General Principles of Endocrinology 9.1

Learning Objectives

- Distinguish among autocrine, endocrine, paracrine, and neurocrine secretions
- List the classical endocrine glands
- Define endocrine gland and hormone
- Describe how the neuroendocrine system affects physiological control
- List those hormones that are polypeptides including
  - **A.** anterior pituitary releasing hormones
  - **B.** anterior pituitary hormones
  - **C.** posterior pituitary hormones
  - **D.** five gastrointestinal hormones
  - **E.** hormones that originate from pancreas, kidney, heart, and parathyroid glands
- List those hormones that are derivatives of amino acids
- List those hormones that are derived from steroid metabolism
- Describe the process of making, processing, storing, and releasing polypeptide hormones
- Describe the process of making, processing, storing, and releasing steroid hormones
- Draw the Scatchard plot, titration curve, and Hill plot. Label the axes and indicate what parameters can be obtained from each plot
- Identify threshold, maximum response, and ED_{50} from titration curves
- Define upregulation and downregulation
- Describe desensitization and distinguish heterologous from homologous desensitization
- Define metabolic clearance rate and how it relates to the first-order rate constant for hormone degradation

ENDOCRINE GLANDS RELEASE SIGNALING MOLECULES INTO THE BLOOD

The classical definition of an **endocrine gland** is: "**A comparatively small, circumscribed organ, devoid of ducts, and having access to a rich blood supply. It releases its products (hormones) directly into the blood stream.**" The classical endocrine glands include the

- Pituitary
- Hypothalamus
- Thyroid gland
- Parathyroid glands
- Adrenal glands
- Pancreatic islets
- Gastrointestinal system
- Gonads (testes, ovaries)
- Placenta.

The classical definition of a hormone is an organic substance that is released into the blood at low concentrations by a ductless gland, and that travels through the circulation to a remote target tissue where it evokes systemic adjustments by engaging specific responses. It is now recognized that a number of cells release hormone-like signaling molecules into the blood but do not fit into the classical definition of comprising circumscribed organs, but are more diffusely located. These cells include neurons, renal cells, cardiac atrial cells, adipocytes, osteocytes, and platelets.

MODERN DEFINITIONS OF HORMONE INCLUDE LOCAL AND DISTANT EFFECTS AND INTEGRATION OF THE ENDOCRINE AND NEURAL SYSTEMS OF CONTROL

It is now recognized that the complicated control systems of the classical endocrine glands probably evolved from more "primitive" systems that control local events in small multicellular animals without circulatory systems. These local control systems are discussed in Chapter 2.8 and are partly recapitulated in Figure 9.1.1. **Paracrine** hormones influence cells that are near the source of the paracrine hormone. **Autocrine** hormones affect the cell that secretes it. If the hormone is released into the blood to travel to its target, it fits the classical definition of an **endocrine** hormone. Neurons release materials that affect only those neighboring cells that have receptors for the neurotransmitters. Thus neurotransmitters are a kind of paracrine secretion, but the axon extends the distance between the cells. In neurocrine systems, neurons release neurotransmitters into the blood, and they exert their effects on distant target tissues that are reached through the blood.

DOI: http://dx.doi.org/10.1016/B978-0-12-800883-6.00084-7

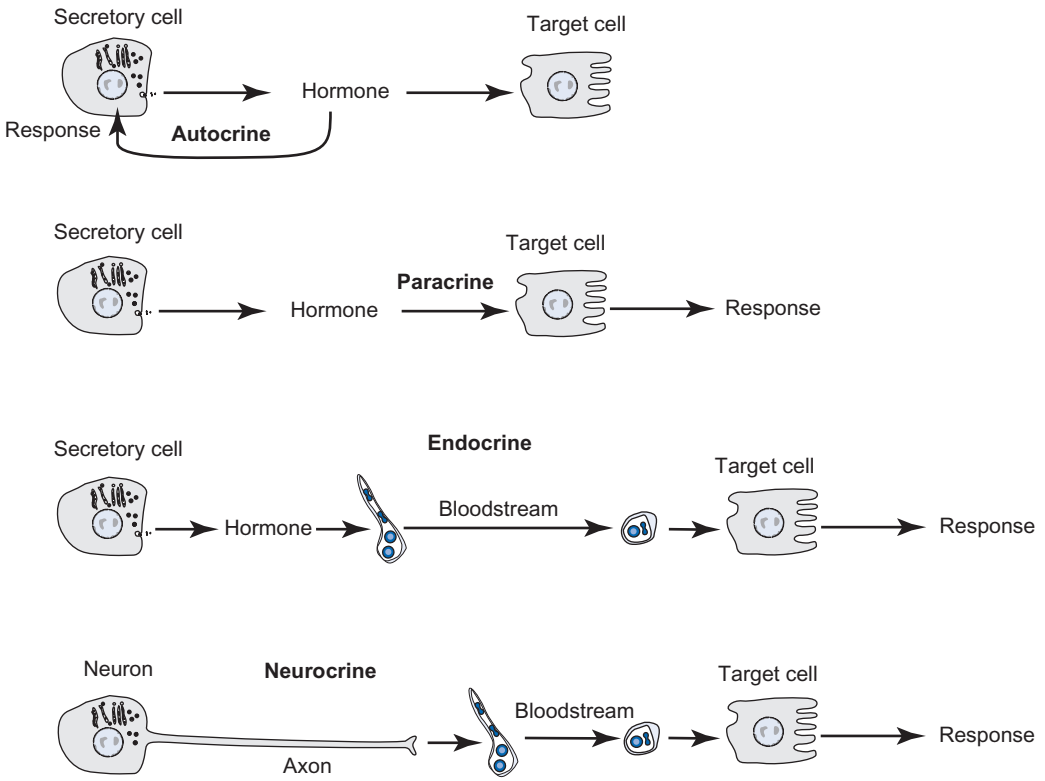

FIGURE 9.1.1 Various classes of hormones. Autocrine hormones affect the secretory cell; this is an autocrine function. Paracrine hormones diffuse through the neighboring extracellular matrix to affect nearby cells. Endocrine hormones are released into the blood to be transported to target cells, where they engage physiological responses. Neurocrine hormones are released into the blood after having been transported down axonal processes and stored in terminals near the point of release. These are released in response to action potentials that invade the nerve terminal, and so nerve cell activity precedes release of hormone.

THE NEURAL SYSTEM PROVIDES FAST, SHORT-LIVED CONTROL; ENDOCRINE CONTROL IS SLOWER AND LONGER LASTING

Both the neural and endocrine systems have components that

- secrete materials by exocytosis;
- generate membrane potentials and can be depolarized;
- affect their target cells by binding to specific receptors on target cells.

In addition,

- Some neurotransmitters are also used as hormones.

The main difference between the neural system of control and the endocrine system is the use of the axon. The axon extends the cell body and allows it to release its neurotransmitter close to its intended target in response to action potential propagation into the axon terminus. The endocrine system relies on the blood to transport its signaling molecules great distances. Both systems derive their specificity from the interaction of the signaling molecules with their receptors. Because of the axon and the extraordinarily fast conduction velocity of nerve impulses, the neural system provides fast control with rapid onset and nearly equally rapid shut-off. Endocrine systems typically produce a signal that is slower in onset but persists much longer. Overlap in the neural and endocrine control systems is illustrated in Figure 9.1.2. The endocrine system is controlled mainly through **negative feedback loops**, in which the response of the target cells restores the internal conditions towards normal, thereby lessening stimulation of the endocrine and neural systems and turning off the activating signal.

HORMONES CAN BE CLASSIFIED BY THEIR CHEMICAL STRUCTURE AND SOURCE

Hormones are chemically diverse but they can be grouped according to their chemical composition. The major hormones are listed in Tables 9.1.1A and 9.1.1B. The three major classes of hormones are:

1. **Modified amino acids**
2. **Polypeptides**
3. **Steroids.**

Hormones that are modified amino acids include **epinephrine**, also called **adrenaline**. It is synthesized from the amino acid, **tyrosine**, and secreted by the adrenal medulla in response to preganglionic sympathetic nervous stimulation. **Thyroxine** is another hormone derived by iodination of tyrosine molecules.

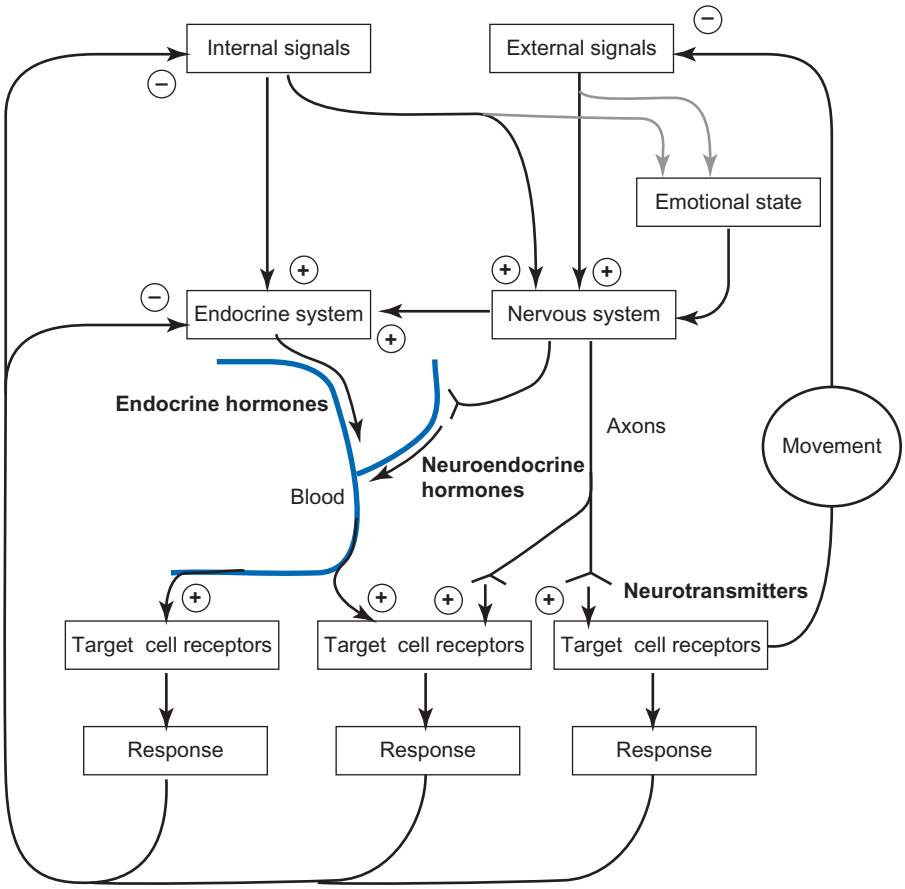

FIGURE 9.1.2 Overall control of body systems by the neuroendocrine systems. Interoreceptors sense the internal environment, and exteroreceptors sense events in the surrounding world. Interoreceptors can activate the endocrine branch directly, or they can activate the neural branch that then activates other nervous components or parts of the endocrine system. Exteroreceptors work exclusively through activation of the nervous system, which may then activate other neural, endocrine, or neuroendocrine responses. External and internal signals also influence the psychological state of the individual, which has profound, neurally mediated effects on the endocrine system. The two systems work together to provide integrated responses to challenges of homeostasis.

TABLE 9.1.1A Summary of the Major Human Hormones

Hormone Name	Source	Chemical Nature	Target Cells	Primary Action
Releasing hormones	Hypothalamus			
TRH (thyrotrophin releasing hormone)		3 aa chain	Thyrotrophs	Stimulates TSH release
CRH (corticotrophin releasing hormone)		41 aa chain	Corticotrophs	Stimulates ACTH release
GHRH (growth hormone releasing hormone)		40 or 44 aa chain	Somatotrophs	Stimulates GH release
GnRH (gonadotrophin releasing hormone)		10 aa chain	Gonadotrophs	Stimulates FSH and LH release
Somatostatin		14 aa chain	Somatotrophs	Inhibits GH release
Anterior pituitary hormones	Anterior pituitary			
TSH (thyroid stimulating hormone)	Thyrotroph cells	92 aa + 112 aa chains	Thyroid follicle cell	Stimulates thyroid hormone production + release
ACTH (adrenocorticotrophic hormone)	Corticotroph cells	39 aa chain	Adrenal cortex	Stimulates glucocorticoid, androgen production + release
GH (growth hormone)	Somatotroph cells	191 aa chain	Bone, liver, muscle	Stimulates growth
LH (luteinizing hormone)	Gonadotroph cells	92 aa + 112 aa chains	Ovarian follicle cells	Triggers ovulation
FSH (follicle stimulating hormone)	Gonadotroph cells	92 aa + 119 aa chains	Sertoli cells, ovarian cells	Stimulates growth and development
PRL (prolactin)	Lactotroph cells	199 aa chain	Alveolar cells of the breasts	Stimulates milk production

(*Continued*)

TABLE 9.1.1A (Continued)

Hormone Name	Source	Chemical Nature	Target Cells	Primary Action
Posterior pituitary hormones	Synthesized in hypothalamus, released in posterior pituitary			
ADH (antidiuretic hormone)		9 aa chain	Renal tubule and blood vessels	Increases water retention; vasoconstriction
Oxytocin		9 aa chain	Uterine muscle and breasts	Stimulates uterine contraction and milk "let-down"
Gastrointestinal hormones				
Gastrin	G-cells of stomach	17 aa or 34 aa chain	Parietal, ECL cells	Stimulates acid secretion
Cholecystokinin	Duodenal I cells	83 aa chain + fragments	Smooth muscle, neurons	Contracts gall bladder, slows gastric emptying
Secretin	S-cells in intestine	27 aa chain	Duct cells	Increases HCO_3^--rich secretion
Motilin	M-cells of duodenum	22 aa chain	Myenteric neurons	Coordinates migrating motor complex
Ghrelin	oxyntic glands	28 aa chain	hypothalamic neurons	stimulates appetite and release growth hormone
Somatostatin	D-cells of stomach	14 or 28 aa chain	Secretory cells	Inhibits gastric, biliary, and pancreatic secretion
Miscellaneous Polypeptide Hormones				
Insulin	β-cells of pancreas	30 aa + 21 aa chain	Liver, muscle, fat	Regulates glucose uptake and lipolysis
Glucagon	α-cells of pancreas	29 aa chain	Liver	Regulates glucose metabolism
Somatostatin	δ-cells of pancreas	14 aa chain	Pancreatic islets	Inhibits insulin and glucagon secretion
Parathyroid hormone (PTH)	Parathyroid gland	84 aa chain	Bone, kidney, lymphocytes	Regulates blood $[Ca^{2+}]$ and $[H_2PO_4^-]$
Calcitonin (CT)	C-cells of thyroid	32 aa chain	Bone	Inhibits bone resorption of Ca^{2+} and $H_2PO_4^-$
Atrial natriuretic peptide (ANP)	Cardiac atrial cells	28 aa chain	Renal tubule cells	Stimulates Na^+ excretion
Erythropoietin	Renal cells		Bone marrow stem cells	Stimulates erythropoiesis

TABLE 9.1.1B Summary of the Major Human Hormones

Hormone Name	Source	Chemical Nature	Target Cells	Primary Action
Thyroid hormone	Thyroid follicle	Modified tyrosine	Nearly all cells	Regulates energy metabolism
Epinephrine (adrenaline)	Adrenal medulla	Modified tyrosine	Circulatory system, liver	Engages "fight or flight" reaction
1,25-$(OH)_2$ cholecalciferol (vitamin D)	Skin, activated in liver and kidney	Secosteroid	Bone, intestine, kidney tubules	Mineralizes the skeleton by keeping plasma $[Ca^{2+}]$ high
Glucocorticoids	Adrenal cortex	Steroid	Fat, muscle, liver	Regulates metabolism
Aldosterone	Adrenal cortex	Steroid	Kidney tubule cells	Decreases Na^+ excretion and increases K^+ excretion
Androgens	Testes and adrenal	Steroid	Reproductive tract	Stimulate growth and development
Estrogens	Ovaries and placenta	Steroid	Reproductive tract	Stimulate growth and development
Progesterone	Ovaries and placenta	Steroid	Uterus and breasts	Stimulate growth and development

A great many of the endocrine hormones are polypeptide hormones, and many of these are glycosylated. These range in length all the way from **thyroid releasing hormone** (**TRH**), which is a tripeptide, to **human chorionic gonadotrophin** (hCG), which is about 34 kDa. Tables 9.1.1A and 9.1.1B lists the major hormones along with their source, target, and major physiological action. The polypeptide hormones can be classified in a variety of ways. These hormones include the following:

ANTERIOR PITUITARY RELEASING OR INHIBITING HORMONES

Cells in the hypothalamus release these hormones into a local circulation that delivers them to the anterior pituitary where they control the release of yet another set of hormones. They include:

- **Thyroid releasing hormone (TRH)**; 3 amino acids
- **Corticotrophin releasing hormone (CRH)**; 41 amino acid chain
- **Growth hormone releasing hormone (GHRH)**; 44 or 40 amino acids
- **Gonadotrophin releasing hormone (GnRH)**; 10 amino acids
- **Somatostatin (SST)**; 14 amino acids.

ANTERIOR PITUITARY HORMONES

The anterior pituitary secretes a set of trophic polypeptide hormones that influence other endocrine glands. The anterior pituitary hormones include:

- **Thyroid stimulating hormone (TSH)**; two chains; glycoprotein
- **Adrenocorticotrophic hormone (ACTH)**; 39 amino acids
- **Growth hormone (GH)**; 191 amino acids
- **Luteinizing hormone (LH)**; two chains; glycoprotein
- **Follicle stimulating hormone (FSH)**; two chains; glycoprotein
- **Prolactin (PRL)**; 199 amino acids.

For most of these, the names are self-explanatory. TSH stimulates the thyroid gland. ACTH stimulates the adrenal cortex. GH stimulates whole body growth and also has particular effects on the skeleton. LH, FSH, and PRL are all hormones involved in sexual reproduction. Each of these hormones will be discussed in later chapters.

POSTERIOR PITUITARY HORMONES

Hypothalamic neurons project to the posterior pituitary where they release neuroendocrine hormones into the general circulation. These include:

- **Antidiuretic hormone** (**ADH**), also known as vasopressin; 9 amino acids
- **Oxytocin**; 9 amino acids.

ADH regulates the excretion of water by the kidney. Oxytocin contracts the uterus during **parturition** and also stimulates the **milk ejection reflex** during breastfeeding of the infant.

GASTROINTESTINAL HORMONES

The gastrointestinal system releases its own set of hormones that mainly regulate gastrointestinal function. The function of these hormones was discussed in Unit 8. These polypeptide hormones include:

- **Gastrin**; 17 or 34 amino acid chain
- **Cholecystokinin**; 83 amino acids
- **Secretin**; 27 amino acids
- **Motilin**; 22 amino acids
- **Somatostatin**; 14 or 28 amino acid chain
- **Ghrelin**; 28 amino acids.

OTHER POLYPEPTIDE HORMONES ARE RELEASED BY INDIVIDUAL GLANDS

A number of other polypeptide hormones are released by a variety of endocrine glands.

- **Insulin**, released by β-cells in the islets of Langerhans in the pancreas
- **Glucagon**, released by α-cells in the pancreas
- **Somatostatin**, released by δ-cells in the pancreas
- **Parathyroid hormone** (**PTH**), released by the parathyroid glands
- **Calcitonin** (**CT**), released by C-cells in the thyroid
- **Atrial natriuretic peptide** (**ANP**), released by cardiac atrial cells
- **Erythropoietin**, released by the kidney
- **FGF-23, fibroblast growth factor**, released by osteocytes
- **IGF-I** and **IGF-II**, released by the liver.

STEROID HORMONES ARE SYNTHESIZED FROM CHOLESTEROL

A variety of steroid hormones are produced in the adrenal cortex, gonads, and placenta. These include:

- **Androgens**, including **testosterone** and **dehydroepiandrosterone** (**DHEA**)
- **Estrogens**, primarily **estradiol**
- **Glucocorticoids**, including **cortisol** and **corticosterone**
- **Mineralocorticoids**, including **aldosterone**.

The skin produces

- **Cholecalciferol**, precursor to its most active form, **1,25-dihydroxycholecalciferol**.

POLYPEPTIDE HORMONES ARE TYPICALLY SYNTHESIZED AS LARGER PRECURSORS

Cells synthesize polypeptide hormones using the same processes for the synthesis of other secretory products such as digestive enzymes and mucin that we have already described (see Chapters 2.3 and 2.4). The process is summarized in Figure 9.1.3.

Briefly, RNA polymerase II transcribes specific regions of the DNA that carries the instruction for synthesizing the hormone. After processing, mRNA leaves the nucleus to attach to ribosomes on the rough endoplasmic reticulum (ER) where it is translated into protein. Generally, the mRNA first directs the synthesis of a larger precursor called

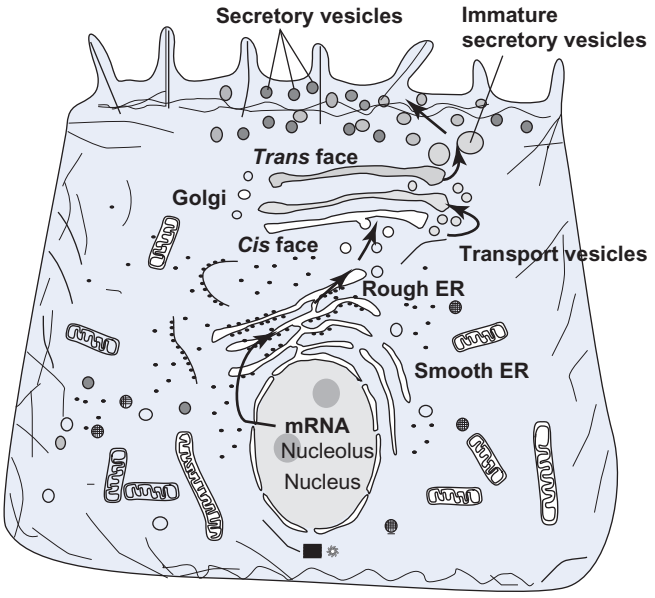

FIGURE 9.1.3 Overall processing of peptide hormones. The mRNA originates in the nucleus and is transported to the rough ER where it specifies the synthesis of a preprohormone. The preprohormone is elongated and transferred to the lumen of the ER. It is cleaved to form a prohormone. From there it travels to the Golgi stack by transport vesicles that bud off the ER membrane. In the Golgi stack, the hormone is further processed by proteolysis and possibly by glycosylation. The Golgi apparatus packages the polypeptide into immature secretory vesicles which concentrate the hormone into secretory vesicles. These vesicles form a storage pool of hormone that can be released rapidly upon stimulation of the secretory cell.

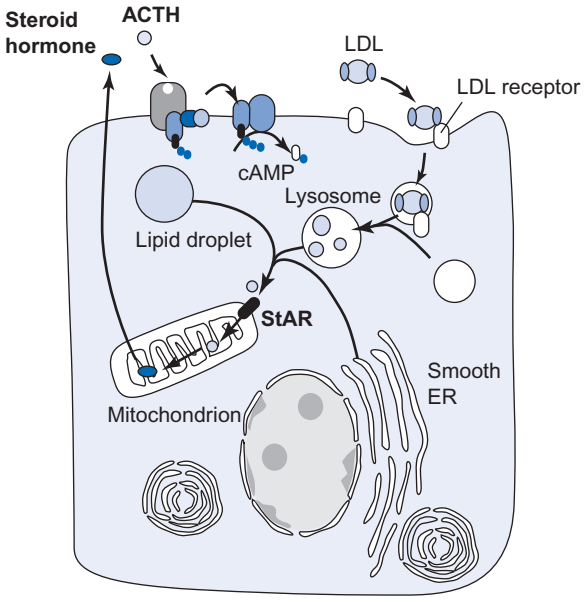

FIGURE 9.1.4 Process of synthesis and secretion of steroid hormones in cells of the adrenal cortex. Synthesis is stimulated by ACTH, or corticotropin, which is coupled to increased cytoplasmic concentrations of 3′,5′cAMP through a heterotrimeric G-protein. The synthesis of the steroid hormones occurs in the inner mitochondria and is controlled by the activity of StAR, a 30-kDa protein that transfers cholesterol from the outer mitochondrial membrane to the inner membrane. The steroid hormones leave the cell by passive diffusion.

a **preprohormone** that has a signal sequence at the N-terminus of the growing peptide chain. This signal sequence is recognized by a **signal recognition particle (SRP)** (see Chapter 2.4), which stops elongation and transfers the peptide chain to a **translocon**, a protein channel in the ER membrane. After SRP dissociates from the ribosome, chain elongation resumes and the growing peptide chain passes into the lumen of the ER via the translocon.

The portion of the ER containing enclosed polypeptide buds off the ER membrane and travels to the *cis*, or forming, face of the **Golgi stack**. The opposite pole of the Golgi stack facing the plasma membrane is the *trans* or maturing face. Vesicles are able to transport materials through the Golgi stack in either direction: from *cis* face to *trans* face or from *trans* face to *cis* face. During transit, proteins are processed in a variety of ways, including proteolytic cleavage, glycosylation, and association of different subunits. The finished product buds off the *trans* face to form an immature secretory vesicle, which matures by concentration of its enclosed contents, probably by removal of excess membrane back to the Golgi. These **secretory vesicles** form a reservoir of hormone that can be mobilized when the secretory cell is stimulated.

STEROID HORMONES ARE METABOLIZED FROM CHOLESTEROL AND ARE NOT STORED

Unlike cells that secrete polypeptides, steroid producing cells do not make secretory vesicles with stores of ready-made hormone that await the signal for secretion. Instead, steroid hormone synthesis and secretion is all part of a single process. The steroid hormones are synthesized from cholesterol that originates either from circulating low-density lipoproteins that are taken up by the secreting cells, from cholesterol stores in lipid droplets within these cells, or from cholesterol that is newly synthesized from acetyl CoA on the smooth endoplasmic reticulum of these cells. The rate-limiting step appears to be the transfer of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane, where the enzymes that synthesize the hormones are located. A protein called **steroid acute regulatory protein (StAR)** carries the cholesterol across. This 30-kDa protein is regulated by cytosolic levels of cAMP. ACTH increases cAMP by binding to its G_s-coupled receptor on the surface of these cells. The overall process of steroid hormone synthesis and secretion is shown schematically in Figure 9.1.4.

BLOOD CARRIES HORMONES IN EITHER FREE OR BOUND FORMS

The polypeptide hormones and epinephrine are readily soluble in water, and so the blood carries them in the dissolved state. These hormones do not readily penetrate cell membranes. Instead, they bind to receptors on the outside surface of the target cells. How this binding is used to affect the target cell has been discussed in general terms in Chapter 2.8. A variety of other hormones are not readily soluble in water. Large carrier

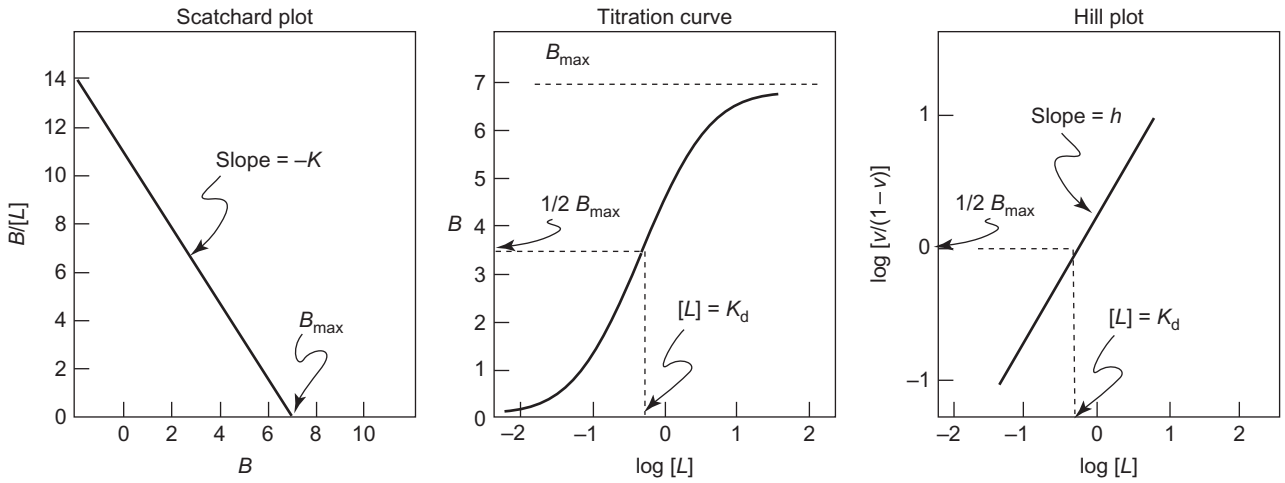

FIGURE 9.1.5 Examples of the Scatchard plot, titration curve, and Hill plot. The Scatchard plot is generally used to determine the affinity of the receptor for its ligand and the number of binding sites; the titration curve best shows how the affinity is determined by points above and below K_d, and shows the whole range of response; the Hill Plot is generally used to determine the cooperativity of the ligand–receptor interaction. See Appendix 9.1.A1 for a full discussion.

proteins bind to these materials and carry them in the plasma. These binding proteins include:

- **Thyroxine binding globulin (TBG)**
- **Corticosteroid binding globulin (CBG or transcortin)**
- **Sex hormone binding globulin (SHBG)**
- **Vitamin D binding protein**
- **Albumin.**

These proteins are made by the liver and secreted into the circulation. Most of these are about 50–60 kDa plasma glycoproteins. They bind the hormones with affinities that depend on the hormone. TBG binds mainly triiodothyronine (T3) and tetraiodothyronine (T4). Corticosteroid binding protein binds both cortisol and progesterone, whereas the SHBG carries testosterone and estrogen. Albumin binds many hydrophobic materials nonspecifically. All of these carrier proteins are in dynamic equilibrium with free hormone. Typically, only 1–10% of the total blood hormone is free in solution; the remaining 90–99% remains bound to the carrier. All of the materials carried by plasma binding proteins are believed to exert their effects by penetrating cells directly and binding to receptors within the target cells. Thus only the free material is biologically active, because only it can penetrate cells to exert biological effects. Most of these hydrophobic hormones alter genomic expression of their target cells, although nongenomic effects also occur.

ONLY TARGET CELLS WITH RECEPTORS TO THE HORMONE RESPOND TO THE HORMONE

Hormones are very much like neurotransmitters in that **only cells that have receptors to the hormone can respond**. Peptide hormones bind to the plasma membrane of their target cells, initiating a signaling cascade to change target cell behavior. This requires receptors for the hormone on the surface membrane. The collection of receptors on the cell's membrane defines its responsiveness to the hormones. The hydrophobic hormones bind to intracellular receptors and only those cells with the appropriate intracellular receptors can respond. Specificity of hormone action derives from the specificity of the molecular interaction of hormone and receptor.

DOSE–RESPONSE CURVES DERIVE FROM THE KINETICS OF HORMONE BINDING AND POST-RECEPTOR EVENTS

The kinetics of ligand binding to receptors can be graphically represented in a variety of ways including the **Scatchard plot**, the **titration curve**, or the **Hill plot**. The mathematical basis for these plots is discussed in Appendix 9.1.A1. The three types of plots are shown in Figure 9.1.5.

THE SCATCHARD PLOT *PLOTS BOUND/FREE VERSUS BOUND*

The Scatchard analysis plots the total amount of bound ligand divided by its free concentration ($B/[L]$) against the total bound ligand, B. For a single kind of receptor, the slope is equal to $-K$, the association constant for the binding of ligand to receptor, and the x-intercept is equal to the number of binding sites for the ligand, B_{max}.

THE TITRATION CURVE *PLOTS THE AMOUNT OF BOUND LIGAND AGAINST THE LOG OF THE FREE LIGAND CONCENTRATION*

The titration curve plots the amount of bound ligand against the logarithm of the free ligand concentration. Its utility is in identifying the concentration of ligand at which one-half of the receptors are occupied. This occurs when the $[L] = K_d$, the dissociation constant for ligand binding.

HILL PLOTS *ARE USED WHEN SCATCHARD PLOTS ARE NOT LINEAR*

Hill plots define a saturation fraction, ν, which is the amount of bound ligand divided by the number of binding sites: $\nu = B/B_{max}$. The value of log $(\nu/(1-\nu))$ is plotted against log $[L]$. The slope of the line between $0.1 < \nu < 0.9$ is the Hill coefficient, h, and is an index of cooperativity. Values of $h < 1.0$ indicate negative cooperativity. This means that binding of ligand makes further binding of ligand more difficult; positive cooperativity occurs with values of $h > 1.0$, meaning that binding of ligand makes further binding easier.

DOSE–RESPONSE CURVES ARE MOST EASILY VISUALIZED WITH TITRATION CURVES

The dose–response curve for a target tissue usually uses some unit of biological response instead of occupancy of the receptors, on the assumption that biological response relates linearly to receptor occupancy.

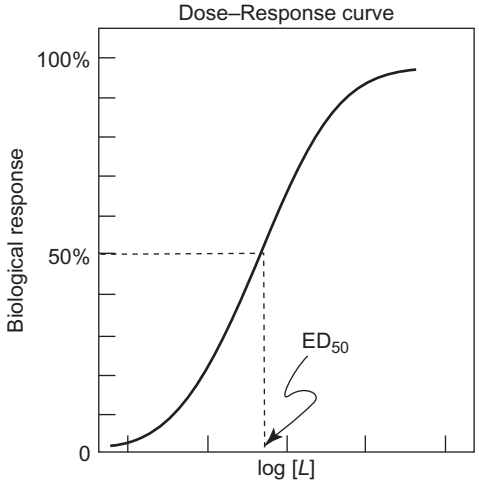

FIGURE 9.1.6 Typical dose–response curve for the effect of a hormone on a target tissue. Generally the dose–response curve has a sigmoid shape. The concentration of hormone at one-half maximal target cell activity is called the ED_{50}, for effective dose at 50% of maximal response.

Typically, targets exhibit some **basal activity** that occurs in the absence of the hormone. The response to increasing hormone passes a **threshold** level, which is the smallest discernible increase in biological response, to eventually reach a **maximal response**. The concentration of hormone required to produce a response halfway between the basal level and the maximal level is called the **median effective dose** or $\mathbf{ED_{50}}$ (the effective dose at 50% response). The overall dose–response curve depends on the kinetics of hormone binding to its receptor and to the kinetics of postreceptor events (see Figure 9.1.6).

DOSE–RESPONSE CURVES CAN BE "UPREGULATED" OR "DOWNREGULATED"

The sensitivity of target tissues to hormones can change with conditions. Two major ways in which the dose–response curve can change is in the maximal response and in the ED_{50}. These are shown diagrammatically in Figure 9.1.7.

Theoretically, the maximal response of a target tissue can be altered by changing

- **the number of active cells in the tissue**
- **the number of receptor molecules in the tissue**
- **the effectiveness of each receptor molecule**.

UPREGULATION *AND* DOWNREGULATION *ALTER THE NUMBER OF ACTIVE RECEPTORS*

Cells continually synthesize and degrade receptor molecules. Exposure to their ligand almost always causes **downregulation**, a decrease in the number of available or responsive receptors. This can occur rapidly by moving receptors from the surface membrane into intracellular sites where the latent sites cannot react to extracellular hormone. Although less common, **upregulation** also occurs. FSH and estradiol, for example, upregulate the number of their receptors in order to amplify their action.

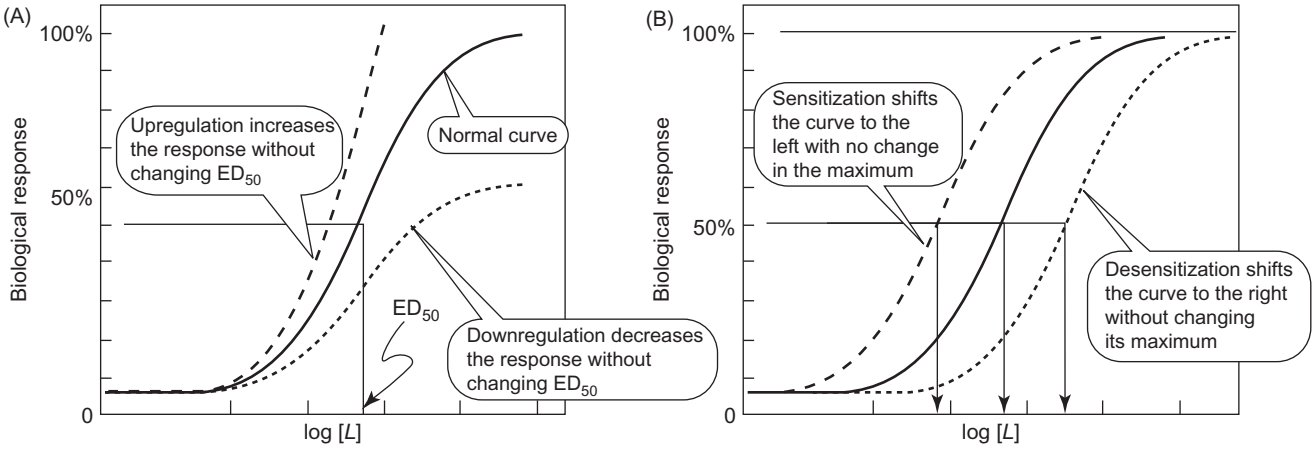

FIGURE 9.1.7 Dose–response curves reveal the mechanism by which target tissue response changes. Target tissue responsiveness may change, indicated by a change in the maximal response with no change in the ED_{50} (A). Target tissue sensitivity may also change (B) as indicated by a change in the ED_{50} without a change in the maximal response.

DESENSITIZATION *CHANGES THE CONCENTRATION DEPENDENCE OF TARGET RESPONSE*

Changes in the sensitivity of the target tissue shift the dose–response curve right or left without changing its maximum (see Figure 9.1.7). Desensitization refers to a shift to the right, so that higher concentrations of hormone are necessary to elicit the same response; **sensitization** indicates a shift to the left. If exposure to a hormone desensitizes the target to itself, the process is **homologous desensitization**. If desensitization results from exposure to some other agent, the process is **heterologous desensitization**.

THE *HALF-LIFE* AND *METABOLIC CLEARANCE RATE* QUANTITATIVELY DESCRIBE HORMONE METABOLISM

Hormones disappear from the circulation by

- **uptake by target cells**
- **metabolism**
- **excretion into the urine or bile.**

The overall process can be described quantitatively by the **metabolic clearance rate (MCR)**, *defined* as:

$$\text{MCR} = \frac{\text{amount of hormone removed per unit time (in units of mg min}^{-1})}{\text{plasma[hormone] (in units of mg mL}^{-1})} \quad [9.1.1]$$

The units of MCR are mL min^{-1}, which are the same units used to express renal clearance. MCR corresponds to the volume of plasma that contains all of the hormone that is cleared in 1 minute. This is usually determined by injecting someone with a small amount of radioactive hormone and determining the rate of disappearance of the labeled hormone from serial blood samples. The rate of disappearance of labeled hormone generally obeys **first-order kinetics**: the rate of hormone loss is proportional to how much hormone is present. This is expressed quantitatively as

$$\frac{dH}{dt} = -kH \quad [9.1.2]$$

where H is the amount of labeled hormone and k is the aggregate rate constant. The description "first-order kinetics" derives from this rate law being proportional to the first power of the substrate. This equation can be easily solved for $H(t)$ by separation of variables and integrating to give

$$H = H_0\, e^{-kt} \quad [9.1.3]$$

First-order plots are plots of $\ln H$ against t. If the process is first order, then plots of $\ln H$ against t should be linear with slope $= -k$. The **half-life** is defined as the time required for H to decay to $H_0/2$. Inserting $H = H_0/2$ into Eqn (9.1.3) we find

$$t_{1/2} = \frac{\ln 2}{k} = \frac{0.693}{k} \quad [9.1.4]$$

Thus the half-life $t_{1/2}$ is inversely related to the rate constant for disappearance of material. If k is large, then the half-life is short and the material disappears rapidly.

THE MCR IS INVERSELY RELATED TO THE HALF-LIFE

The MCR tells us what volume of plasma is cleared of hormone per minute. If this is large, it means that the hormone is rapidly cleared, which means that the half-life is short. Thus the half-life is inversely proportional to the MCR. The exact relationship between MCR and $t_{1/2}$ is apparent when we recognize from its definition that

$$\text{MCR} = \frac{-dH/dt}{H/V_d} \quad [9.1.5]$$

where dH/dt is an amount per unit time; since it is disappearing, dH/dt is negative and $-dH/dt$ is positive; H is the amount of hormone, and V_d is its volume of distribution; H/V_d is its concentration. We substitute in from Eqn (9.1.2) to obtain

$$\text{MCR} = \frac{kH}{H/V_d} = kV_d \quad [9.1.6]$$

Stated another way, we can write

$$k = \frac{\text{MCR}}{V_d} = \frac{\text{MCR}[H]}{V_d[H]} \quad [9.1.7]$$

Note that MCR$[H]$ is the size of the pool that turns over every minute, and $V_d[H]$ should be equal to the size of the pool of hormone (in units of amount) that is distributed in the volume V_d. The ratio of these two is the **fractional turnover rate**: the fraction of the entire hormone pool that turns over per minute. Thus Eqn (9.1.7) tells us that **the first-order rate constant for disappearance of hormone is equal to the fractional turnover rate**.

Half-life of hormones must be established using radioactive labels or some other technique because the total amount of hormone in the body does not obey Eqn (9.1.2). It obeys the equation:

$$\frac{dH}{dt} = -k\,H + S \quad [9.1.8]$$

where kH is the rate of degradation and S is the rate of secretion. The rate of secretion can be highly variable and episodic. If S is constant, then the amount of hormone in the body, and therefore its concentration, would be constant, set so $dH/dt = 0$ and $H = S/k$. Normally this is not the case. The rate of secretion is generally episodic and dependent on other variables that change with physiological condition, and the hormone levels fluctuate within some normal limits.

A VARIETY OF TECHNIQUES CAN MEASURE HORMONE LEVELS

THE EARLIEST HORMONE ASSAYS WERE BIOASSAYS

Many hormones were initially discovered by their effects on live animals or people, often through "experiments

of nature" in which the circulating hormone concentrations were either excessive or insufficient. Because of this, many of the early hormone assays were biological assays, or **bioassays**, in which a biological response was noted after injection of a sample. Biological assays have the disadvantages of lack of specificity, lack of sensitivity, slow response time, and the need to use many animals. They have been replaced by physical and immunological techniques.

CHROMATOGRAPHY *SEPARATES MATERIALS BASED ON PARTITIONING BETWEEN PHASES*

Chromatographic assays for hormones involves **high performance liquid chromatography (HPLC)** systems. These generally involve a solid phase column of material that interacts with dissolved materials as they flow past in a liquid phase. There are two general kinds: **normal phase** and **reverse phase** HPLC. The column packing in normal phase HPLC contains polar groups such as amino or nitrile ions. In reverse phase HPLC, the solid phase presents a nonpolar stationary stage. The liquid phase can be constant (**isocratic elution**), changed stepwise (**step elution**), or changed gradually in **gradient elution**. The trick is to choose the columns and elution solvents that separate the compounds of interest. The compounds can be detected in the eluate by a variety of techniques including light absorption, fluorescence, electrochemical properties, and mass spectroscopy. The advantage of HPLC is that it can allow the simultaneous detection of several related materials, such as the steroid hormones, in a single sample.

MASS SPECTROMETRY

Mass spectrometry detects materials on the basis of their charge to mass ratio. After cleaning up the samples by HPLC, for example, the sample is fragmented into charged ions. This fragmentation occurs in specific ways, depending on the chemical nature of the material, and so the resulting spectrum of fragments with mass/charge ratios allows qualitative and quantitative identification of the material.

RADIOIMMUNOASSAYS

Radioimmunoassays measure hormone concentration by competing with radioactively labeled material for

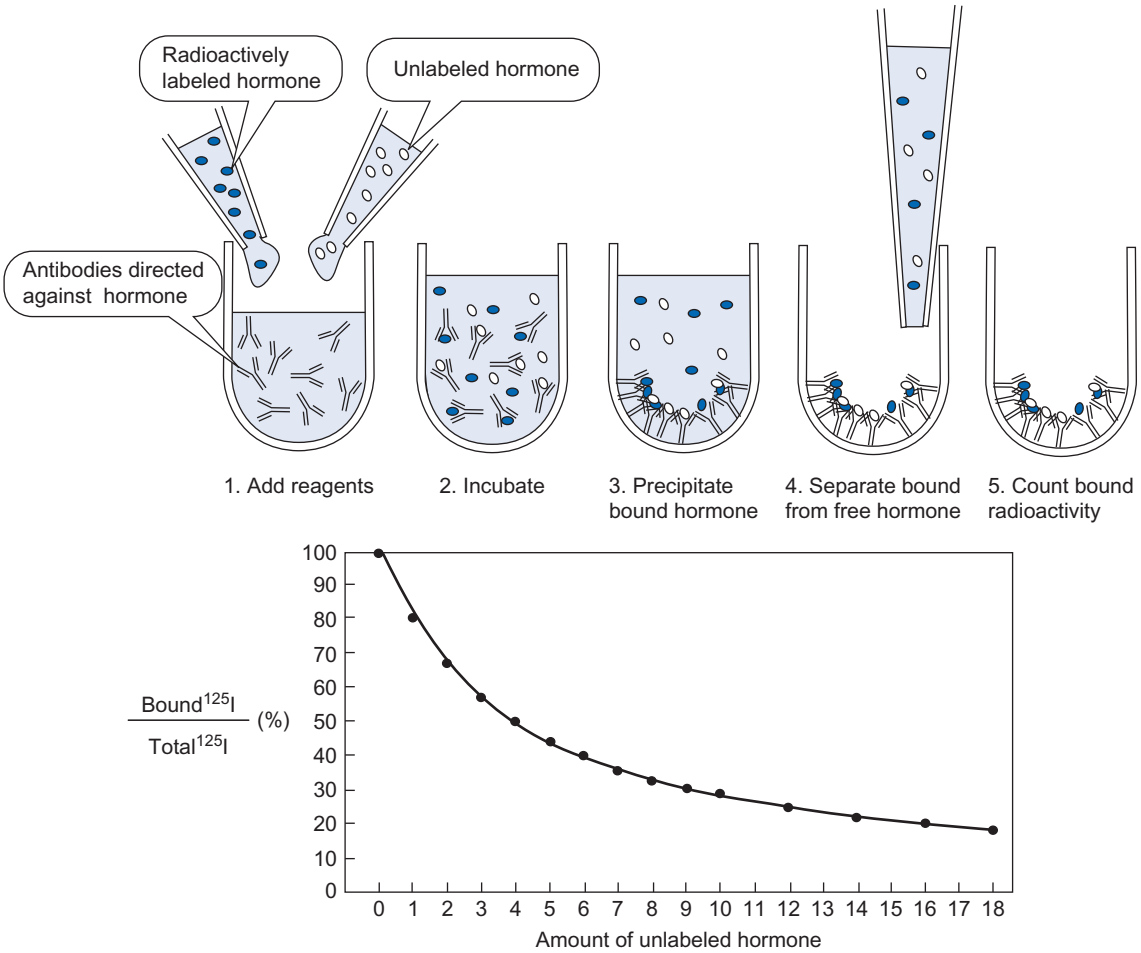

FIGURE 9.1.8 Basic principles of radioimmunoassay. Antibodies against the hormone are incubated in a series of tubes containing a constant amount of radioactively labeled hormone and various amounts of standard, unlabeled hormone or samples containing unknown amounts of hormone. The labeled and unlabeled hormones compete for binding sites on the antibodies. When more unlabeled hormone is present, less radioactivity binds to the antibody. After equilibration, the antibody–hormone complex is precipitated and collected into a pellet by centrifugation. The free unlabeled and radioactive hormone is removed and the bound radioactivity is measured using a radiation counter. Plots of the percent of radioactivity bound against unlabeled hormone concentration are highly nonlinear. The amount of hormone in the unknowns is determined by interpolation.

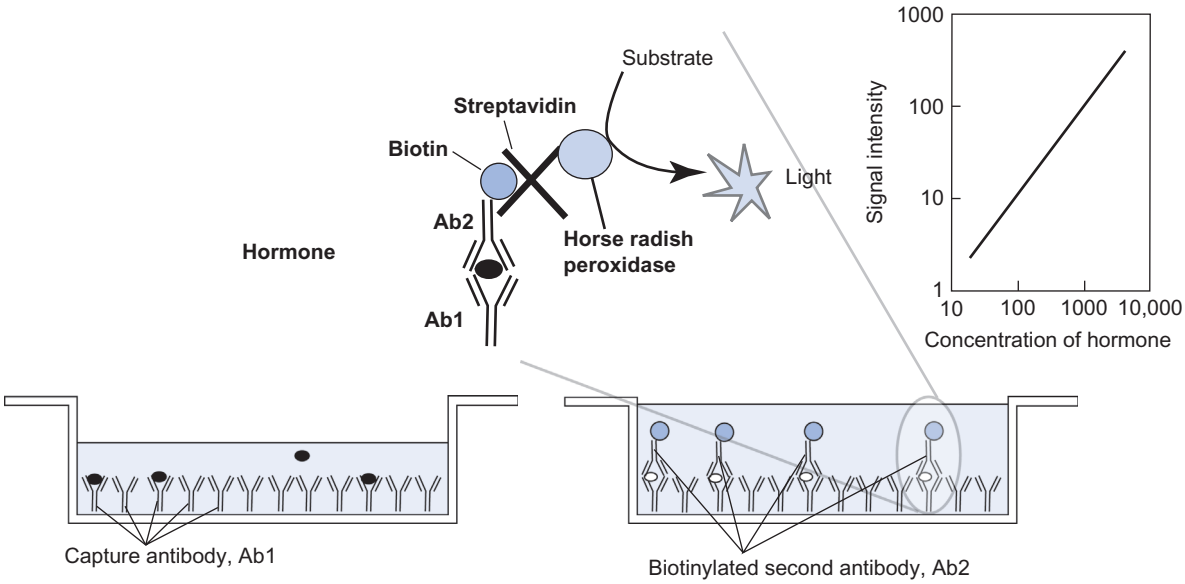

FIGURE 9.1.9 Basic principles of ELISA. The assay is conducted in plastic wells coated with a capture antibody, Ab1, present in great excess of hormone. Hormone binding to Ab1 is therefore proportional to its concentration. Incubation of a second antibody (Ab2) covalently linked with biotin makes an antibody–hormone–antibody sandwich. The biotin is a convenient handle to link Ab2 to an enzyme, HRP, by incubating the sandwich with streptavidin conjugated with HRP. Streptavidin has a very high affinity for biotin. Incubation of the resulting complex with appropriate substrates causes a chemiluminescent reaction. The intensity of the emitted light is proportional to the concentration of hormone over three orders of magnitude.

binding sites on antibodies that bind both labeled and unlabeled hormones. If there is little "cold" hormone, then most of the radioactivity will be bound. A lot of "cold" hormone will compete for the hormone binding site and less radioactivity will be bound. Standard curves are prepared by using a series of known amounts of cold hormone in separate tubes (see Figure 9.1.8).

ENZYME-LINKED IMMUNOSORBENT ASSAY

This assay is sometimes called the sandwich assay because the hormone is sandwiched between two antibodies. The assay is usually conducted in a 3×5 inch plastic plate containing 96 wells that are coated with a **capture antibody** (Ab1) that extracts hormone from the standards containing various known amounts of the hormone or the samples. The capture antibody is used in large excess over the hormone to be detected. The hormone–Ab1 complex is then reacted with a second antibody (Ab2) that can be linked to a detection system. One method of doing this is to complex Ab2 with **biotin**. Biotin is a water-soluble vitamin that is required as a cofactor for carboxylase reactions in the body. Its utility is that it can be easily covalently linked to protein molecules, and it is bound extremely tightly by **streptavidin**, a 70-kDa protein. The biotin on Ab2 serves as a link to streptavidin conjugated to **horseradish peroxidase** (HRP). Addition of substrates for HRP produces a signal that can be detected photoelectrically. This entire process is illustrated in Figure 9.1.9.

SUMMARY

Endocrine hormones are a class of signaling molecules that originate from a comparatively small and localized part of the body. They travel through the blood stream and reach distant target tissues where they exert specific effects. These classical endocrine hormones have been supplemented by a variety of signaling molecules that affect only nearby cells (paracrine hormones) or affect the cells that made them (autocrine hormones). In addition, nerve cells release neurocrine hormones. These signaling molecules work together with the nervous system to regulate normal bodily function.

Hormones can be grouped according to their chemical structure as (1) modified amino acids; (2) polypeptides; and (3) steroid derivatives. Each group contains a large number of examples.

Amino acid derivatives include epinephrine and thyroxine.

Polypeptide hormones include hypothalamic releasing factors, including TRH, CRH, GHRH, GnRH, and SST. The anterior pituitary hormones, all polypeptides, include TSH, ACTH, GH, LH, FSH, and PRL. The posterior pituitary hormones include ADH and oxytocin. The name of a polypeptide protein often describes its action.

Gastrointestinal polypeptide hormones include gastrin, cholecystokinin, secretin, motilin, ghrelin and SST. Other polypeptide hormones, secreted by individual glands, include insulin, glucagon, SST, parathyroid hormone, CT, ANP, and erythropoietin.

The steroid hormones include androgens, estrogens, glucocorticoids, mineralocorticoids, and cholecalciferol.

Peptide hormones are typically synthesized as larger prohormones and stored in secretory vesicles awaiting signals for their release. Steroid hormones are typically secreted as they are synthesized.

The response of target cells to circulating hormones depends on the hormone receptors. Lipid soluble hormones have intracellular receptors; polypeptide hormones bind to receptors on the cell surface. Binding of hormone to its receptor can be analyzed using Scatchard plots, titration curves, or Hill plots. The titration curve is often most useful because the full range of ligand concentration is easily visualized.

Secretion of most hormones is regulated whereas degradation follows simple first-order kinetics. The MCR is the volume of plasma completely cleared of hormone per minute. It is equal to the fractional turnover rate times the volume of distribution of hormone.

Hormone assays include radioimmunoassay, in which radioactively labeled hormone competes with "cold" hormone for binding sites on antibodies directed against the hormone. Enzyme-linked immunosorbent assay (ELISA) involves sandwiching the hormone between two antibodies, one of which is linked through biotin to streptavidin-linked HRP. Reaction of the HRP produces light that is proportional to the amount of captured hormone.

REVIEW QUESTIONS

1. What is a hormone? How is it distinguished from a neurotransmitter?
2. Name three major chemical classes of hormones. What is different about their synthesis and storage?
3. What are the two major types of hormone receptors?
4. What is a Scatchard plot? What is a titration curve? What is a Hill plot? What information can you derive from each?
5. What is a dose–response curve? What is downregulation? Upregulation? Desensitization?
6. What is the metabolic clearance rate? How is it related to fractional turnover?

APPENDIX 9.1.A1 ANALYSIS OF LIGAND BINDING

THE LANGMUIR ADSORPTION ISOTHERM

It was observed long ago that a finely divided solid exposed to a dye solution or a gas at low pressure caused a reduction in the dye concentration or a reduction in the gas pressure. In these cases the dye or gas was **adsorbed** onto the surface of the solid. The fine division of the solid ensured that there was a great total surface area of varying shapes and sizes to accommodate the dye or gas. This adsorption can be represented by a chemical equilibrium

[9.1.A1.1] $$A + S \rightleftharpoons A \cdot S$$

where A is the material being absorbed and S is the vacant site on the surface of the solid. We cannot write the equilibrium in the usual way by considering the concentration of A and S, because the concentration of the vacant sites is poorly defined: S is distributed on an area, not throughout a volume. The concentration of S would have different units than the concentration of A. However, these different units will actually cancel in the definition of the equilibrium. Let Θ be the **fraction** of sites occupied by A. Then $1 - \Theta$ will be the fraction of sites which remain vacant. Then we can write

[9.1.A1.2] $$K = \frac{\Theta}{(1-\Theta)A}$$

to define the equilibrium in the heterogeneous phase of liquid solution and solid absorbant. Here K is an association constant. This can be rearranged to give

[9.1.A1.3] $$\Theta = \frac{KA}{1+KA}$$

THE SCATCHARD PLOT

Consider a protein which has a single site for binding a ligand. The word "ligand" originates from the Latin "ligo" meaning "to tie." The protein may be soluble or itself bound to a membrane fraction. We define the **intrinsic association constant** to be

[9.1.A1.4] $$K = \frac{[L \cdot P]}{[L][P]}$$

where $[L]$ is the free ligand concentration in equilibrium with the binding site, $[P]$ is the concentration of the protein with the site vacant, and $[L \cdot P]$ is the concentration of protein with ligand bound. In strict analogy to the Langmuir Adsorption Isotherm, we define the **saturation fraction**, ν, as the proportion of the site which is occupied by L:

[9.1.A1.5] $$\nu = \frac{[L \cdot P]}{[L \cdot P] + [P]}$$

By Eqn (9.1.A1.4),

[9.1.A1.6] $$[L \cdot P] = K[L][P]$$

Substitution into Eqn (9.1.A1.5) and rearranging, we obtain:

[9.1.A1.7] $$\nu = \frac{K[L][P]}{K[L][P] + [P]} = \frac{K[L]}{1 + K[L]}$$

This may be rearranged to give

[9.1.A1.8] $$\begin{aligned} \nu + \nu K[L] &= K[L] \\ \frac{\nu}{[L]} &= K(1-\nu) \end{aligned}$$

This equation is the foundation of the Scatchard plot. In this plot, the values of $\nu/[L]$ are plotted against the values of ν. According to Eqn (9.1.A1.8), this plot should result in a straight line intersecting the vertical, $\nu/[L]$ axis at K and intersecting the horizontal, ν axis at 1.0, the slope of the line being $-K$.

The real experimental situation is hardly ever so clear as this theoretical analysis. First, the binding reaction

being investigated may be occurring in a heterogeneous mixture consisting of more than one protein. The total number of binding proteins, and the number of binding sites on each protein, may be unknown. According to the definition of the saturation fraction, it is limited to the range $0 \leq \nu \leq 1.0$. In the experiment, some quantity of protein containing the binding site is incubated with a known concentration of radioactively labeled ligand. After incubation for sufficient time to ensure equilibration, as determined by previous experiments, the free ligand is separated from the bound ligand, and the amount of bound ligand is determined. The binding is then expressed as the amount of bound ligand per unit of protein, typically as pmol/mg or some similar unit. This is the amount bound, B. In the Scatchard plot, the axes are $B/[L]$ against B; the experimental value can be treated as ν, even though it is clear that it is no longer restricted to values between 0 and 1.0. In the Scatchard analysis using the dimensionless values of ν, the slope is $-K$ in units of M^{-1}. Using the experimental values of B gives the identical slope, but the intercept on the B axis is now the B_{max}, the maximum binding at saturation.

The above description holds for a mixture of proteins, each of which binds at a single site and which are independent of each other. Suppose now that we introduce the notion of multiple sites. We define the intrinsic association constant of the ith site to be

$$[9.1.A1.9] \qquad \nu_i = \frac{K_i[L][P]}{K_i[L][P] + [P]} = \frac{K_i[L]}{1 + K_i[L]}$$

Suppose now that there are more than one sites on the protein, and that the ligand binding can be modeled as a set of j independent sites, each with the identical intrinsic association constant, K_i. Now we define the **gross saturation fraction** as

$$[9.1.A1.10] \qquad \nu_i = \sum_{i-1}^{j} \frac{K_i[L][P]}{K_i[L][P] + [P]}$$

If all of these j independent sites are characterized by the same intrinsic association constant, then Eqn (9.1.A1.10) becomes

$$[9.1.A1.11] \qquad \nu_i = \frac{nK[L]}{1 + K[L]}$$

where n is the number of binding sites. This equation may be rearranged into the Scatchard form:

$$[9.1.A1.12] \qquad \frac{\nu}{[L]} = K(n - \nu)$$

In using the experimental values for the ligand binding, this is

$$[9.1.A1.13] \qquad \frac{B}{[L]} = K(B_{max} - B)$$

The result of the plot of $B/[L]$ against B, then, is a slope equal to $-K$, and an intercept on the B axis equal to B_{max}, and an intercept on the $B/[L]$ axis equal to $B_{max}K$.

The discussion above pertains to the situation in which there is a single set of noninteracting sites with identical association constants. Consider now the case in which there are two sets of ligand-binding sites. Consider for now that the sites do not interact and each set of sites is characterized by one association constant, but the two sets have different K_s. Each set of sites is described by its own saturation fraction, which we express here in terms of the experimentally observable B, the amount of ligand bound per unit total protein:

$$[9.1.A1.14] \qquad B_1 = \frac{B_{max\ 1}K_1[L]}{1 + K_1[L]} \qquad B_2 = \frac{B_{max\ 2}K_2[L]}{1 + K_2[L]}$$

and each of these relations can be transformed to the Scatchard equation:

$$[9.1.A1.15] \qquad \frac{B_1}{[L]} = K_1(B_{max\ 1} - B_1) \qquad \frac{B_2}{[L]} = K_2(B_{max\ 2} - B_2)$$

Now the binding of ligand to these two sets of sites occurs in a single reaction medium which has a single free ligand concentration $[L]$. The total amount of ligand bound is just the sum of the amounts bound to the different sets of sites, as given by Eqn (9.1.A1.10). We may divide the total amount bound by the free ligand concentration to obtain the relations:

$$[9.1.A1.16] \qquad B = B_1 + B_2 \qquad \frac{B_1}{[L]} = \frac{B_1}{[L]} + \frac{B_2}{[L]} \qquad \frac{B}{[L]} = K_1(B_{max\ 1} - B_1) + K_2(B_{max\ 2} - B_2)$$

According to Eqn (9.1.A1.15), each set of sites independently plotted as $B/[L]$ against B gives an intercept on the $B/[L]$ axis of K_1B_{max1} or K_2B_{max2}, and an intercept on the B axis of B_{max1} or B_{max2}, with a slope of $-K_1$ or $-K_2$. For a protein (or mixture of proteins) containing both sets of sites, the resulting Scatchard plot is the vector sum of the individual plots. Figure 9.1.A1.1 shows a Scatchard plot for two sets of sites determined separately. The resulting Scatchard plot when the two sets of sites are present in a single mixture can be constructed from the individual graphs by realizing that all points on a line passing through the origin are characterized by the same free ligand concentration. If the two sets of sites are present in the mixture and are at equilibrium, they must be simultaneously in equilibrium with a single $[L]$. Along a line passing through the origin, $B = B_1 + B_2$ and $B/[L] = B_1/[L] + B_2/[L]$. The composite B and $B/[L]$ for the two sets of sites is the sum of the individual values along the lines of equal $[L]$. Figure 9.1.A1.1 shows the way in which the composite Scatchard results from the vector addition of the two component sets of sites.

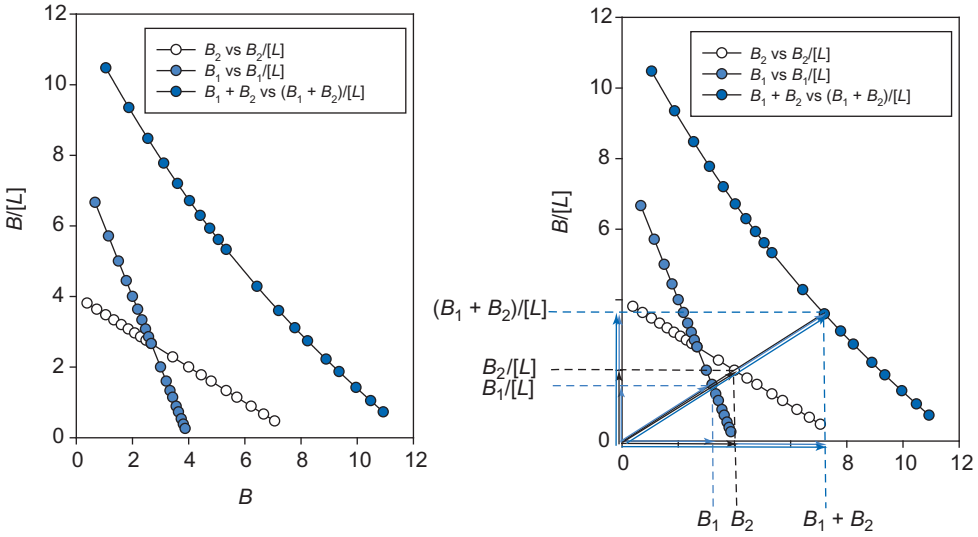

FIGURE 9.1.A1.1 Scatchard plot for two independent sites and their resulting composite plot.

This theoretical analysis shows how the binding constants and number of binding sites produce a composite Scatchard plot. The experimental problem, however, is this: how does one determine the affinities (association constants) and number of binding sites given an experimentally derived Scatchard plot? This is generally called the **inverse problem**. Predicting the behavior of a complex system given its characteristics is generally much easier than uniquely determining what those characteristics are, given the behavior of the system. Taking the theoretical results of Figure 9.1.A1.1, for example, the resulting Scatchard plot for two sets of sites is nearly linear. Given experimental error, it would be a common error to assume there is only one set of binding sites because the two sites do not differ enough in their association constants to produce a sufficiently curved composite plot. Even if there is only one set of binding sites, the analysis of the Scatchard plot can be complicated. Application of linear regression to such a plot, for example, is inappropriate because linear regression assumes no error in determination of the abscissa value, and all the error is in the ordinate value. In the case of the Scatchard plot, it is generally assumed that the free ligand concentration is known exactly, and that the error is entirely in the determination of B. In this case, the error bars would lie on a line passing through the origin. In practice, however, there is error in both B and $[L]$. Computer programs such as LIGAND are available which peel the Scatchard plot into its component parts, while minimizing the global error in fitting the binding parameters to the data. There are a number of cautions which must be exercised in this process. Basically, the cautions boil down to one piece of advice: do not waste powerful mathematics on bad data. It is better to spend the time to get good binding data.

THE TITRATION CURVE

The Scatchard analysis given above used the association constant, K, as one descriptor of the binding reaction. As noted above, the association constant has the units of M^{-1}. The dissociation constant, K_d, is the inverse of the association constant, with units of M. For the case of a single set of noninteracting sites with identical K, we write:

$$[9.1.A1.17] \qquad B = \frac{B_{max}K[L]}{1 + K[L]}$$

The **titration curve** is a plot of B against log $[L]$. It is analogous to the alkaline titration of acid, in which the amount of added strong alkali (NaOH) is plotted against the pH. The curve is S-shaped. Its maximum slope occurs when $[L] = K_d$. To see this, we differentiate Eqn (9.1.A1.17) with respect to log $[L]$:

$$\begin{aligned} \frac{dB}{d\log[L]} &= [L]\frac{dB}{d[L]} \\ &= [L]\left[\frac{B_{max}K}{1 + K[L]} - \frac{B_{max}K^2[L]}{1 + K[L]^2}\right] \\ &= B_{max}\left[\frac{K[L]}{1 + K[L]} - \left(\frac{K[L]}{1+K[L]}\right)^2\right] \\ &= B_{max}[\nu - \nu^2] \end{aligned} \qquad [9.1.A1.18]$$

where ν is the saturation fraction defined by Eqn (9.1.A1.7), whose range is restricted to values between 0 and 1.0. The slope of the titration curve can be seen from this relation to be zero when $\nu = 0$ and when $\nu = 1.0$, and to have a maximum when $\nu = 0.5$. The maximum slope occurs at an inflection point in the S-shaped curve. The inflection point at $\nu = 0.5$ can be derived by setting the second derivative of B against log$[L]$ equal to zero. When $\nu = 0.5$, from the definition of ν, $[L] = 1/K = K_d$; this is the point at which the free ligand concentration is equal to the dissociation constant for the sites. An example of the titration curve is shown in Figure 9.1.A1.2.

The utility of the titration curve is that it allows all of the binding data to be represented in a single plot and it visually allows judgment of an important point: do the binding data encompass values of B both above and

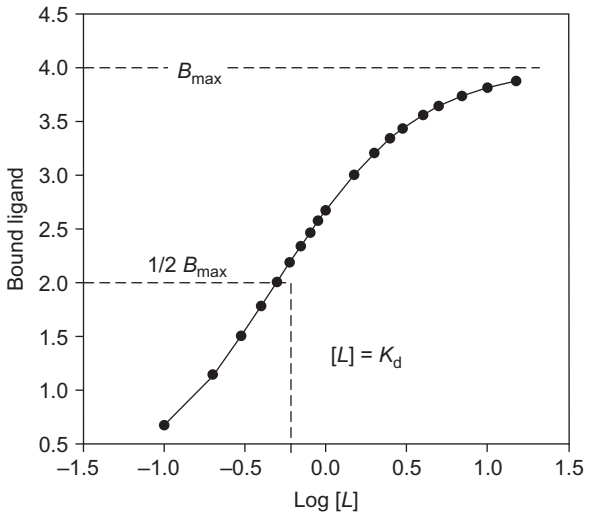

FIGURE 9.1.A1.2 Titration binding curve with $B_{max} = 4$ and $K_d = 0.5$.

below 0.5 B_{max}? This is important quality control in determining if the binding data are up to the task of characterizing K_d and B_{max}. Unless the inflection point is well defined, how is it possible in the titration curve to predict where the binding will level off to B_{max}? Both K_d and B_{max} are best defined when there are B, $[L]$ data taken above and below $\nu = 0.5$. (See I.M. Klotz, Number of receptor sites from Scatchard graphs: facts and fantasies. *Science* **217**:1247–1249, 1982.)

COOPERATIVITY AND THE HILL PLOT

According to the above discussion, there are three plots one can make to represent ligand-binding data: (1) the adsorption isotherm in which the degree of binding, B (or, alternatively, the saturation fraction, ν) is plotted against the free ligand concentration, $[L]$; (2) the titration curve, in which the degree of binding is plotted against the logarithm of $[L]$; and (3) the Scatchard plot, in which the ratio of the degree of binding to $[L]$ is plotted against the degree of binding. According to the analysis presented so far, the Langmuir adsorption curve should show a hyperbolic curve, the titration curve should be *S*-shaped, and the Scatchard plot should be linear. The analysis so far, however, has been limited to identical sets of **noninteracting** sites. Sometimes, there is interaction between the sites so that the binding at one site affects the binding at another. This interaction is referred to as **cooperativity**. If binding ligand at one site increases the association constant at a vacant site, then the cooperativity is positive; if binding the first ligand decreases the association constant at the vacant site, the cooperativity is negative. A quantitative index of cooperativity is the **Hill coefficient**.

Hill attempted to explain the binding of oxygen to hemoglobin by postulating empirically that h molecules of oxygen were bound in a single step:

[9.1.A1.19] $$P + hL \rightleftharpoons P \cdot L_h$$

with an association constant given by

[9.1.A1.20] $$K = \frac{[P \cdot L_h]}{[P][L]^h}$$

Here the fractional saturation is given by

[9.1.A1.21] $$\nu = \frac{[P \cdot L_h]}{[P] + [P \cdot L_h]} = \frac{K[L]^h}{1 + K[L]^h}$$

Hill proposed this equation purely on an empirical basis, and the equation is now known as the **Hill equation**, with the variable Y usually being used instead of ν. This equation can be rearranged to give

[9.1.A1.22] $$\frac{\nu}{1-\nu} = K[L]^h$$
$$\log\left[\frac{\nu}{1-\nu}\right] = \log K + h \log[L]$$

Thus a plot of $\log(\nu/(1-\nu))$ against $\log[L]$ should give a straight line with slope h. This plot is the **Hill plot**, and it allows evaluation of K and h. This plot may also be applied to enzyme reactions, where ν is usually calculated as v/V_{max}, where v is the enzyme velocity and V_{max} is the maximum enzyme velocity.

The exponent h is referred to as the **Hill coefficient**. It is an index of the cooperativity as the larger h, the greater the degree of cooperativity. Its interpretation will become clearer after we consider the Adair equation.

Adair considered that the binding of oxygen to hemoglobin could be described as a four-step process (because the hemoglobin molecule was a tetramer), rather than a single concerted step as required by the Hill equation. In this case, we write:

[9.1.A1.23] $$P + L \rightleftharpoons P \cdot L$$
$$P \cdot L + L \rightleftharpoons P \cdot L_2$$
$$P \cdot L_2 + L \rightleftharpoons P \cdot L_3$$
$$P \cdot L_3 + L \rightleftharpoons P \cdot L_4$$

We suppose that the binding sites are identical, but they may be influenced identically by prior occupation of adjacent sites. Thus we define an **intrinsic or site association constant**, K_1, K_2, K_3, and K_4 for the binding to the first, second, third, and fourth sites, respectively. The rate of binding to the protein molecule, however, is affected by the number of vacancies. To convert the site constants to **molecular constants**, we must multiply by the number of sites involved in the reaction per molecule. Equilibrium is attained when the forward rate is equal to the reverse rate. So for the binding of ligand to the first site, we write:

[9.1.A1.24] $$Q_{on\ 1} = 4\ K_{on\ 1}[P][L]$$
$$Q_{off\ 1} = K_{off\ 1}[P \cdot L]$$

where Q_{on1} and Q_{off1} are the rates of the on reaction and off reaction at the first site, respectively. The molecular on rate constant is $4\ K_{on1}$ because there are four

empty sites on each protein molecule. At equilibrium, the on and off rates are equal, so we define:

$$[9.1.A1.25] \quad \begin{aligned} Q_{\text{on }1} &= Q_{\text{off }1} \\ 4\,K_{\text{on }1}[P][L] &= K_{\text{off }1}[P\cdot L] \\ \frac{[P\cdot L]}{[P][L]} &= \frac{4K_{\text{on }1}}{K_{\text{off }1}} = 4K_1 \end{aligned}$$

In analogy, we write the relations as

$$[9.1.A1.26] \quad \begin{aligned} \frac{[P\cdot L_2]}{[P\cdot L][L]} &= \frac{3K_{\text{on }2}}{2K_{\text{off }2}} = \frac{3}{2}K_2 \\ \frac{[P\cdot L_3]}{[P\cdot L_2][L]} &= \frac{2K_{\text{on }3}}{3K_{\text{off }3}} = \frac{2}{3}K_3 \\ \frac{[P\cdot L_4]}{[P\cdot L_3][L]} &= \frac{K_{\text{on }4}}{4K_{\text{off }4}} = \frac{1}{4}K_4 \end{aligned}$$

In this formalism, all four constants would be equal ($K_1 = K_2 = K_3 = K_4$) if the four sites were identical and acted independently.

In Adair's model, the concentration of the various bound forms is given by Eqns (9.1.A1.25) and (9.1.A1.26) as

$$[9.1.A1.27] \quad \begin{aligned} [P\cdot L] &= 4K_1[P][L] \\ [P\cdot L_2] &= \frac{3}{2}K_2[P\cdot L][L] = 6K_1K_2[P][L]^2 \\ [P\cdot L_3] &= \frac{2}{3}K_3[P\cdot L_2][L] = 4K_1K_2K_3[P][L]^3 \\ [P\cdot L_4] &= \frac{1}{4}K_4[P\cdot L_3][L] = K_1K_2K_3K_4[P][L]^4 \end{aligned}$$

We now use these results to calculate the saturation fraction as

$$\begin{aligned} \nu &= \frac{[P\cdot L] + 2[P\cdot L_2] + 3[P\cdot L_3] + 4[P\cdot L_4]}{4([P] + [P\cdot L] + [P\cdot L_2] + [P\cdot L_3] + [P\cdot L_4])} \\ &= \frac{\begin{aligned}&4K_1[P][L] + 12K_1K_2[P][L]^2 + 12K_1K_2K_3[P][L]^3 \\ &+ 4K_1K_2K_3K_4[P][L]^4\end{aligned}}{\begin{aligned}&4[P] + 16K_1[P][L] + 24K_1K_2[P][L]^2 \\ &+ 16K_1K_2K_3[P][L]^3 + 4K_1K_2K_3K_4[P][L]^4\end{aligned}} \end{aligned}$$

[9.1.A1.28]

Factoring out 4[P] from the numerator and denominator, we have

$$\nu = \frac{K_1[L] + 3K_1K_2[L]^2 + 3K_1K_2K_3[L]^3 + K_1K_2K_3K_4[L]^4}{1 + 4K_1[L] + 6K_1K_2[L]^2 + 4K_1K_2K_3[L]^3 + K_1K_2K_3K_4[L]^4}$$

[9.1.A1.29]

This last equation is known as the **Adair equation** for four sites. If all four intrinsic association constants are equal, then this equation simplifies as

$$[9.1.A1.30] \quad \nu = \frac{K[L](1+K[L])^3}{(1+K[L])^4} = \frac{K[L]}{1+K[L]}$$

which is the expression derived for noninteracting sites. If all four association constants are equal, the adsorption isotherm will be a hyperbola, and the Scatchard plot will be linear. Conversely, deviations from a hyperbola on the adsorption isotherm or from linearity on the Scatchard plot mean that the association constants cannot all be equal. In particular, if $K_4 >> > K_1$, K_2, and K_3, then Eqn (9.1.A1.29) becomes

$$[9.1.A1.31] \quad \nu = \frac{K_1K_2K_3K_4[L]^4}{1 + K_1K_2K_3K_4[L]^4}$$

which is the Hill equation (Eqn 9.1.A1.21) with $K = K_1K_2K_3K_4$ and $h = 4$. There is no way to simplify Eqn (9.1.A1.29) to give a Hill coefficient greater than 4. Also, when [L] is very small, [L] dominates the terms in higher orders of [L], and the observed Hill coefficient tends to 1.0. In general, the Hill coefficient approaches unity at both extremes of [L] and cannot exceed the number of binding sites in the linear region of the curve from $0.1 \le \nu \le 0.9$. Thus the interpretation of the Hill coefficient, h, is that it is a minimum estimate of the number of binding sites.

COMPETITIVE INHIBITION OF BINDING

Here we consider that a second ligand, an inhibitor, I, is able to bind to the same binding site, and in so doing blocks the binding of the first. The reactions are written as

$$[9.1.A1.32] \quad \begin{aligned} P + L &\rightleftharpoons P\cdot L \\ P + I &\rightleftharpoons P\cdot I \end{aligned}$$

In competition experiments, it is customary to use the dissociation constants rather than the association constants. The dissociation constants are defined as

$$[9.1.A1.33] \quad \begin{aligned} K_{\text{d}} &= \frac{[P][L]}{[P\cdot L]} \\ K_{\text{I}} &= \frac{[P][I]}{[P\cdot I]} \end{aligned}$$

where both K_{d} and K_{I} are in units of M. We define the saturation fraction as before:

$$[9.1.A1.34] \quad V = \frac{B}{B_{\max}} = \frac{[P\cdot L]}{[P] + [P\cdot L] + [P\cdot I]}$$

substituting in for $[P\cdot L]$ and $[P\cdot I]$ from Eqn (9.1.A1.33), we obtain

$$[9.1.A1.35] \quad \begin{aligned} \nu &= \frac{[P][L]/K_{\text{d}}}{[P] + ([P][L]/K_{\text{d}}) + ([P][I]/K_{\text{I}})} \\ &= \frac{[L]/K_{\text{d}}}{1 + ([L]/K_{\text{d}}) + ([I]/K_{\text{I}})} \\ &= \frac{[L]}{K_{\text{d}}(1 + ([I]/K_{\text{I}})) + [L]} \end{aligned}$$

There are several possible ways to evaluate K_{I}. One is to choose a single concentration of free ligand, [L], and

determine the inhibitor concentration at which the binding is inhibited 50%. This concentration of inhibitor is the IC_{50}, the inhibitor concentration at 50% inhibition. Taking the ratio of ν in the absence and presence of inhibitor at the IC_{50}, we have

$$\frac{B \text{ with}[I]=0}{B \text{ with}[I]=IC_{50}} = 2 = \frac{[L]/(K_d+[L])}{[L]/(K_d(1+([IC_{50}]/K_I))+[L])} \quad [9.1.A1.36]$$

Canceling out the identical free ligand concentration in the two experiments and rearranging, we obtain

$$2 = \frac{K_d(1+(IC_{50}/K_I))+[L]}{K_d+[L]} \quad [9.1.A1.37]$$

$$K_d + \frac{IC_{50}}{K_I} + [L] = 2K_d + 2[L]$$

$$K_I = \frac{IC_{50}}{1+([L]/K_d)}$$

This last equation enables us to determine K_I for the inhibitor from the free ligand concentration used to determine IC_{50} and from the K_d for the ligand binding alone.

THE DIXON PLOT

A second method for determining the K_I for an inhibitor does not require prior knowledge of K_d. Equation (9.1.A1.35) can be rewritten as

$$B = \frac{B_{max}[L]}{K_d(1+([I]/K_I))+[L]} \quad [9.1.A1.38]$$

This can be inverted and rearranged to give

$$\frac{1}{B} = \frac{K_d(1+([I]/K_I))+[L]}{B_{max}[L]} \quad [9.1.A1.39]$$

$$\frac{1}{B} = \frac{K_d+[L]}{B_{max}[L]} + \frac{K_d/K_I}{B_{max}[L]}[I]$$

According to this equation, plots of $1/B$ against $[I]$ at a constant $[L]$ should be linear with an intercept on the $1/B$ axis of $(K_d+[L])/(B_{max}[L])$ and a slope of $(K_d/K_I)/(B_{max}[L])$. This plot of $1/B$ against $[I]$ is the **Dixon plot**. If we experimentally determine two lines at differing $[L]$, $[L]_1$, and$[L]_2$, then the two curves will intersect at $1/B_1 = 1/B_2$. It can be seen from Eqn (9.1.A1.39) that this condition is met when $[I] = -K_I$. That is, the point of intersection of the lines in a Dixon plot is $(-K_I, 1/B_{max})$.