated muscle.) Lymphocytic infiltrations of muscle tissue were also found. But the interrelationships were difficult to define, since the disease symptoms involve the myoneural junction primarily, rather than contractile elements of muscle.

A hypothesis for etiology of myasthenia gravis which fits available facts, and an experimental model have been presented. It was shown that animals injected with muscle antigen subsequently develop a thymitis resembling the condition seen clinically. In animals with intact thymus glands, a delayed impairment of neuromuscular function emerges. Thymectomized animals do not develop myasthenia-like symptoms, although they form the same humoral antibodies found in intact animals. It was shown further that a substance first named *thymin* and later *thymopoietin*, can be extracted from the thymus glands and that it can, after repeated administration, affect both the myoneural junction and contractile elements of striated muscle. It has therefore been proposed that the clinical condition involves development of an autoimmune thymitis associated with production of abnormal amounts of thymopoietin. It has been proposed further, that smaller amounts of thymopoietin are released by healthy thymus glands, and that the substance may be a true hormone. Influences

of thymopoietin on the lymphatic system have been demonstrated.

Relief from myasthenia symptoms following thymectomy has been attributed to removal of the source of thymopoietin. When thymectomy is ineffective, it is suggested that either damage to the neuromuscular system has already reached an irreversible stage by the time of surgery (as is likely in cases of long duration), or that thymic remnants remote from the main body of tissue continue to release thymopoietin. No explanation has been offered for the higher remission rates in young females with short-term disease as compared with young males presenting similar symptoms. It is possible that effects are related to secretion of gonadal steroids; estrogen influences may be different from those exerted by androgens. But no hormonal connection has been established, and differences related to chromosomal make-up which are not mediated via the endocrine system have not been ruled out.

THE THYMUS GLAND AND THE ENDOCRINE SYSTEM^{4, 13}

General Considerations

The question of whether the thymus gland secretes hormones not directly implicated in development and functions of the immune system (or otherwise participates in hormonal regulation) remains unanswered after more than a century of investigation. A comprehensive review of the early literature on the subject was published in 1938.⁴ Less extensive surveys describing more recent work are also available.¹³ Interest in this area has waned in the United States, but work has continued in European countries, especially in Hungary, Czechoslovakia, Rumania, Poland, and Italy.

Two very different kinds of explanations can be offered for the failure to define an endocrine role of the thymus gland. They are considered below.

Concept #1: The Thymus Is an Important Component of the Endocrine System, but Special Features of Its Function Require a Different Design of Experiments than Has Been Utilized in the Past

Structure. The thymus originates in close proximity to, and from the same

kinds of tissue that give rise to thyroid, parathyroid, and ultimobranchial structures. It looks like a developing endocrine gland in its earliest stages. Although it later becomes heavily colonized with lymphocytic elements, it retains medullary epithelial cells which are metabolically active and in contact with a capillary blood supply.

It is possible that during certain stages the lymphocytic elements perform functions that dominate or mask more subtle endocrine activities; and that procedures which markedly affect lymphocytic functions (*e.g.*, thymectomy) affect the experimental animals in ways that make endocrine functions difficult to demonstrate.

Temporal Patterns. It was noted that the role of the thymus in development of the immune system assumes greatest importance quite early in life—during fetal stages in some mammals and continuing into perinatal periods in others. Neonatal thymectomy can have devastating effects on the immune system of rats and mice; but removal of the thymus just a few days later leads to only minor disruptions. A crucial role of the thymus in maintenance of the immune system of *adult* animals becomes obvious only when the latter are subjected to special kinds of insults, *e.g.*, X-irradiation or administration of antilymphocytic agents. It seems unlikely that a structure as large and metabolically active as the thymus gland, and one which is present in every known vertebrate from lamprey eels to humans, is maintained throughout evolution for the sole purpose of protecting animals against possible exposure to X-rays, cyclophosphamide, or antilymphocytic sera.

The thymus gland continues to grow rapidly long after the critical period for development of the immune system is over. It acquires exquisite sensitivity to gonadal steroids which are not secreted in substantial quantities until after the onset of puberty. It responds dramatically to glucocorticoids at all postnatal stages, participates obviously in bodily changes associated with stress, and regenerates rapidly following withdrawal of exogenous glucocorticoids or stressing stimuli. It also exhibits statistically significant diurnal variations in weight.⁷⁷

The thymus undergoes a remarkable series of changes in morphology which

begin in the very young embryo and continue throughout most of the life span. The changes are affected by such factors as nutritional status and the plasma concentrations of numerous hormones (peptide and protein as well as steroid). It is quite possible that endocrine functions vary along with morphology, and that *timing* of experiments is critical. This seems especially likely in the face of evidence that certain substances derived from the thymus gland exert biological actions which are opposite in direction to those seen when chemically different thymus gland derivatives are administered. It may be absolutely necessary for investigators of endocrine functions to breed their own animals, and to select only those identical in age and body weight, ones reared in similar-sized litters, weaned at a specific time, and fed a known diet afterward. The time of the year in which the study is performed may also be of greatest importance. It is not at all uncommon for investigators to order from supply houses "rats 6 weeks old" and to disregard the possibility that they may range from 5.5–6.5 weeks, and that some weighing 120 g may be older than others weighing 140 g.

The Nature of Thymus Endocrine Functions. Animals thymectomized when they are past the neonatal stage usually look healthy, grow at normal rates, reproduce, lactate, and otherwise can appear very similar to sham operated animals or unoperated controls. This suggests that the thymus gland does not affect "vital" functions. If the thymus performs an endocrine function, it is likely to be one of "fine adjustment." Rather than seeking out some drastic change in any specific direction, it may be more reasonable to look for possible influences of the thymus gland on *ability to adjust* to changing situations. Some indications will be presented below that thymectomized animals exhibit wider fluctuations on *both* sides of normal values when they are presented with unusual situations, *e.g.*, diets excessively rich in sodium.^{50c}

Evidence for Hormone Production by the Thymus Gland. A very large number of influences on the endocrine system have been described following administration of thymus gland extracts and following thymectomy. The thymus is also capable of developing many different kinds of tumors,

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including ones producing and releasing ACTH-like, glucagon-like, insulin-like and calcitonin-like substances.

Concept #II: The Thymus Functions only in Development and Maintenance of the Immune System; Apparent Influences on the Endocrine System are Without Physiological Significance

Structure. It is possible that early resemblances of the embryonic thymus to "typical" endocrine glands may have no greater physiological significance than early indications that the mammalian embryo starts to develop gill-like structures.

The Presence in the Thymus of "Hormone-like" Principles. No "hormone" component of the thymus has ever been shown to have physiological actions different from those exerted by known and established endocrine structures. All unique components seem to be related to immune functions.

Significance of the Presence of "Hormone-like" Principles. The thymus contains a remarkable assortment of cell types, including fibroblasts, mast cells, myoid cells, adipocytes, reticulo-endothelial elements, lymphocytes of several sizes, and epithelial components which seem to be different in the cortex as compared with the medulla. The thymus is believed to engage in "immune surveillance" and in destruction of cell types which, if released, could inflict damage to previously healthy tissue. In order to carry out such activity, it requires mechanisms for recognition of "acceptable" cell types; the assortment of components may be needed for such a purpose. The resident cells may release very small quantities of hormones which have no influence on the endocrine balance of a normal animal; but special experimental conditions may elicit abnormal activity on the part of certain ones.

Reproducible effects of thymectomy on the endocrine system do not necessarily demonstrate that the thymus is an endocrine gland. Thymectomy is a traumatic experience for most animal types, since it involves deep anesthesia, incision of the sternum, exposure of the contents of the thoracic cavity to atmospheric pressure, temperature and humidity, and a rela-

tively long recovery period in which animals suffer pain and sometimes also transient difficulties with respiration and swallowing. The profound influences of sham thymectomy point up some of the nonspecific effects; but sham operated animals may experience less stimulation of, or injury to nerves, and they do not have a sizable chunk of tissue suddenly removed from the thoracic cavity (or replacement of normal tissue with some other component).

Since it has been so difficult to define effects of thymectomy, investigators have scrutinized pituitary glands, adrenal glands, thyroids, gonads, blood plasma, bone ect. It is likely that changes in the preceding occur after extirpation of many organs, but there has been little reason to empirically examine so many tissues after removal of other structures.

THE THYMUS GLAND AND SOMATIC GROWTH

4, 18, 24, 28, 58b

The thymus gland is large during the period of rapid growth in all mammals, and it tends to be greatest in size in the most robust members of litters (in which genetic differences are minimized by inbreeding). The thymus hypertrophies in response to administration of growth-promoting hormones (STH, PRL and small amounts of TSH); and it shrinks when growth rate is retarded because of inadequate nutrition, stress, or administration of growth-retarding hormones (glucocorticoids and large doses of thyroxine).

Various authors have proposed that (1) the thymus is a true "target organ" for STH, and that pituitary somatotrophs and thymus gland cells engage in mutual feedback regulation of STH and thymus hormone secretion; (2) the thymus gland mediates actions of STH; (3) the thymus participates in metabolism of STH (or its effectors) and protects young animals against effects of excessive STH (this concept has been invoked to explain the lesser tendency of young animals, as compared with older ones, to develop diabetes-like symptoms following STH injection); and (4) thymus hormones directly promote growth. It has also been suggested that STH is absolutely essential for development and function of the thymus gland.

The study of possible thymus gland

influences on growth is fraught with special difficulties. If, as suggested above, the thymus gland influence is one of "fine adjustment," it would be expected that animals already growing at an optimal rate would be least responsive to the administration of additional growth-promoting factors. On the other hand, if animals with sub-optimal growth rates are studied, comparisons between thymectomized and sham-operated animals may be difficult to interpret since the "controls" will have abnormally small thymus glands.

In one of the earlier studies, it was shown that young rats fed bits of thymus tissue grew at a more rapid rate.⁶⁰ But comparisons of "control" rats of that study with more recent data suggest that the animals not receiving thymus supplements were decidedly undernourished by today's standards. It is possible, therefore, that the accelerated growth rate following thymus feeding is wholly attributable to dietary enrichment. In another study from the same laboratory, it was reported that tadpoles fed bits of thyroid tissue underwent precocious metamorphosis, while tadpoles fed bits of thymus grew rapidly and exhibited delayed metamorphosis. Since the thyroid treated groups had a shorter larval period in which to grow and store up nutrients, they developed into very small frogs, while thymus-treated tadpoles developed at a later time into relatively large frogs. It is possible that the thymus supplements provided a diet rich in many nutrients but low in iodine, and that no hormonal influence was involved.^{60b}

Studies from several laboratories are consistent with the concept that thymectomy of young animals past the neonatal stage leads to at least transient stimulation of the adenohypophysis, with relatively greater influences on somatotrophs than on other cell types.^{13, 22} Histological evidence for early stimulation of somatotrophs, thyrotrophs, gonadotrophs and possibly also corticotrophs includes increased cell number and size, increased incidence of mitotic figures, and degranulation. Delayed effects (which may take days or weeks to develop, depending on ages and species of animals, and also on cell types examined) are consistent with ultimate exhaustion of the cells. Nonspecific effects of anesthesia, surgical trauma and events of the healing

period on the central nervous system must be considered when evaluating the findings. But reproducible differences between thymectomized and sham thymectomized animals have been observed.

No obvious growth spurt seems to have been associated with early stimulation of the somatotrophs. Explanations offered include the following: (1) Pituitary effects are too transient to affect a long-range process. (2) Histological changes of somatotrophs are not associated with great increases in STH release. (3) Thymectomy leads to excessive release of ACTH and glucocorticoids, and these counteract effects of STH on growth. (4) Thymectomy removes a mediator of STH influences on growth.

If the thymus does, in fact, somehow enhance effectiveness of STH, then simultaneous loss of the thymus principle and increased function of the somatotrophs would be expected to cancel each other out, and to result in near-normal growth rates of thymectomized animals. In one study, thymectomized-hypophysectomized animals exhibited insensitivity to STH preparations which stimulated growth of hypophysectomized controls. Administration of a thymus gland preparation (homoeostatic thymic hormone, HTH) restored the ability of thymectomized animals to grow in response to the STH. HTH was also observed to prevent changes in pituitary morphology which otherwise occurred in animals subjected to just thymectomy.

It is usually reported that young animals that recover from thymectomy without complications of unusual trauma or obvious infection soon begin to consume adequate quantities of food, and that they grow as fast as unoperated siblings.^{56, 57} (And hundreds of rats of three different strains have grown nicely in the writer's laboratory.) But rabbits, guinea pigs, dogs, mice, and tadpoles which do not develop "wasting disease" sometimes go through a period of retarded growth before catching up with controls. The possibility that they suffer from mild infections or poor appetite has not been ruled out. Puppies seem to be especially susceptible to both infection and appetite impairment. It has been reported that guinea pigs that continue to nurse after the surgery do not show retardation, while the effects on growth are common in

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guinea pigs raised on solid food. It has also been noted that mice handled excessively after surgery are more susceptible to growth impairment than are unhandled thymectomized or handled sham operated controls.

There are numerous descriptions of accelerated growth of young animals following administration of thymus gland preparations, including HTH and something named thymoresin. It has even been proposed that human infants that "fail to thrive" during the first few post-natal months might benefit from injections of the extracts. But the few reports of good responses to such extracts have not been confirmed by independent observers.

What is interesting about the reports as a whole is that several authors state that thymus preparations promote growth, while others find no effect after administration of similar preparations. But there are few indications that thymus extracts retard weight gain or body elongation in either intact or thymectomized animals.

Experiments in which thymus extracts were administered to several successive generations of rats, and said to lead to progressively greater birth weight, accelerated growth rate and vigor of the young, and precocious sexual maturation¹³ were mentioned in the introductory part of this chapter. It was noted that repeated attempts to reproduce the studies in independent laboratories were consistently unsuccessful¹⁴, and that doubt was cast upon the validity of the findings for this reason and also because of questions concerning the ability to transmit acquired physiological superiority to the offspring. However, some observers noting the failures continued to wonder if there was not something special about the particular extracts employed, and whether substances prepared from a tissue so very rich in nucleic acids could not indeed exert unusual actions.

THE THYMUS AND THE ADRENAL CORTEX^{4, 13, 23c, 27, 50d, 67}

The exquisite sensitivity of the thymus gland to an outpouring of adrenocortical steroids when animals are stressed, and the rapid thymic involution following injection of glucocorticoids, make it reasonable to seek out some function of the thymus in

regulation of adrenocortical secretion or in mediation of responses to stress.^{3ac, 3ec, 50b} It is known, however, that animals deprived of the adrenal cortex readily succumb to a wide variety of noxious stimuli, whereas those deprived of just the thymus gland exhibit resistance which at least superficially resembles that of the intact animal.

There have been numerous reports that thymectomy leads to elevation of plasma corticosterone concentrations, increases in adrenal gland size and weight, and transient reduction of adrenal ascorbic acid and cholesterol content.^{13, 27, 3ec} It has also been reported that thymectomized-hypophysectomized animals are more sensitive than hypophysectomized controls to exogenous ACTH, and that the sensitivity can be reduced by administration of HTH.¹³ All of the preceding are consistent with some kind of inhibitory influence exerted by the thymus gland on adrenocortical functions.³⁴ It has been proposed that the thymus functions as an "antistressor", protecting against potentially harmful effects of too much glucocorticoid.

On the other hand, the findings have not been consistent. Some investigators found no evidence of changes in adrenocortical steroid hormone secretion following thymectomy. Contradictory data raise the possibility that the timing of the measurements may be critical; this seems reasonable when one considers the wide circadian fluctuations in plasma steroid levels. It is also important to choose the right parameters for measurement. Adrenal gland weights do not provide an accurate index of hormone secretion. One group of investigators reported no large changes in plasma corticosterone concentrations in adrenal venous blood of thymectomized Wistar rats, but they did find a 6.5-fold increase in concentrations of 18-OH-deoxycorticosterone.²⁷ There are other data suggesting that the thymus may modify the responses to, rather than the concentrations of, glucocorticoids.

There are indications (and some are cited below) that while thymectomy does not threaten survival of animals exposed to stress, it does affect their ability to cope with administration of high sodium diets, excessive amounts of parathyroid, glucocorticoid and thyroid hormones, and the administration of histamine. It is also

known that thymectomized animals have poor tolerance to sublethal irradiation, and that not all of the difficulties can be directly related to known immune functions of the thymus gland.

Destructive influences of adrenocortical hormones on the thymus gland have been widely discussed. But the adrenal cortex may also play an essential role in thymus gland development. In addition, the thymus seems to be involved in early differentiation of the adrenal gland.^{50a}

THE THYMUS AND THYROID FUNCTION^{4, 13, 33a, 35, 50f}

Several different kinds of observations point to some kind of thyroid-thymus antagonism. But the effects may be indirect and involve participation of gonadal hormones.

In addition to influences on appearance of pituitary thyrotrophs described above, there have been several reports that thymectomized guinea pigs have increased rates of ¹³¹I uptake by thyroid glands and of iodine incorporation into thyroid gland proteins, as well as greater height of thyroid follicular cells, with depletion of colloid. The concentrations of protein bound iodine of the serum, of pituitary gland TSH and of urinary TSH are elevated, and the basal metabolic rates are above control levels. It has also been reported that the negative nitrogen balance and high urinary excretion of creatine which follows thymectomy, can be reversed in a dose-related manner by administration of HTH, and that the latter also effectively counteracts actions of exogenous TSH and thyroxine.

It was found in one laboratory that either thymectomy or castration of guinea pigs led to increased urinary creatine excretion, and that the creatine excretion was further elevated by administration of thyroid hormones. Moderate doses of thymus extracts reduced the creatinuria in such animals, but excessive amounts enhanced it. Creatinuria could be consistently reduced by administration of testosterone and increased with estradiol.¹³

It was also noted that thymectomy, castration, combined thymectomy-castration, or thyroidectomy each led to increased urinary excretion of TSH; but the combination of thyroidectomy-castration-thymectomy did not induce high TSH

secretion. Administration of HTH, testosterone or thyroxine could reduce the urinary TSH of singly or doubly operated animals.

The data are all consistent with thymus gland and testicular hormone suppression of TSH secretion, but also with severe disruption of feedback mechanisms if animals are simultaneously deprived of thyroid, thymus and gonadal hormones. The findings are of interest by themselves, but more so in the light of observations presented below.

HTH was also found to counteract thyroxine stimulation of glucose uptake by isolated rat diaphragm, to reduce plasma concentrations of protein bound iodine, and to prevent thymectomy influences on the pituitary gland. (It will be recalled that the same thymus gland preparation was reported to correct thymectomy influences on the immune system and to protect neonatally operated animals against wasting disease.)

Effects of HTH on thyroxine actions have been attributed to enhancement of the activity of a factor in the liver which promotes catabolism of thyroid hormones. Degenerative changes in thyroid glands of guinea pigs were described following prolonged treatment with thymus extracts.

Changes in thyroid gland histology suggestive of transient stimulation, sometimes followed by exhaustion, and increased effectiveness of both endogenous and administered thyroid hormones have been described by different investigators working with thymectomized animals of other species. Thyroidectomized animals are also said to be adversely affected by doses of HTH which promote growth in intact animals. There are numerous indications that thymus gland preparations can antagonize actions of thyroid hormones. Thyroxine-induced elevation of metabolic rate in mice was reportedly reduced by administration of a thymus homogenate. Feeding of dried thymus antagonized influences of thyroxine on feather growth in chickens. Addition of bits of thymus to tadpole food was said to prevent premature induction of metamorphosis resulting from treatment with thyroid gland preparations.³⁵

In an unrelated study, it was found that TSH is inactivated *in vitro* by thymus and thyroid glands. Some limited activity was

also found in lymph nodes, but not in a variety of other structures including liver and gonads.⁶⁰

The thyroid system of the rat has been said to be less sensitive than that of several other species to thymectomy or administration of thymus extracts, while the guinea pig system may be especially responsive.

Male hooded rats thymectomized at 6½ weeks had significantly elevated rates of ¹³¹I uptake by thyroid glands 24 hr after administration of the isotope, when tests were performed 21 days after the surgery; but no differences in values were found (when compared with unoperated controls) at 1, 7, 14, 18, or 28 days. Sham-thymectomized animals had significantly reduced rates of ¹³¹I uptake at 18 days only. Some indirect influence on thyroid function is suggested.

Small to moderate dosages of thyroid hormones, or of TSH, promote growth of the thymus and extrathymic lymphoid tissues. Effects are most pronounced when the hormones are tested on lymphoid tissue which is regenerating following steroid hormone-induced atrophy. It has been suggested that thyroid influences are effected by an enhanced rate of catabolism of steroid hormones, or through stimulation of somatotrophs. But actions of thyroid hormones can be demonstrated in hypophysectomized, gonadectomized or adrenalectomized animals. On the other hand, thyroxine treatment reduces the mass of lymphoid tissue when administered to thymectomized animals. It has therefore been proposed that the thymus exerts "permissive actions" required for expression of thyroid hormone stimulation of the lymphatic system.^{32a, 25}

Toxic doses of thyroxine diminish thymus gland size (and also impair appetite and growth). The thymic atrophy is very different from that seen following administration of glucocorticoids. Narrowing of cortical width has been attributed to outward migration of cortical lymphocytes (rather than to pycnotic degeneration); the lymphocyte population of the medulla and the numbers of Hassall's corpuscles are increased.

Doses of thiouracils which promote thyroid gland enlargement but do not totally suppress thyroid hormone synthesis induce thymus gland hypertrophy; but doses large

enough to shut off thyroid hormone secretion eventually elicit thymic atrophy. Effects of surgical thyroidectomy are difficult to assess, since thymus glands atrophy in response to deprivation of parathyroid glands which are usually simultaneously removed. It has been reported that administration of thyroxine lessens the thymus atrophy otherwise seen after thyroparathyroidectomy.

Hypertrophy of both the thymus and the lymph nodes is a common feature of clinical hyperthyroidism (Grave's disease).⁶¹ It is not clear whether large thymuses are related to thyroid hormone levels or to autoimmune processes. Thymectomy has been performed in hyperthyroid patients, but it usually has not proven beneficial for alleviation of the condition.

Additional indications of complex interrelationships between the thymus, thyroid and gonads are suggested by reports that thyroidectomy prevents the thymic hypertrophy which otherwise results from castration. It has also been stated that thyroidectomy exacerbates the effects of thymectomy in guinea pigs and increases the incidence and severity of wasting disease, while castration seems to afford some protection to thymectomized and thyroidectomized-thymectomized animals.¹³

In a study of tolerance to near-lethal doses of histamine-phosphate, no differences in survival were found when intact male rats were compared with intact females. Histamine tolerance was very much increased by thymectomy in males but only slightly (yet significantly) in females⁶²; however, thyroidectomy very greatly increased the tolerance of females and only increased the tolerance of males to a small extent. Influences of thyroidectomy plus thymectomy were additive in both sexes; doubly operated animals of both sexes had LD₅₀ values twice as great as those of unoperated or sham operated controls.

While gonadectomy in either sex increased histamine tolerance, it totally reversed effects of combined thyroidectomy-thymectomy. The pattern of responses shows some resemblances to effects of similar procedures on creatine excretion of guinea pigs. An additional curious observation was that, while survivors of all other groups regained health after recovery from the effects of histamine injection, only thyroidectomized-thymectomized animals

went into a state of cachexia, became emaciated over a period of a few weeks and died. Triply operated (thyroidectomized-thymectomized-gonadectomized) animals did not do this (C. Martin, unpublished observations).

Similar interrelationships among the thymus, thyroid, and gonads may underlie findings of a very different nature. When 2-month-old rats were subjected to renal obstruction they developed secondary hyperparathyroidism, and this led in turn to necrosis and calcification of the myocardium and the smooth muscle of the arterial tree and gut. Thymectomy very markedly exacerbated the damage in male rats, but only slightly increased it in females. Gonadectomy alone increased the damage in animals of both sexes, but it totally counteracted effects of thymectomy, so that gonadectomized-thymectomized animals responded much like sham operated controls.^{50d}

All of the preceding are consistent with some kind of "fine adjustment" function of the thymus gland on the endocrine system.

THE THYMUS GLAND AND PARATHYROID FUNCTION^{4, 13, 80d, 86, 88}

Again, it is possible to compile evidence for an antagonistic relationship between the thymus and a known endocrine gland, but only when findings are interpreted with great care.

In early studies, thymectomy was reported to lead to onset of rickets and thinning of the bones of puppies; but in at least some cases there are indications that the animals were maintained in cages too small to permit adequate exercise for normal bone growth. (Decalcification of the bones is common in intact animals when activity is severely restricted. It has been observed in bed-ridden patients and also in astronauts confined to small quarters and subjected to reduced gravitational pressures.)⁴

Weak, fragile skeletons have been found in thymectomized mice and tadpoles under conditions in which activity was not restricted. And immature thymectomized rabbits have been reported to suffer from delayed maturation of the skeleton, reduced weight, volume and calcium content of the bones, reduced plasma calcium, and elevated plasma phosphate concentrations.

A high molecular weight protein derived from the thymus gland (thymus protein, TP) is said to reverse such effects of thymectomy in the rabbit, while similarly prepared materials from muscle or spleen are ineffective.¹¹

All of the preceding, and studies cited in the section on the thyroid, are consistent with the concept that thymectomy removes a physiological brake on parathyroid hormone secretion. It has been proposed that the greater tendency for older individuals to develop hypercalcemia under certain conditions can be related to smaller thymus glands.

On the other hand, the thymus may contain substances which affect calcium metabolism directly rather than via the parathyroid glands. It has been reported that young salamander larvae go into tetany when fed thymus tissue, but that salamanders old enough to develop parathyroid gland function are resistant.^{4, 88a}

Calcitonin has been positively identified in thymus glands, but there is no clear evidence that it is secreted. In addition, substances of considerably higher molecular weight which lower plasma calcium concentrations and affect phosphate excretion have been found in extracts of thymus glands.⁷⁸ Animals deprived of calcitonin have difficulty restoring their plasma calcium ion concentrations following calcium salt infusion. It was observed that thymectomized animals injected with calcium gluconate maintained elevated plasma calcium values for longer periods than intact (sham operated) animals given the same dose.⁷

Glucagon has been identified in the thymus gland. Glucagon stimulation of calcitonin secretion was described in Chapter 6.

Influences of thymectomy or administered of thymus gland preparations on plasma and urinary concentrations of calcium and inorganic phosphate have been described in experimental animals; and patients with certain thymic disorders, including myasthenia gravis, sometimes exhibit disturbances in calcium and phosphate metabolism.

The thymus may affect calcium metabolism indirectly via influences on secretion of STH, thyroid and adrenocortical hormones, or on the gonads. However, most differences between young and older ani-

mals in responsiveness to administration of calcitonin, vitamin D and other agents affecting metabolism can probably be explained by mechanisms not dependent upon the thymus.

THE THYMUS GLAND AND THE REPRODUCTIVE SYSTEM^{4, 13, 16, 20}

No aspect of thymus gland interrelationships with the endocrine system has received as much attention, or is riddled with as much controversy, as the question of possible influences on reproductive functions.

It is firmly established that testosterone and estrogens promote thymus gland involution, that gonadectomized animals have larger thymus glands than intact ones, and that thymus glands tend to be larger before puberty than afterward in intact animals of many species. None of this provides positive proof that the thymus affects the gonads.

Relatively recent evidence has been presented for development of a condition of "ovarian dysgenesis" in which hormone-secreting ovarian stromal tissue functions after puberty but in which ripe follicles and corpora lutea do not appear, when mice are thymectomized at 2-4 days of age.¹⁶ It has also been reported that sterility of female rats which would otherwise result from neonatal androgen administration can be prevented by administration of thymus gland cells.¹⁶ On the other hand, neonatally thymectomized male rats are apparently capable of engaging in normal spermatogenesis after puberty.¹⁶

There are many descriptions of thymectomy influences on the male reproductive system. The highly controversial nature of the findings suggests that either the influences are indirect,¹⁷ or else that they are strongly affected by other conditions associated with thymus gland deprivation.

In a study on male hooded rats subjected to surgery at 6½ weeks of age, it was found that sham thymectomy delayed growth of ventral prostate glands, seminal vesicles, and coagulating glands, while thymectomy did not. Significant differences between sham-operated and unoperated animals did not become apparent until 18 days after surgery; and by the 28th day, the sham-operated animals had caught up with the unoperated controls. The same

influences on a group of Wistar rats (which normally enter puberty later) could be elicited only if surgery was delayed by more than an additional week.¹⁶ No effects of either thymectomy or of sham operation were found in the hooded strain if the surgery was performed when the animals were 4½ weeks, or older than 8 weeks.

The interpretation presented was that animals cannot cope simultaneously with the demands of the onset of puberty and the trauma of thoracic surgery, and that animals with intact thymus glands (*i.e.*, the 6½-weeks-old sham-operated group) have some means for temporary diversion of their metabolic energies into such processes as wound healing. This would account for the delayed onset of puberty. Thymectomized animals, on the other hand, lack some regulatory device, and start reproductive development at times when it would be more appropriate to utilize resources for coping with the stress situation. The concept was backed up by later studies which showed that reduction of adrenocortical responses to stress (by performance of a unilateral adrenalectomy at the time of sham thymectomy) blocked the pubertal delay. Further investigation revealed that growth of accessory reproductive structures could be delayed in intact, sham-thymectomized, and thymectomized rats, if all were given injections of cortisone acetate. But reproductive structures were again smaller in sham-operated than other cortisone-injected groups, while only thymectomized animals exhibited markedly delayed surgical healing.

The studies were pursued by a different investigator who measured citric acid content and enzyme activities as well as weights of accessory reproductive structures of hundreds of hooded rats subjected to surgery at 31-33 days of age.¹⁸ At first it appeared that sham thymectomy consistently accelerated the onset of puberty, and that thymectomy was even more effective than sham operation. It was tentatively concluded that nonspecific stimulation of the central nervous system brought on by the surgery led to premature release of gonadotrophins, and that the responses were exaggerated in thymectomized rats in which some regulatory device had been impaired.

But in time it became apparent that highly significant influences of thymec-

tomy and sham thymectomy emerged only when experiments were performed between May and October. Unoperated controls studied during "winter" months had smaller accessory reproductive structures than did unoperated "summer" animals; and neither sham operation nor thymectomy had consistent influences. An interaction between thymus and pineal glands in determining the timing of onset of puberty, and in susceptibility of the system to external disruption was considered.¹⁶

Once animals have established their adult reproductive functions, the thymus seems to exert little influence. It is well known that adult thymectomized females demonstrate no deficiencies in lactation or maternal behavior. However, male rats thymectomized at 6½ weeks and permitted to follow usual laboratory lives afterward, developed very large prostate glands many months later. Differences in zinc uptake by several tissues, and differences in responsiveness of older rats to administration of gonadotrophins were also observed to be related to deprivation of thymus glands.¹²

THYMUS GLANDS AND HEPARIN^{53b, 56b, 56c}

It was noted above that thymus glands of young mice pick up radioactive sulfate and radioactive glucosamine, and incorporate these substances into a mucopolysaccharide or glycoprotein which then seems to be secreted.³¹ The findings are consistent with (but do not clearly demonstrate) heparin secretion by the thymus gland.

It has been reported that heparin concentrations in the blood plasma are lowered by thymectomy, and that plasma heparin levels are affected differently by adrenalectomy in thymectomized as compared with intact animals.^{56b} According to some observers, thymocytes can be converted to heparin-containing mast cells in the rat; and the process is strongly influenced by glucocorticoid and thyroid hormones. Impaired abilities of thymectomized animals to cope with administration of inflammatory agents has been related to their inability to form mast cells in this way.⁵⁵

Physiological functions of heparin are poorly understood. The mucopolysaccharide has been implicated in activation of

lipoprotein lipases, and thymectomy also affects this parameter.^{56a} In addition, heparin influences bone calcification and sodium metabolism, and the thymus has been linked with such functions.

In one study, thymectomized, sham-thymectomized, and unoperated rats excreted similar quantities of sodium, potassium, chloride, and water when all were maintained on standard laboratory food and tap water. The quantities of electrolytes excreted showed seasonal variations in all groups.

When the drinking water was replaced by 1% sodium chloride, thymectomized animals retained excessive amounts of sodium during times of the year when excretion rates were low for intact animals, and they excreted excessive amounts during times of the year when excretion rates for controls were high. It appeared, therefore, that deprivation of the thymus gland led to loss of some kind of "fine adjustment." In these experiments, sham surgery was without apparent influence.

The effects of thymus gland removal could not be attributed to changes in mineralocorticoid secretion, since influences of thymectomy were actually exaggerated by simultaneous adrenalectomy. (Thymectomized-adrenalectomized rats differed markedly from sham-thymectomized-adrenalectomized animals.)⁴⁵

Injections of heparin had a "normalizing" influence on thymectomized animals. They reduced excessively high rates of potassium excretion in March, and increased the very low rates of sodium, potassium, and chloride excretion in January.^{56c}

THE THYMUS AND PINEAL GLAND^{11, 13, 74}

Some of the many similarities in reports on the thymus and pineal glands were mentioned at the beginning of this section. Both structures are found in every class of vertebrate including the cyclostomes. Both are large in young animals and exhibit evidence of age-related changes including involution after puberty with persistence of some active cells well into old age, and both undergo circadian variations in size that can be related to the photoperiod. Both have been implicated in regulation of somatic growth, reproduction, responses to stress, thyroid and parathyroid function, and water and electrolyte balance. Pep-

tides, carbohydrate-containing molecules and lipid-extractable components derived from both have been thought to affect the endocrine system. An interaction of pineal, thymus, and thyroid systems in production of mast cells has been described.

A possibility worth considering is that the two glands perform similar functions, but that one predominates at certain times of the year or during certain developmental stages, while the other is temporarily relatively quiescent. Additional separate roles, e.g., of the pineal in pigmentation and of the thymus in development of the immune system, are not incompatible with the concept.

Pineal gland regulation of the reproductive systems of some mammals provides for synchronization of activities with environmental conditions, and therefore for birth of the young at times of the year most favorable for survival. It may also delay growth and maturation of young born relatively early for that species, so that metabolic demands associated with the onset of puberty do not arise before the food supply becomes abundant. In many species, the pineal receives its major cues from changes in environmental lighting; but when nutrition is inadequate, reproductive maturation is delayed in animals with intact pineal glands. During times of the year when both light and food are available, it is conceivable that some of the controls over growth and maturation are taken over by the thymus gland. In one study cited above, thymus gland influences over the timing of puberty were seen only during times of the year when the pineal might be expected to be relatively quiescent.

In the heparin study,^{50c} seasonal variations in electrolyte excretion patterns were mentioned. During January, when the pineal might be expected to dominate, all groups excreted very small quantities of salts and water. This could be useful at times when water and food are scarce. A role of the pineal in water and electrolyte conservation is indicated by many kinds of observations (Chapters 11 and 25); and pineal glands of rats have been shown to contain high concentrations of renin-like substances which can be depleted in response to administration of hypertonic saline.⁵¹

While conservation of water and salts is

desirable, excessive retention must be avoided. Later (in the springtime) it may be physiologically useful to excrete larger amounts, but this too must be held within limits. Regression of the pineal may permit greater salt loss. It was noted that animals deprived of thymus glands seemed to lack fine control over the functions; they retained too much fluid during January, and lost too much in March. Modifying influences of the thymus gland exerted during the winter months do not contradict the concept of reciprocal influences of the thymus and pineal. Mineralocorticoid administration promotes thymic hypertrophy, and excessive salt retention may directly activate the thymus at times when its function is usually minimized.

A further hint at possible interactions between the two glands was noted when drinking and renal excretion rhythms were studied in adrenalectomized rats maintained under conditions of continuous illumination (which would be expected to suppress certain pineal functions). It was found that injections of melatonin significantly affected rhythms of animals that were also thymectomized, but did not have this influence on animals with intact thymus glands.⁵⁰

It is possible that many of the observations described in the preceding sections will prove to have explanations very different from the ones proposed. Further investigation may provide some insights into unexplained toxicities of agents which affect the thymus gland, e.g., oral contraceptives and the dosages of steroid hormones utilized in suppression of inflammatory reactions or transplant rejection.

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