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With few exceptions, the photographed models used in this book are Corey-Pauling space-filling atomic models with Koltun connectors. These were kindly loaned by Dr. J. Schellman. The photographs are the result of the patience and skill of Stephen L. Lowe, a doctoral candidate at the University of Oregon.

The photographed model accompanying Fig. 22.1 was assembled by Dr. J. A. Rupley, Department of Chemistry, University of Arizona. It is based on the X-ray data of Dr. D. C. Phillips, Molecular Biophysics Laboratory, University of Oxford. The photos were taken by my colleague Dr. R. G. Wolfe, and are reproduced by the consent of each person named.



# 1

## biochemistry 1964-1966

The developmental processes, either in living cells or in bodies of knowledge, defy accurate concurrent reporting. By the time a statement has been carefully formulated, the conditions will have changed. Any book, therefore, must report the author's view of his subject as of the publication date; this view will be composed of ideas, facts, and data antedating publication by varying periods of time.

This book is an attempt to present what I believe to be worth discussing at this time; the reader himself is expected to emend some of the topics and add new evidence. For a subject such as biochemistry no text or general review can be more than an agendum for discussion. This small volume presents the ideas and concepts that require a year of lecturing in my course in biochemistry. The course attracts seniors and graduate students from both chemistry and biology, and it presumes some knowledge of biology and of organic and physical chemistry. No apology is made for the numerous omissions. There is no attempt at complete coverage, since the book is intended, not as a reference book, but as a stimulus to discussion, contemplation, and further reading. There is an attempt to display some of the perplexities of biochemistry as well as the achievement of the biochemist.

In a recent book, *Man and Science*, Heitler, a noted physicist, discusses the working rules of the exact sciences. The characteristic common to all, he concludes, is *measurement*—quantitative and precise. It was the conviction of Newton, of Descartes, and of many who followed that accurate predictions of behavior could be made if a *complete description of the initial state* were available. The development of quantum theory modified this view with the revelation that the act of measurement itself is a perturbation and thus a source of indeterminacy. In general, biochemists have not shrunk from perturbing the systems under investigation. They have subscribed wholeheartedly to Bacon's dictum that "Nature must be tortured to reveal her secrets" without worrying very much about the strict accuracy of the revelations.

Despite the philosophical imperfections, biochemistry (and the closely related molecular biology) has accumulated outstanding results during the past twenty years. So outstanding and continuing have these results been that many believe most aspects of biological activity can be described in chemical and physical terms. This belief is a working hypothesis that should be challenged from time to time. Occasionally a newspaper or magazine contains the dubious statement that Dr. ——— is about to reveal the "secret of life." It is difficult to know what this means in view of the obscurity of the term *life*. However, it is certain that the entire body of knowledge presently available

to the scientist is quite insufficient for the purpose of synthesizing or analyzing life however that term is defined.

Biochemistry may be considered the chemist's view of biology. It should be developed or pursued with the attitude that it will not supply the answers to many biological questions but that it will lead to the construction of some interesting models, and that these models will incite us to fruitful experimentation. Molecular biology shares some of biochemistry's character, but there is much greater emphasis on biological macromolecules and the epigenetic system.

To the biochemist all biological processes are of interest, and there is a constant endeavor to define such processes by using the hypotheses current in chemistry and physics. But the hypotheses in these disciplines are based on very simple models; because of their simplicity, explanations arising from their use can have only limited validity. In short, biological phenomena are such a web of interactions that comprehension may necessitate studying the behavior of clusters of interrelated models. This will require a much more sophisticated physical science than we now possess. Until we develop the necessary tools, it would be well to suppress our *hubris*.

In the initial sections of this book I have tried to establish the compartmentalization of chemical reactions within the cell. Those reactions that tend to be common to all cells have been discussed first, and this has also allowed the relating of structure to function. As the discussion moves from a concern with small molecules to concern with macromolecules, there is a marked increase in content of molecular biology. I have felt it necessary to emphasize the differences between the bacteria, the favorite experimental subjects of molecular biology, and nucleated cells. This leads easily to the treatment of processes which are species-specific and to a brief consideration of cell membranes and cell walls. Finally, there is a brief essay on the mechanisms of biochemical control.

This book was intended to be succinct, readable, and occasionally provocative. I have not hesitated to include some ideas that I find exciting even if they are not firmly established. It is my hope that you will enjoy both the unity and the diversity of biochemistry.

## references

The references listed at the end of each section are not meant as "supplementary reading," but they are meant to provide background or elaboration, whichever will ensure the fullest comprehension of the material. The depth and extent of such reference consultation will depend upon the circumstances in which this book is used.

The student should note the kinds of journals that have carried the important papers. He should take very seriously the references to older papers; their survival is an index to the perspicacity of the investigators. The references have been made as specific as possible, and an effort has been made to include

as much recent material as possible. It may be that some of the later references will hardly become classic, but at least they will reflect current thought.

In some institutions the student will have difficulty in gaining access to reference materials. In such cases he should not hesitate to purchase available specialized paperbacks, to request either duplicated copies of journal articles or reprints, and to acquire a minimal library. No text, including this one, should ever serve as a major source of information; the best it can do is to suggest the topics worth pursuing.

**enzyme nomenclature** Throughout this book the name of an enzyme, when introduced, will be followed by a numerical-code designation of the Enzyme Commission of the International Union of Biochemistry. The Commission has recommended the use of its classification for all enzymes:

**group 1** Oxidoreductases

**2** Transferases

**3** Hydrolases

**4** Lyases

**5** Isomerases

**6** Ligases (synthetases)

In Appendix I will be found a list of all the enzymes referred to in the text, together with information not given in the text, such as the systematic nomenclature. For a complete catalog of all enzymes considered by the Commission see its 1964 report (REF. 3).

The use of this reference system is recommended even to the neophyte. Enzyme nomenclature has developed idiosyncracies: many synonyms exist, and the multitude of names obscures the generalizations that can be made. Then, too, that an enzyme is in the recommended list gives assurance that it has been reasonably well characterized by someone. It should be noted that an occasional enzyme name in the text is unaccompanied by a code number, indicating that the enzyme is newly discovered or, as yet, poorly characterized. That a reference to an enzyme is the first one is indicated by the use of small capital letters for printing the name of the enzyme. Further references are set in ordinary type.

**organisms used for experimentation** Appendix III contains a discussion of the organisms referred to in this book. Very few students will have sufficient biological knowledge to understand experimental choices. Some choices were based on extrascientific factors such as convenience and tradition. The number of organisms referred to is small compared to the number of organisms that have been used in some investigation or other, and the purpose of the appendix is simply to identify the materials most frequently used and to indicate their biological nature.

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# 2

## chemical reactions in a cellular environment

A central assumption in biochemistry is that all the reactions occurring in a living cell are catalyzed by enzymes. Even reactions known to reach equilibrium spontaneously, such as the hydration of  $\text{CO}_2$  to carbonic acid, are included. In addition, the cell is conceived as a structure much lower in entropy than its environment. Since this structure can be maintained only under a narrow range of conditions, efficient control mechanisms must be operative.

How many separate, or distinguishable, reactions take place simultaneously in a living cell is unknown, but the possible number must be very large—many hundreds, perhaps thousands. This network of interrelated reactions is termed *intermediary metabolism*. Simple coupling of reactions due to common intermediates must provide some measure of control; but if this were the only type of control, the activities of the cell would be dominated completely by the environment. The present working hypothesis is that the rate of each reaction is determined by the enzyme that catalyzes the reaction. The flow of small molecules in the cell is profoundly affected by relatively small numbers of macromolecules, having molecular weights of 10,000 to 500,000, synthesized by the cell. How the production of these macromolecular catalysts is accomplished will be considered in Sec. 20, on protein synthesis.

In order to assess properly the effects of temperature changes, changes in  $\text{H}^+$  concentration, and the action of inhibitors or of activators, one must examine enzyme-catalyzed reactions individually. General statements which result from such studies are included in the topics comprising enzyme kinetics, a subdivision of general solution kinetics. The study of the reactions of intermediary metabolism and the corresponding enzymes has occupied the attention of a majority of biochemists from the last decade of the nineteenth century until recently. An important stimulus to this type of investigation was the work of the Buchner brothers in Germany. They found, in 1897, that it was possible to ferment sugar to alcohol with the contents of the yeast cell—that the intact cell was unnecessary. There ensued fifty years of biochemical exploration during which hundreds of enzymes were isolated, and many were prepared, as well-characterized proteins. During the early part of the period, physiologists were highly critical of this experimental approach. In their view, no catalog of enzymatic reactions, however complete, could be adequate for a description of the life process. Even among biochemists there arose a group whose working hypothesis was that enzymes are coordinated in assemblages within the cell, that these assemblages form the active substance of the subcellular structures, and that studies on single reactions are less fruitful than studies of groups of reactions.

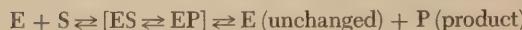
Both types of research have been necessary; both have contributed heavily to modern biochemistry. The understanding of control mechanisms requires

the consideration of subcellular assemblages; the understanding of the catalytic process requires the study of single enzymes. One is *biochemistry*, the other *biochemistry*.

In the following discussion of enzyme kinetics the models and concepts are those of physical chemistry. Although they are not representative of the actual cell, simple systems will be considered because they are easy to describe. The only bridge to biological reality is the enzyme involved—a protein that is a hallmark of living systems.

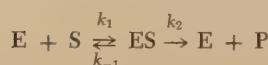
### **Michaelis-Menten hypothesis**

In a word-association game a biochemist might well associate the word *enzyme* with the name *Michaelis*. Michaelis and Menten propounded their well-known hypothesis on enzymic reaction rates within a few years of the founding of the *Biochemische Zeitschrift* (REF. 1), in which their paper appeared.<sup>6</sup> Their hypothesis stated simply that an enzyme E was a reactant which was not used up and that the substance undergoing change in the catalyzed reaction, the *substrate* S, actually combined with the enzyme. The symbolism now employed is this:



It must be emphasized that this is *not* an equation but a brief statement of a postulated mechanism. It implies that the rate of the enzyme-catalyzed reaction may be determined by the rate of decomposition of the enzyme-substrate complex, symbolized as ES. During the past twenty years, several ES complexes have been demonstrated directly; hence it is no longer necessary to consider such complexes hypothetical.

Patient examination of the velocities or rates of enzyme-catalyzed reactions will reveal a general behavior as shown in Fig. 2.1, *provided the study is made with a high substrate concentration*. Therefore it is justifiable to write the expanded statement



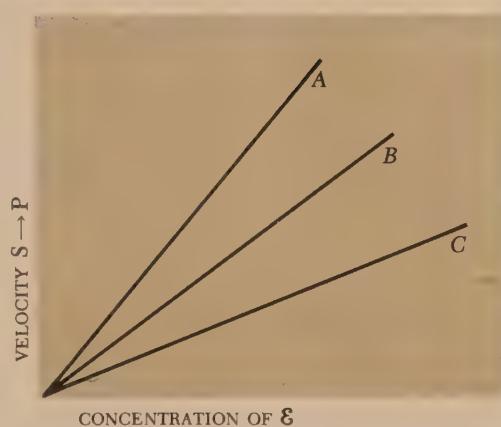
and, moreover, to note that

$$v = k [E]$$

If, in contrast, the concentration of S is varied while the concentration of E is held constant, the reaction velocity will vary as in Fig. 2.2.

<sup>6</sup> In part, their conclusions were as follows: "Sucrose unites with invertase to form a compound whose dissociation constant is 0.0167. . . . Since the decomposition of the sucrose-invertase compound must be a monomolecular reaction, then the rate must be proportional to the concentration of the sucrose-invertase compound. From all of these assumptions there may be derived a differential equation, for the course of sucrose hydrolysis, whose integral corresponds well to the observed values." By permission of Springer-Verlag. From L. Michaelis and M. L. Menten. *Biochem. Z.*, **49**, 333–369, Berlin: Springer (1913), 368–369.

**FIGURE 2.1**  
**relation of velocity of reaction to enzyme concentration.**  
 A, B, and C represent different enzymes. Substrate concentration, temperature, and  $H^+$  concentration must be kept constant. At high substrate concentrations  $[S]$  is effectively constant and  $[E] = [E]$



It was proposed that the maximum velocity  $V$  is reached when all of the enzyme molecules are complexed with substrate molecules, so that  $V = k [E] = k [E]$ .

Since Michaelis and Menten had assumed an equilibrium condition to exist, it followed that

$$\frac{[ES]}{[E][S]} = K = \frac{k_1}{k_{-1}}$$

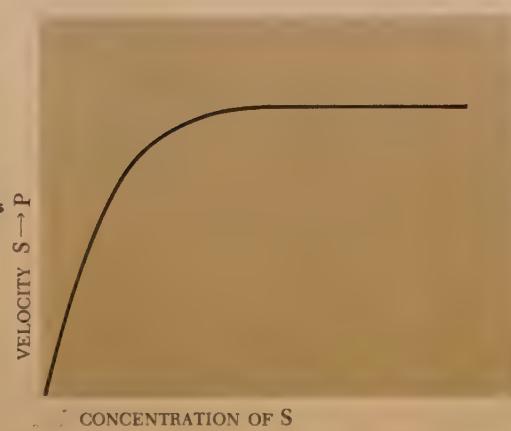
according to the usual mass-action relationship. And, by definition, the amount of enzyme added to an experimental system,  $\epsilon$ , can be considered to exist in solution as free, uncombined enzyme  $E$  and as a complex  $ES$ . Thus,

$$[\epsilon] = [E] + [ES]$$

Simple substitution will then result in the relation

$$\frac{[ES]}{([\epsilon] - [ES])[S]} = K \quad \text{or} \quad [ES] = \frac{K[\epsilon][S]}{1 + K[S]}$$

**FIGURE 2.2**  
**relation of velocity of reaction to concentration of substrate.** Note that each point on the curve represents an initial velocity. Thus the only variable is  $[S]$



Since  $k_2[\text{ES}]$  determines the initial velocity,

$$v_0 = \frac{k_2 K [\mathcal{E}] [S]_0}{1 + K [S]_0}$$

Here the equilibrium constant,  $K$ , is the reciprocal of the one employed by Michaelis and Menten (symbolized in the literature as  $K_m$ ). Therefore,

$$v_0 = \frac{k_2 [\mathcal{E}] [S]_0}{K_m + [S]_0}$$

Assuming that  $V = k_2[\mathcal{E}]$ , which seems reasonable when the substrate concentration is high, this substitution provides

$$v_0 = \frac{V [S]_0}{K_m + [S]_0} \quad \text{and} \quad \frac{v_0}{V} = \frac{[S]_0}{K_m + [S]_0}$$

In the special case where the initial velocity is one-half the maximal velocity,

$$\frac{v_0}{V} = \frac{1}{2} = \frac{[S]_0}{K_m + [S]_0}$$

and it is obvious that  $K_m = [S]_0$  when the velocity is  $V/2$ . It is not always appreciated by beginning students that the Michaelis "constant" (usually expressed in molarity) is really not very constant. The value changes with the  $\text{H}^+$  concentration and, of course, with the substrate. Therefore,  $K_m$  is a property of a system and is characteristic of an enzyme only in order of magnitude. It is a measure of the tendency of the enzyme and substrate to form a complex under specified conditions.

In the years since the above relations were formulated, a large literature on enzyme kinetics has accumulated. Every sort of refinement has been made. An example of an early amendment is the objection of Briggs and Haldane (REF. 2) that  $K_m$  could not be a simple equilibrium constant. Only if  $k_2$  were small would an equilibrium constant be undisturbed. A "steady-state" formulation would seem more adequate. This would result in the following set of relations:

$$\begin{aligned} k_1[\text{E}] [\text{S}] - k_{-1}[\text{ES}] - k_2[\text{ES}] &= 0 \\ k_{-1}([\mathcal{E}] - [\text{ES}]) [\text{S}] - (k_{-1} + k_2)[\text{ES}] &= 0 \\ [\text{ES}] &= \frac{k_1[\mathcal{E}] [\text{S}]}{k_{-1} + k_2 + k_1[\text{S}]} \\ v &= k_2[\text{ES}] \end{aligned}$$

and thus,

$$v_0 = \frac{k_2 k_1 [\mathcal{E}] [S]_0}{k_{-1} + k_2 + k_1[S]}$$

In order to simplify this expression, let

$$K = \frac{k_1}{k_{-1} + k_2}$$

Then

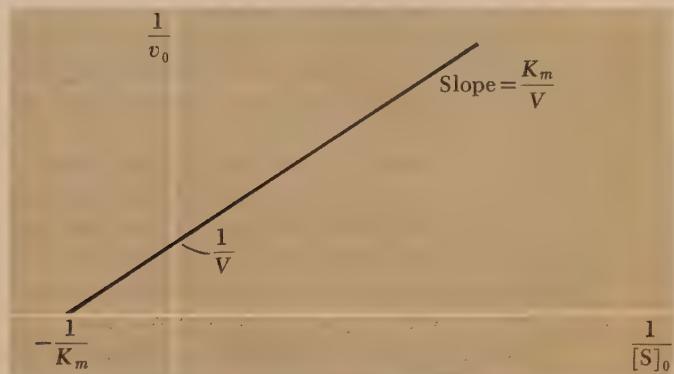
$$v_0 = \frac{k_2 K [\mathcal{E}] [S]_0}{1 + K [S]_0}$$

If  $k_2$  is much smaller than  $k_{-1}$ , this relation will be seen to be identical with that given by Michaelis and Menten.

In order to present enzyme data for ease of extrapolation and evaluation of unknowns, several types of plots have been suggested. The earliest suggestion was that of Lineweaver and Burk (REF. 3), who recommended the use of reciprocals:

$$\frac{1}{v_0} = \frac{K_m}{V} \cdot \frac{1}{[S]_0} + \frac{1}{V}$$

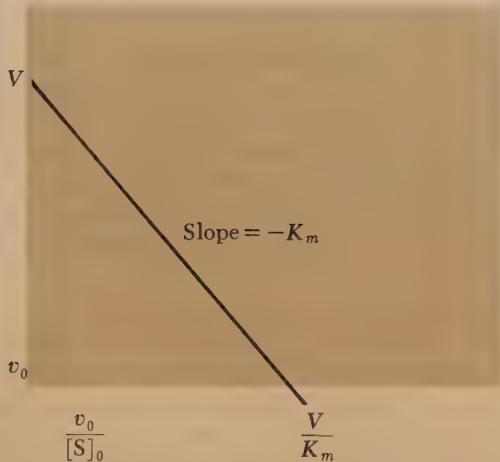
**FIGURE 2.3**  
a Lineweaver-Burk plot



The suggestion of Augustinsson (REF. 4) depends on the relation

$$v_0 = V - K_m \left( \frac{v}{[S]_0} \right)$$

**FIGURE 2.4**  
Augustinsson plot



The previous considerations imply that all reacting substances are in solution, but this is not a necessary condition for enzymic catalysis. Many enzymes remain active in the insoluble state; indeed, in the cell it is difficult to know whether or not an enzyme is truly in solution. Many enzymes in the living

cell form part of a subcellular structure; hence specification of an enzyme "concentration" would not have the usual simple connotation of solution kinetics.

Catalytic macromolecules respond to changes in the environment. Changes in the concentrations of ions are reflected by changes in activity. In some cases, an ion *must* be present for activity and thus is termed an activator without reference to mechanism. Variation in the H<sup>+</sup> concentration of the environment affects the activity of all enzymes, but the profile of the activity-ion concentration curve is not predictable, either in detailed shape or range. In general, the curve is bell-shaped.

It is improper to refer to the optimum pH of an enzyme, since this value is characteristic of a *system*. It is possible to choose a condition of specified temperature, enzyme and substrate concentration, and pH in which a maximum catalytic velocity may be observed. This is to say that the "optimum pH" is an extensive property: its value varies with the quantity of enzyme in the system. The Michaelis constant is also an extensive property. (In contrast, and by way of illustration, the specific refractive index is an intensive property.) The behavior of every enzyme derives from the properties of many dissociable groups—many both in number and in kind. Moreover, the substrate itself may be a dissociable compound. Hence, pH-activity plots represent the response of many groups to a given set of conditions.

The determination of temperature effects must also remain an experimental procedure. The rate of a chemical reaction may be predicted to increase if the temperature is raised. As a practical matter, the majority of enzyme catalysts rapidly become inactive near or above 50°. There are exceptions. A few enzyme structures are so stable that even boiling does not cause irreversible disruption. Clearly, there can be no genuine optimum temperature for an enzyme—adding heat increases the rate at which the reactants are converted to products, but the extent of destruction of the enzyme protein structure eventually nullifies the increase. There can be an optimum temperature for the enzyme-catalyzed reaction, however. Just why warm-blooded (homoiothermic) animals have a mechanism that holds the temperature near 37° is not clear. In any case, no enzyme optimum is involved.

The empirical nature of much of our knowledge of enzyme behavior has been pointed out by Niemann (REF. 5) in the following excerpt:

*The rates of α-chymotrypsin-catalyzed reactions are dependent upon reaction parameters other than enzyme and substrate concentrations. The additional parameters commonly include temperature, hydrogen ion concentration, presence or absence of added electrolytes, and solvent system. Other parameters may be involved in particular cases. These reaction parameters do not necessarily function independently, hence alteration in the magnitude of any one will frequently lead to perturbation of several others. Faced with such a complex situation we are limited at present to a qualitative or empirical description of the effects associated with these parameters. We are far from a rational quantitative description of any one of them and are still farther away*

from a grand rate equation applicable to all of them.\*

Enzyme activity may also reflect the presence of organic compounds, some of them very complex, often referred to as *coenzymes*. (See Sec. 29 for examples.) This term was applied during the period when the nature and function of these compounds were obscure. Coenzymes are normally present at a low concentration in the cell, are involved directly in the enzyme-catalyzed reaction as acceptors or donors of groups or atoms, can be treated kinetically as substrates, and help to define the specificity of the enzyme system. From the above discussion it is understandable that we should be cautious in making assumptions about enzyme activity *in vivo*. Until we are more certain about the exact microenvironment in the cell, we must assume that *in vitro* experiments furnish us with only a range of possible values of activity.

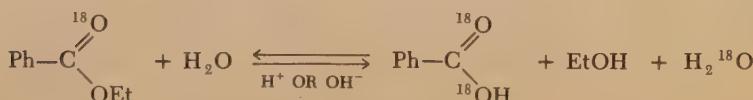
### **enzyme mechanisms**

For the chemist, the study of enzymes leads in two directions: either to a preoccupation with mechanism or to a preoccupation with protein structure. Consideration of the latter will be deferred to Sec. 22. At present (and for some time past) some investigations of enzyme mechanism have taken a rather indirect approach. The intent is to study the behavior of simplified compounds that may be viewed as enzyme models or analogs, with the hope that the mechanism may be deduced.

It is appropriate here to set forth only an abridged discussion of esterification and hydrolysis, two reactions that are often involved in the contact between the organism and the environment. In general, macromolecules do not enter live cells, nor do they become metabolically available to an animal by way of the intestine. Moreover, size is not the major consideration since a relatively small molecule such as sucrose does not cross cellular membranes without hydrolysis either. Usually the substance to be found in the cell is the smallest product that can result from the hydrolysis of the substrate being assimilated.

The study of enzyme models requires careful studies of organic mechanisms. As an example of the relation of an experimental observation to a working hypothesis, the following may be cited:

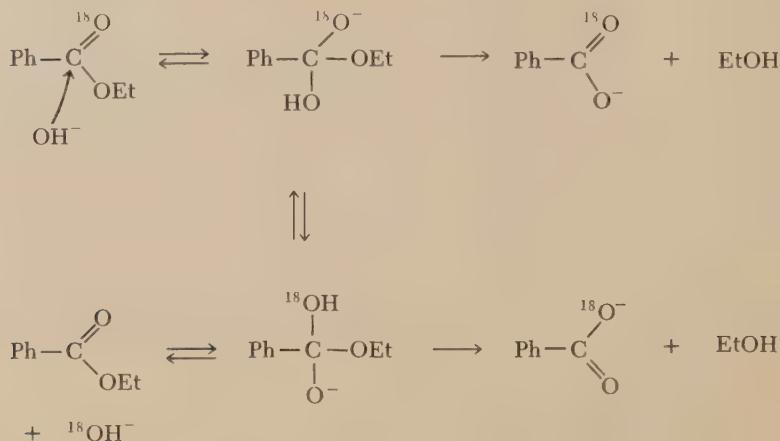
- Observation:



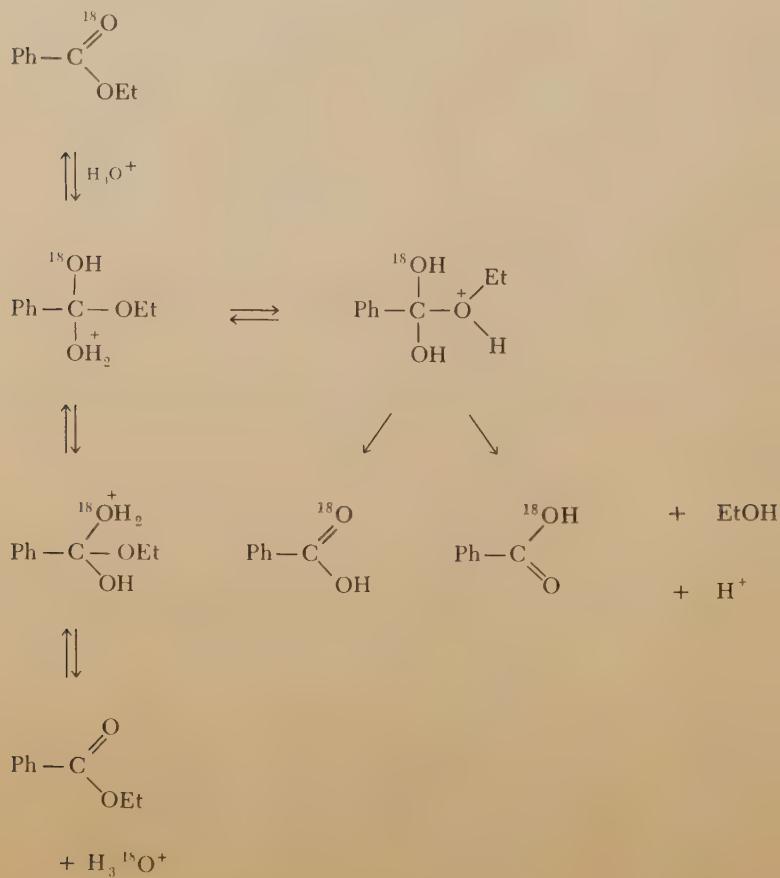
What sort of mechanism will allow the oxygen-18 to become distributed in this way during the hydrolysis of ethyl benzoate? If the water becomes labeled, why not the ethanol also? Might not an intermediate that permits an exchange rate differing from the hydrolysis rate be formed?

\*By permission of *Science*. From C. Niemann, *Science*, 143, 1287 (1964). Copyright 1964 by the American Association for the Advancement of Science.

■ Hypothesis: For OH<sup>-</sup> catalysis,



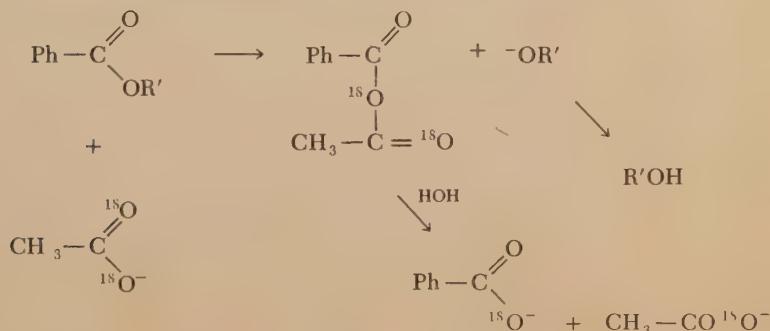
■ Hypothesis: For H<sup>+</sup> catalysis,



The molecular arrangements accounting for the original observations during  $H^+$  or  $OH^-$  catalysis in aqueous solution are adequately described by this symbolism. Current opinion is that enzymic hydrolyses proceed according to similar mechanisms but that a catalytic macromolecule may possess some additional attributes. Enzymes are remarkable catalysts, and the rates of enzyme-catalyzed reactions may be orders of magnitude higher than the rates of the same reactions catalyzed in free solution. How to account for this difference is part of the challenge of the subject. Presumably both acid and basic groups can be present simultaneously and in such spatial arrangement that each mechanism complements the other with a resultant reinforcement. For example, in the above reaction, if a proton were added to the oxygen atom of the carbonyl group, the initial attack of the  $OH^-$  would be facilitated. It might also be guessed that the pertinent groups would be spatially disposed on the macromolecular structure to favor the maximum possible rate.

Even though both type and position of the groups involved are to be considered, it is still possible that multifunctional compounds that are as effective as catalytic proteins can be synthesized. If they can be, the smaller size alone will facilitate studies, and detailed knowledge of the structure of the catalyst will be invaluable.

In addition to the ionic catalysis described above, it has been found that hydrolysis can be catalyzed by other ions—by acetate, for example. In the following reaction, the rate is independent of a pH between 4 and 9.



Such a reaction is termed a *double displacement reaction*, and the mechanism may be distinguished by its lack of sensitivity to  $H^+$  concentration in the pH range 4 to 9. The mechanism cannot be ruled out for enzyme systems, although most enzyme-catalyzed reactions are sensitive to the  $H^+$  environment.

- the active site** Closely allied to studies of enzyme models are the “active site” investigations. It follows from the Michaelis-Menten formulation that each molecule of enzyme must interact with at least one substrate molecule. When an enzyme molecule contains more than one active site, E (in the Michaelis-Menten formulation) represents the concentration of *active sites*. In enzyme-coenzyme systems there must be at least two parts or subsites in the active site since, kinetically, two substrate molecules are interacting with the enzyme at the

same time. As the reader will find, it is not yet easy to specify just what one molecule of enzyme is. (This will be discussed as part of the subject of protein structure in Sec. 22.) It is tempting to postulate that there is one active site per polypeptide chain; since most proteins consist of more than one peptide chain, it would follow that there is generally more than one active site. In the few examples that have been tested, this hypothesis does not seem valid.<sup>o</sup> The active site appears to be a special conformational complex which may require only one polypeptide chain, or it may require more than one in juxtaposition with others.

Occasionally asked is whether there are multifunctional enzymes—enzymes that can catalyze more than one kind of reaction. They are certainly uncommon, but examples can be given. Liver ALCOHOL DEHYDROGENASE (1.1.1.1)<sup>†</sup> catalyzes the dehydrogenation of ethanol to acetaldehyde and also the isomerization of several aldehydes to ketones.

The concept of an active site has varied from enzyme to enzyme, and it has also varied with the investigator. As noted, enzyme-catalyzed reactions generally proceed at high rates. High rates indicate that the mechanisms lower the activation energies. Presumably there occur, in the transition states, peculiarly favorable steric conformations that permit mechanisms that are unlikely to occur in free encounters in solution. If certain steric conformations are involved, then selectivity for the substrate is an expected consequence. Certainly, selectivity, or specificity, is a notable characteristic of enzymes. In many cases it seems probable that the orientation of the substrate involves a group not *directly* related to the reaction mechanism. This group is often termed a "binding site." But it is possible that the binding site interacts with the catalytic site in some manner, since the product of the reaction dissociates and there has been no chemical change at the binding site. Rather recently it has been suggested that the enzyme protein undergoes a conformational change as a result of the formation of an ES complex and that completion of the reaction allows the protein to revert to its original form, with a concurrent dissociation of the product. This *allosteric theory* (Sec. 31) appeals to the imagination, but so far it has not led to unambiguous results. From kinetic studies it seems certain that enzymes can form many kinds of activated complexes, and the concept of the active site will remain a loose one until more knowledge has been accumulated.

In the literature of active site research there are frequent references to several groups but especially to sulphhydryl and imidazole groups. Chronologically the sulphhydryl group was the first to be examined intensively. Many enzymes were found to be inhibited by chemicals which interacted with —SH groups. For example,  $\text{Ag}^+$ , *p*-mercuribenzoate (PMB), and iodoacetate (IAA) react with the —SH groups of enzymes to varying extents, usually resulting in partial or total loss of activity. Several interpretations of the phenomena

<sup>o</sup> It has been reported (REF. 8) that ALDOLASE (4.1.2.13) consists of three peptide chains, but only one binding site for the substrate is demonstrable.

<sup>†</sup> The author suggests that the reader consult Appendix I whenever a systematic number is given. The exact reaction is recorded, and information about the enzyme is given.

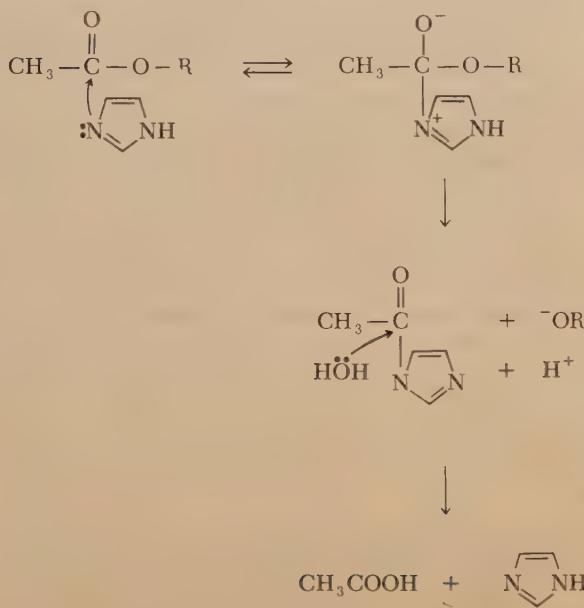
are possible; one is that the —SH group is part of the active site. The detailed chemistry of this group will be considered in Sec. 12.

CHYMOTRYPSIN (3.4.4.5) is a hydrolytic enzyme that contains histidine, an imidazole derivative. It has been found possible to alter the two histidine residues present *in situ* by photooxidation, with the consequence that the enzymic activity is destroyed. Moreover, it has been found that chymotrypsin catalyzes the hydrolysis of *p*-nitrophenyl acetate—a hydrolysis also catalyzed by imidazole. Imidazole is a base with a  $\text{pK}_a$  value of 7, and it may be expected to exist in the following ionic forms:



#### NUCLEOPHILIC SPECIES

The following mechanism has been established for the imidazole-catalyzed hydrolysis of *p*-nitrophenyl acetate in aqueous solution.



Chymotrypsin also is acetylated during the hydrolysis of this substrate, but the acetate group is found attached not to the histidine, the amino acid that is the imidazole derivative, but to serine. It may be concluded that the mechanism differs in detail. Perhaps the fairest judgment is that of Bender and Breslow (REF. 6):

*Obviously the pathway of the enzymatic reaction must differ in some respects from simple organic reactions, for the former in general proceed much more readily than the latter. But for the proper interpretation of a complex en-*

zymatic reaction it is essential that the background of its simpler organic counterparts be understood and used.\*

### relaxation spectrometry

During the past few years, a new experimental approach has been used to study the rapid reaction rates found in enzymically catalyzed reactions. By this technique, relaxation spectrometry, we can measure the rate of the re-establishment of equilibrium after a very rapidly produced disturbance. Suppose, for example, that a reaction  $A + B \rightleftharpoons AB$  is allowed to go to equilibrium. If some parameter, such as temperature, is changed rapidly, a new equilibrium must be established, and the rate at which the reestablishment occurs will reflect the reaction rate. Thus,

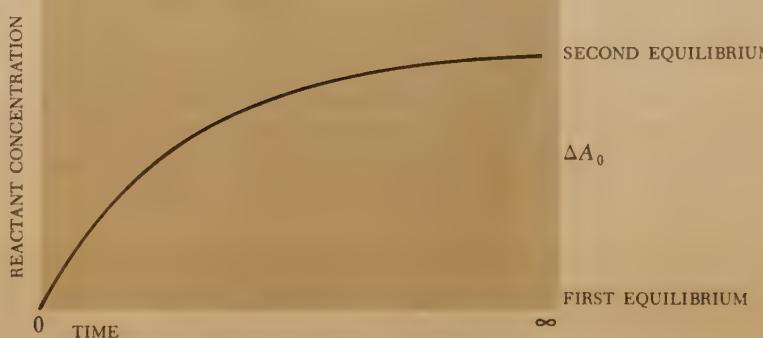
$$-\frac{d\Delta A}{dt} = \frac{1}{\tau} \Delta A \quad \text{and} \quad \Delta A = \Delta A_0 e^{-t/\tau}$$

$$\frac{1}{\tau} = k_1([\bar{A}] + [\bar{B}]) + k_{-1}$$

Here  $\Delta A_0$  is the total change in the concentration of A between the two equilibria and  $\Delta A$  is the change in concentration of A from time  $t$  to the second equilibrium. The relaxation time is symbolized by  $\tau$ . If the reactant whose concentration is changing can be detected by spectrophotometry, the change itself can then be recorded and will appear as in Fig. 2.5. If the total difference between the concentrations at the two equilibria is small, the differential equations are linear. Methods employing temperature changes are effective in measuring reaction times of 1 to  $1 \times 10^{-6}$  sec. Modifications employing sound waves or electric pulses can be used to measure reaction rates down to  $5 \times 10^{-10}$  sec.

\*By permission of M. L. Bender and the Elsevier Publishing Co., Amsterdam. From M. L. Bender and R. Breslow, in *Comprehensive Biochemistry*, M. Florkin and E. Stotz (eds.), 2, 1 (1962).

**FIGURE 2.5**  
relaxation spectrometry. The relaxation curve shows the re-adjustment of equilibrium after a rapid change of parameter at time 0. The exact position of  $\tau$  is defined in the text



One of the advantages of relaxation spectrometry is that high concentrations of reactants may be used; hence reaction intermediates whose existence would not be suspected in dilute solution may become evident, since more than one relaxation value can be observed.

The familiar neutralization reaction  $H^+ + OH^- \rightleftharpoons H_2O$  has a bimolecular rate constant of  $1.3 \times 10^{11}$  liters/(mole)(sec). The reciprocal of this number is the half-time of the reaction at a concentration of 1 M, that is, about  $7 \times 10^{-12}$  sec ( $k = 0.693/t_{1/2}$ ).

For a variety of enzyme-catalyzed reactions, rate constants ( $k_1$ ) of  $10^5$  to  $10^9$  moles $^{-1}$  sec $^{-1}$  have been found. Determination of such rates is of interest as such, but it has also been possible to separate the steps involved. In turn, identification of the steps has made possible evaluation of the times for the formation of the enzyme-substrate complex, conformational changes of the protein, proton or electron transfer, and metal activation.

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# 3

## the mitochondrion and biological oxidation

The study of mechanisms has its adherents, and so has the study of structure. Perhaps it is in the latter that biology and chemistry can most profitably overlap, since the central theme of this approach is the relation of form to function. There is no finer introduction to this subject than the work, now a classic, by D'Arcy Thompson [REF. 3].

It is something of a thrill to find that both gross and fine structures are a direct expression of the component molecules. The shell of the crayfish must exhibit the restrictions of chitin; the character of hair reflects the nature of its fibrous protein. The cell as a whole, in contrast, has no such architectural specialization, and therefore the microscopic appearance is a resultant of several forces. At the subcellular level, however, form is again related to function. The current view of the mitochondrion is a great accomplishment, the kind of scientific synthesis of which biochemists can be proud and which they may hope to repeat.

### **the mitochondrion**

The advent of really good microscopes just before the beginning of the twentieth century was followed by great activity among cytologists. Various "cytoplasmic granules" were described; among them was a type that came to be known as mitochondria (thread granules) or chondriosomes. Much confusion developed in the description of these subcellular particles because of the great variability in their form, number, and appearance. This variability was noted not only by comparing different cells but also by examining a single type of cell in various stages of development or activity. Despite the changes in shape, number, and position, the total amount of mitochondrial substance per cell was found to alter but little.

Mitochondria became characterized as subcellular structures by their peculiar staining properties. As an example, Janus green G (an oxidation-reduction indicator) is first reduced and then reoxidized to stain the particles a characteristic blue. The reactions, however, are complex and not readily reproducible in all tissues. In about 1935 it was shown that material similar in appearance and stainability could be prepared by differential centrifugation. Centrifuged preparations could be examined with the electron microscope and compared with the analogous particles in intact cells. Today there is general agreement that there is fine structure common to all mitochondria regardless of differences in shape and size. The phase microscope and cine-micrography have verified the original impressions that mitochondria can move within the cell and change in shape in a most remarkable fashion.

Mitochondria may be described as subcellular particles which, when properly fixed and sectioned, show a smooth outer membrane and an infolded inner membrane. The external membrane is about 150 Å thick, and three

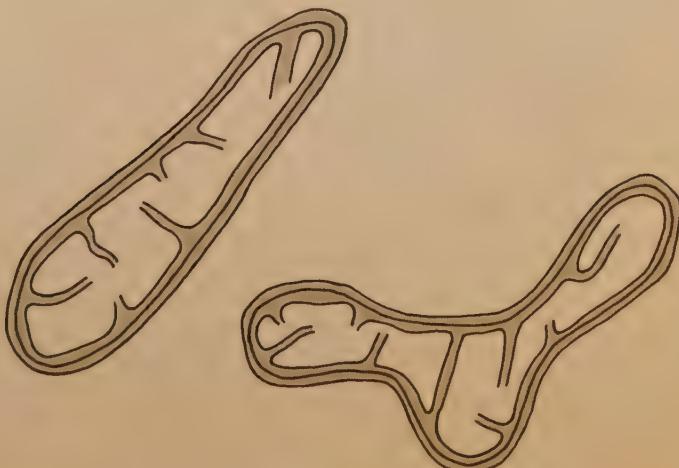
layers appear to be present. The internal membranes (cristae) are somewhat less thick, about 90 Å, and in some cases the two membranes seem to be continuous. Figure 3.1 is a diagrammatic sketch of mitochondria, but you are urged to examine good electron micrographs as found in references 1 and 2.

Heart mitochondria have many cristae, or internal membranes, and it has been found that only about 20% of the protein is easily soluble in aqueous media. Liver mitochondria seem to contain only a few cristae, and more than 50% of the protein seems soluble. The latter particles have many more functions than the former. It is noteworthy that more than 30% of the particle is lipid. As an indication of the abundance of these organelles, 35% of the protein of rat liver is mitochondrial. Similar analytical data have been obtained from other tissues.

Advances in the techniques of isolating well-characterized mitochondrial preparations have been accompanied by increasingly precise enzyme tests. These tests in turn have continued to influence isolation techniques. For example, cell fractionation procedures using 0.25 M sucrose as a medium often resulted in a preparation that appeared to consist of partially disrupted mitochondria. Yet these preparations were enzymically quite active. In contrast, preparations resulting from isolation procedures using 0.88 M sucrose were pleasing to the electron microscopist but required special treatment to release enzymic activity. Despite such problems, the enzymic properties of the mitochondrion have been well cataloged and there is reasonable agreement among investigators using very different techniques, such as the spectrophotometry of the intact cell as compared with the chemical dissection of the particle.

Properly isolated mitochondrial preparations can catalyze the oxidation of a majority of the molecular species that normally furnish energy to the cell, which supports the conclusion that the mitochondrion is the prime locus of biological oxidation. Oxidations do occur elsewhere in the cell, but the central position of the mitochondrion in energy exchange is undisputed.

**FIGURE 3.1**  
diagram of mito-  
chondria (sectioned)  
drawn at a magni-  
fication of about  
30,000 $\times$ , where  
 $1\mu$  ( $10^{-3}$  mm) is  
represented by  
30 mm in the  
illustration



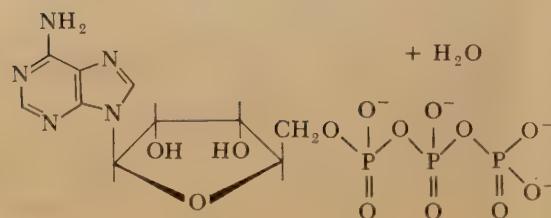
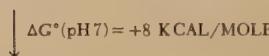
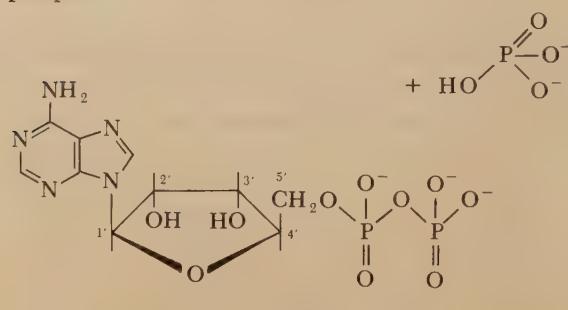
The study of mitochondria has been enormously productive of fact and theory about biological oxidation. Much of the study, however, has been of isolated mitochondria, and it should be stressed that oxidations in isolated sub-cellular particles may not be identical in all details with those in the cell. The isolated particle is an analog, a model, which is convenient for study but cannot represent the entire system.

### ATP formation

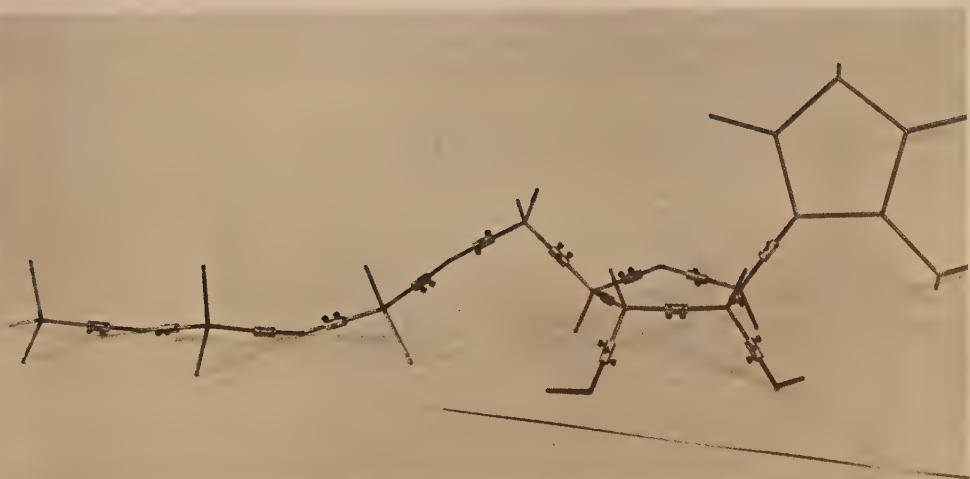
Many types of oxidation reactions have been discerned in living cells. Some, such as the oxidation of  $\text{H}_2\text{S}$  to S and the oxidation of nitrite to nitrate, are severely restricted to certain microorganisms. The type of oxidation most commonly occurring in cells is the transfer of H, bonded to C, O, N, or S in organic compounds, to molecular oxygen with the formation of the very stable compound, water. The transfer takes place in a series of reaction steps, and often the steps are coupled with a reaction that conserves the chemical energy in a form exploitable by the cell. It has been demonstrated that oxidation drives the conversion of adenosine diphosphate to adenosine triphosphate. This is usually symbolized as  $\text{ADP} + \text{P}_i \rightarrow \text{ATP}$ , or more explicitly as in Fig. 3.2. As noted in the diagram, the standard free-energy change is large and positive in sign; therefore, the driving force must be supplied by a coupled reaction.

Adenosine 5'-triphosphate is a derivative of adenosine monophosphate (adenylic acid), a nucleotide which is a structural unit in ribonucleic acid (Secs. 17 and 19). The unphosphorylated compound, or adenine *N*-riboside, is called adenosine (a nucleoside), and hence the names adenosine di- and triphosphate.

**FIGURE 3.2**  
the synthesis of  
adenosine triphosphate



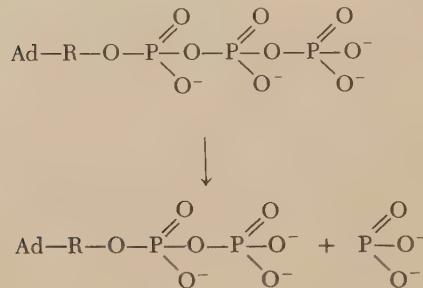
Dreiding model  
of ATP



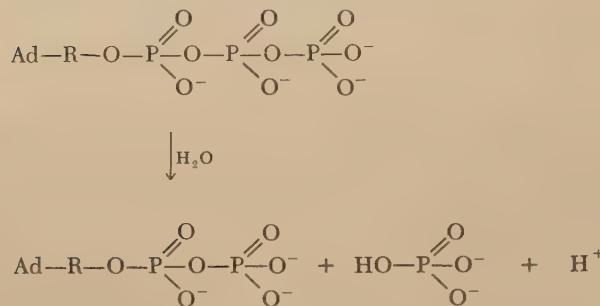
space-filling  
model of ATP



ATP is one of a group of biologically reactive compounds that are called "high-energy-bond compounds" or are referred to as containing "high-energy" phosphate. These terms must not be taken literally. As professional jargon they are useful; as scientific terms execrable. If the bond energy itself were in question, the process would be



and the energy change ( $\Delta E$ ) would be 50 to 100 kcal/mole. The biochemist is concerned with the phosphate transfer (or group) potential, which may be conveniently measured with water as acceptor.



In this process the *free-energy* change ( $\Delta G^\circ$ , pH 7) is large, about -8 kcal/mole,<sup>\*</sup> and hence the notion of "high energy."

During the period 1940 to 1960, much attention was devoted to an explanation of the factors which contribute to this large free-energy value. It has been agreed that one component is the higher resonance stabilization characteristic of the hydrolysis products. Another factor is the density of charges. Energy is required to bring the four charged oxygens together in ATP, and their separation during hydrolysis correspondingly releases energy. Finally, an inspection of the hydrolysis equation reveals an unionized OH group in the inorganic phosphate produced. This means that the hydrolysis mechanism has effected a change energetically equal to neutralization.

Thermodynamically it is obvious that ATP is potentially a vigorous reagent. Kinetically it appears surprisingly stable. Hydrolysis of the two terminal

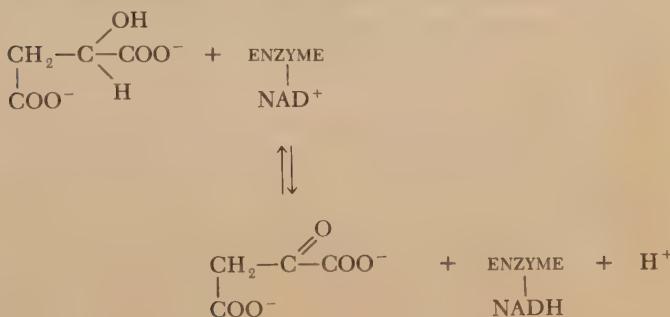
\* The value cited for the free-energy change of ATP hydrolysis will be found to vary disconcertingly. Many values are found because the exact value depends on several experimental parameters. The student is referred to a lucid discussion of the issue in reference 18.

phosphate groups requires 7 to 10 min at 100° in *N* HCl. The product is adenosine 5'-monophosphate (AMP). In aqueous solutions near pH 7, ATP hydrolyzes only slowly. Nevertheless, in the cell ATP is involved in a very large number of chemical reactions both as a phosphorylating agent and as an adenylating agent. It will be seen that specific enzymes are required in each case.

It has been remarked that many reactions involving ATP also require Mg<sup>++</sup> as a component of the system. Mg<sup>++</sup> is known to form complexes with ATP in free solution, and it has been suggested that Mg<sup>++</sup>-ATP-enzyme complexes also form. Several of the complexes that are possible would change the charge distribution and might potentiate a rapid phosphate transfer within the enzyme substrate complex. Chemical models have helped in visualizing the possibilities of such complexing, but nothing less than the whole enzyme complex must eventually be studied.

### hydrogen and electron transfer

As stated before, the biological oxidation process coupled with the ADP→ATP conversion occurs in several steps. Such molecules as pyruvate, malate, α-ketoglutarate, and isocitrate are sources of energy and hydrogen. Dehydrogenation of malate, for example, is catalyzed by the enzyme MALATE DEHYDROGENASE (1.1.1.37), and the hydrogen is transferred to the coenzyme acceptor nicotinamide adenine dinucleotide, NAD.<sup>\*</sup>



Several details should be noted. For one thing, it is inconvenient to show all the ionic forms actually involved in a biochemical reaction. Usually there is a mixture of ionic forms. The second pK value of malic acid is about 5, and therefore the acid is fully dissociated at pH 7. Although pH 7 is often referred to as physiological pH, there is a substantial range of hydrogen-ion concentration inside cells. Values of pH varying from 6 to 8 are not uncommon, but we shall assume a value of 7 as the average expected condition. Another detail is that this is a reversible oxidation-reduction reaction and may be studied in vitro, that is, outside the cell and as an isolated reaction. Finally, the H<sup>+</sup> produced is adequately buffered in vivo and there is very little change in pH.

\* In the older literature this compound is referred to as diphosphopyridine nucleotide, DPN. For formulas and the details of hydrogen transfer, refer to the end of this section. Reduced NAD is symbolized NADH. The oxidized form has a positive charge.

In the cell a steady state exists and a malate/oxaloacetate ratio, as well as an NAD/NADH ratio, reflects that state. If malate is added to the system, it will be oxidized at some rate that is a function of the concentration of the electron acceptor. Actually, the NAD/NADH ratio rarely drops to a very low value, because another coupled reaction acts to restore it to the steady-state value. The steady state is a consequence of the coupling of oxidation-reduction reactions, and it is reflected in the electrode potential. The potential is an intensity factor, and it does not reflect the amount of substrate being oxidized; instead, it reflects the potential level at which electrons are becoming available to the system. Only when the quantities involved begin to embarrass the system or when some catalyst is inhibited or inactivated will the potential be altered.

Presumably the student knows that the following relationship exists in oxidation-reduction systems.\*

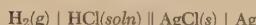
$$E \text{ (volts)} = E^\circ \text{ (volts)} + \frac{RT}{nF} \ln \frac{\text{[OXIDIZED FORM]}}{\text{[REDUCED FORM]}}$$

If the reference electrode is the hydrogen electrode, if the temperature is 30°, and if the number of electrons involved in the transfer,  $n$ , is unity, then

$$E = E_0 + 0.06 \log \frac{\text{[OXID]}}{\text{[RED]}}$$

Physical chemists have found it convenient to use the hydrogen electrode at pH 0 as a point of reference, and  $E_0$  values thus refer to that condition. In bio-

\*The sign conventions used in expressing potentials can be a source of confusion for the student. If the cell is written as



the cell reactions are

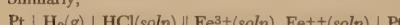


$$E = E^\circ - \frac{RT}{nF} \ln \frac{a_{\text{H}^+} a_{\text{Cl}^-} a_{\text{Ag}}}{a_{\text{H}_2} a_{\text{AgCl}}}$$

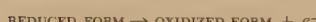
When  $a_{\text{H}}$ ,  $a_{\text{Ag}}$ ,  $a_{\text{AgCl}}$ , and  $n$  are all equal to 1,

$$E = E^\circ - \frac{RT}{F} \ln a_{\text{HCl}}$$

Similarly,



This equation refers to the oxidation process,



In this case  $nFE = -\Delta G^\circ$ , a measure of the tendency to react.  $E$  is an oxidation potential and refers to the left-hand electrode. The potential at the right-hand electrode is a reduction potential; it has a numerical value that is identical with but of sign opposite to the potential at the left. For a reduction potential the cell would be designated



The electrode system with the more negative potential reduces, or donates electrons. In the biochemical literature these are the potentials usually recorded, and they are usually referred to as oxidation-reduction potentials.

logical systems it is more informative to compare data which refer to events at pH 7. Hence,

$$E = E'_0 + 0.06 \log \frac{[\text{OXID}]}{[\text{RED}]}$$

On this scale the reference hydrogen electrode at pH 7 has the value shown in Table 3.1. The reference electrode is still the hydrogen electrode at pH 0. As noted, the potential varies only with the ratio of oxidant to reductant and not with the absolute concentrations. Simple arithmetic will show that, for the NAD/NADH system, ratios of 1/10 and 10/1 yield potentials of -0.35 and -0.29, and the system will be operative within this potential range. If the malate/oxaloacetate system has a value of  $E'$  which is more negative than this range, then it will react and will be oxidized. Some find it convenient to say that electrons "flow" from that system to the NAD/NADH system.

Therefore, if malate enters the cell at some reasonable rate, it will tend to change the ratio of malate/oxaloacetate; this change will be offset by oxidation mediated by malate (malic) dehydrogenase, and the ratio NAD/NADH will tend to change. However, there is properly situated in the cell another oxidation-reduction coupled reaction involving another coenzyme, another nucleotide. Actually, there are usually two similar flavin nucleotides, FMN (flavin mononucleotide) and FAD (flavin adenine dinucleotide), both having an  $E'_0$  value of -0.06 volt. Were we to mix the system NAD/NADH (ratio 1/10,  $E = -0.29$ ) and the system FAD/FADH<sub>2</sub> (ratio 1/10,  $E = -0.09$ ) in the presence of the proper catalyst, the value of  $\Delta G^\circ (-nF\Delta E)$  would be 9230 cal, over 9 kcal.

An enzyme that can catalyze such a reaction is a flavoprotein. Flavoproteins occur in most cells, but it is not to be assumed that an enzyme from one species will resemble that from another in detail. The term *flavoprotein* applies to such a wide variety of enzyme systems that it may be misleading. One specific example is **SUCCINATE DEHYDROGENASE** (1.3.99.1). Succinate is another substrate that generally can provide utilizable electrons to the cell in the oxidation reaction:



In this reaction  $E'_0 = 0.0$  volt; hence the NAD/NADH system with a more negative potential cannot act as an electron acceptor. Instead, there is a reaction between the substrate system and the flavoprotein. The enzyme does not catalyze the oxidation of the NAD/NADH system, however, as might be expected from the mere examination of the potentials. Here the specificity of

**TABLE 3.1**  
**biochemical**  
**oxidation-reduction**  
**potentials**

system	half-cell reaction	$E'_0 (\text{pH } 7.0, 30^\circ)$ , volts
oxygen electrode	$\frac{1}{2}\text{O}_2 + \text{H}^+ + 2e^- \rightarrow \text{OH}^-$	+0.81
NAD/NADH	(2e <sup>-</sup> shift)	-0.32
hydrogen electrode	$\text{H}^+ + e^- \rightarrow \frac{1}{2}\text{H}_2$	-0.42

the enzyme permits the oxidation of the succinate/fumarate system but does not permit interaction with the nicotinamide (pyridine) nucleotides.

In what follows in this section it will be convenient to confine our attention to the consideration of electron transport in the *mitochondrion* (liver or heart). In Fig. 3.3 is a sketch of the oxidation-reduction sequences. The flavins in the two series are not identical, and the cytochromes are not specified in this general statement. A study of Table 3.2 will show that the electron transport mediators are present in ratios not too far from unity. (Discussion of the specific details of the mediators will be found at the end of this section.) I wish to emphasize that within this potential span of 1.12 volts occur all of the biological oxidations characteristic of most cells. Substances that furnish electrons and hydrogen ions are continually flowing into the cell; electrons are continually being transferred from a negative potential to  $O_2$ , which may be considered at a positive potential. In the steady state both oxidized and reduced forms of the transfer mediators are present, and it must be remembered that each mediator is associated with a protein. For example, each type of cytochrome is characterized by a different protein.

It may be worth repeating that the oxidation-reduction reactions being considered here are quite reversible but that there is no equilibrium in the chemical sense. Reversible reactions in the equilibrium state cannot be a source of energy. Biological systems are "open" in contrast to the classical chemical systems, which are "closed." Living cells continually maintain an entropy<sup>\*</sup> lower than that of the environment. As long as the cell is alive, a magnificent complexity of organization is maintained, with energy derived from the irreversible flow of electrons just described. Another view is that life is maintained by degrading low-entropy photon energy to heat. Death is accompanied by the unrelenting increase of entropy, a well-known feature

<sup>\*</sup>No attempt is made here to discuss entropy. A study of the second law of thermodynamics will provide one kind of understanding. Anyone interested in biological science should consult references 6 and 7.

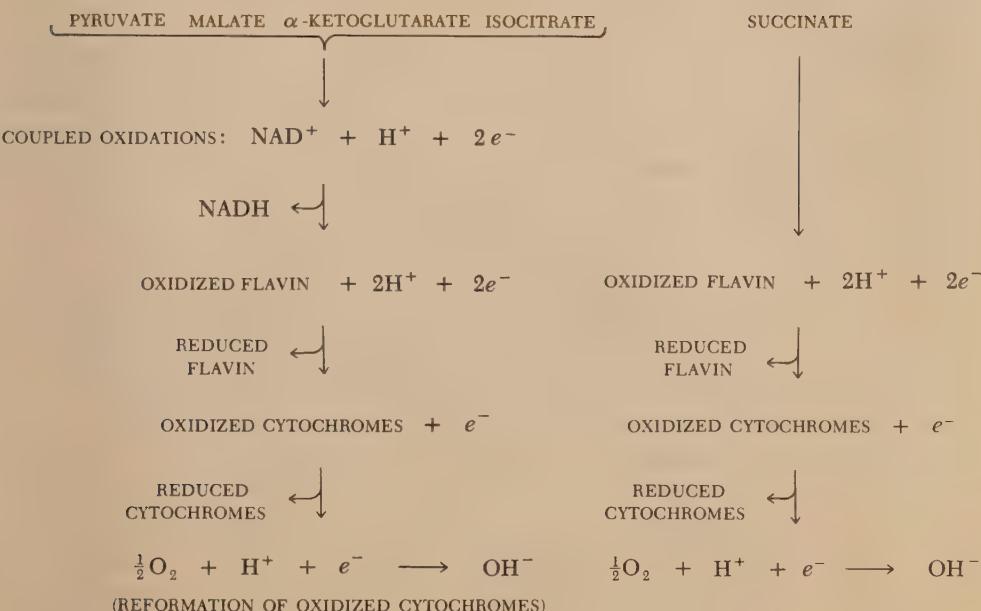
**TABLE 3.2**  
**oxidation-reduction**  
**potentials of electron**  
**transfer systems in**  
**the mitochondrion**

system	$E'_0, pH\ 7$	$E$ corrected for ratio existing in intact mitochondrion <sup>°</sup>
NAD/NADH	-0.32	-0.32
oxid/red flavoprotein	-0.06	-0.03
oxid/red cytochrome b	0.00	+0.04
oxid/red cytochrome c	+0.26	+0.33
oxid/red cytochrome a	+0.29	+0.37
$\frac{1}{2}O_2/OH^-$	+0.82	+0.80

<sup>°</sup>It has been shown that oxidase action preponderates over dehydrogenase action. The cytochromes exhibit an oxidation/reduction ratio greater than 1, whereas the ratio NAD/NADH is about 1. Interruption of the electron transfer mechanism at any step results in an increase in ratio on the "oxygen side" of the interruption and in a decrease on the "substrate side." This information is obviously useful in determining the site of inhibitor action.

**FIGURE 3.3**  
oxidation-reduction  
sequences

## SOURCES OF HYDROGEN AND ELECTRONS:



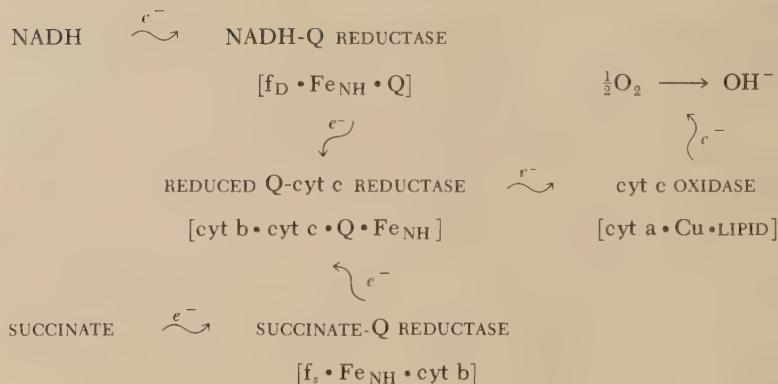
of the universe and characteristic of spontaneous reactions. A mummy may be considered a classic example of the difference between thermodynamic and kinetic stabilities.

**submitochondrial electron transfer** It is possible to isolate from mitochondria, by ultrasonic disruption, a particulate preparation named the electron transport particle, ETP. This particle appears not to be a mere fragment but to possess structure and to mediate the oxidation of substrate quantities of NADH by  $\text{O}_2$ . Some features of its composition are listed in Table 3.3.

**TABLE 3.3**  
composition of ETP  
from beef heart  
mitochondria

components	abbreviation	molecular or atomic ratios
flavin/NADH dehydrogenase	f <sub>D</sub>	1
flavin/succinate dehydrogenase	f <sub>S</sub>	1
nonheme iron	Fe <sub>NH</sub>	27
coenzyme Q	Q	15
cytochrome b	b	2
cytochromes c and c <sub>1</sub>	c, c <sub>1</sub>	2
cytochrome a (cytochrome oxidase)	a	4
copper		8

**FIGURE 3.4**  
participation of co-enzyme Q in the oxidation-reduction sequences. Symbols in brackets are components known to exist in the enzyme complex



There is an accumulation of evidence that the electron transport system contains two interacting but separable reaction sequences as noted in Fig. 3.3. One of these sequences transfers electrons arising from NADH, and since NADH-containing dehydrogenases exist for many substrates, it would appear that this sequence must exist in most cells. The other sequence transfers electrons from succinate. Succinic dehydrogenase is a flavoprotein containing four atoms of iron per molecule of flavin, present as riboflavin phosphate (FMN). REDUCED NADH DEHYDROGENASE (1.6.99.3) is also a flavoprotein, and it has also been isolated as a homogeneous protein.

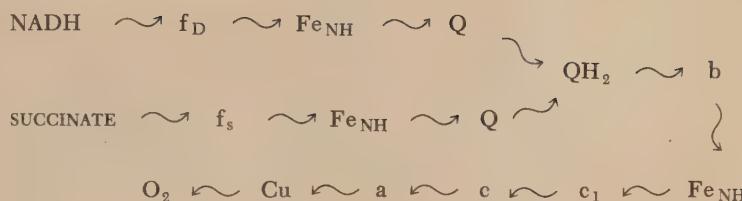
The place of coenzyme Q in Table 3.3 is equivocal. Coenzyme Q is a substituted benzoquinone present in mitochondria and necessary for the electron transfer function. It is not yet certain to which protein it is attached. One of the current proposals is pictured in Fig. 3.4. The NADH dehydrogenase is thought to be in a lipid complex containing coenzyme Q. Similarly, a cytochrome-lipid complex is thought to contain coenzyme Q. However, the exact mechanism in which the quinone participates is anything but clear. There is evidence for the serial transfer of electrons as sketched in Fig. 3.5, but even this is controversial.

It has been recognized for many years that substrates reduce nicotinamide nucleotide, which then reduces flavins, which in turn reduce the iron atoms of various cytochromes (see Sec. 28), and that a special cytochrome, cytochrome oxidase,<sup>o</sup> potentiates the reduction of molecular oxygen. The rather recent discoveries in mitochondria of coenzyme Q, nonheme iron, and copper have posed some problems, as has the exposition of the structural relations of the several components.

Studies using the ETP particle as a model of the oxidative apparatus of the mitochondrion have reinforced the evidence for an enzyme complex. This complex juxtaposes a number of substances that can be oxidized and

<sup>o</sup>To the nonspecialist, enzyme terminology can be troublesome. A *dehydrogenase* is descriptively sufficient, since it refers to oxidation by the removal of hydrogen ions and electrons. A *reductase* indicates catalysis of electron transfer. An *oxidase* is also a catalyst for the transfer of electrons, but the name implies that oxygen is the acceptor. An oxidase may be distinguished from an *oxygenase*, which catalyzes the direct incorporation of molecular oxygen into the substrate without specifying electron transport.

**FIGURE 3.5**  
proposed electron  
transfer sequences



reduced—nucleotides, flavins, a quinone, and organically bound metal ions. It has been suspected that these oxidation-reduction substances are organized spatially in such a way that there is a maximally effective *sequential* action. This notion, commonly held for nearly twenty years, would have it that the flavoprotein dehydrogenase molecules are situated physically closer to the protein containing coenzyme Q than to that containing cytochrome c. In some nonreversible biological processes—the secretion of gastric juice, for example—such a linear arrangement of enzymes would fit in beautifully. In biological oxidation, however, it is not necessary to postulate such an arrangement, since the driving force makes for a thermodynamically irreversible process. It is true that the separation of oxidation-reduction pairs of different potentials seems to call for some type of special structure, but not simple linearity.

Recently a unit of electron transfer has been proposed (REF. 8). Electron micrographs have revealed that the membranes of mitochondria (both internal and external) resemble paired arrays of particles about 100 Å in diameter. It was reported that the particles were isolated and that they were found to consist of the four enzyme complexes shown in Fig. 3.4. The molecular weights and ratios are shown in Table 3.4. Some investigators have been reluctant to consider this particle as *the* unit of electron transfer, so that the matter cannot be considered settled.

It is possible to obtain another type of particle from mitochondria by a different treatment. This particle, designated the ETP<sub>H</sub> particle by University of Wisconsin workers, has been found to mediate electron transfers of the type just discussed. The important difference is that it contains the components necessary for oxidative phosphorylation.

### oxidative phosphoryla- tion

Our detailed knowledge of the mechanism by which “food” enters a cell and yields energy in a “usable” form is very imperfect. The steps in oxidation have been described, but the usable product appears to be ATP. This thermodynamically unstable molecule can enter into a large percentage of the synthetic as well as the motion-producing reactions of the cell. Our

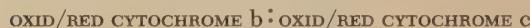
**TABLE 3.4**  
components of the  
ETP particle

enzyme complex	molecular weight	ratio
succinate-Q reductase	230,000	1
NADH-Q reductase	530,000	1
reduced Q-cyt c reductase	200,000	1
reduced cyt c oxidase	430,000	1

dissatisfaction lies in our ignorance of the mechanism by which oxidation results in the production of ATP. The mechanism is called *oxidative phosphorylation*, a purely descriptive term.

The essential phenomena of oxidative phosphorylation were described by Kalckar in 1939. Oxidation in the cell was understood to be accompanied by an uptake of inorganic phosphate ( $P_i$ ) and its incorporation into an organic compound. The organic compound involved was found to be ATP. Many experimentalists devoted effort to the determination of the P/O ratio, the number of  $P_i$  atoms assumed (taken up) per 2H oxidized, and eventually the view developed that this ratio is 3. Many experimental difficulties militated against obtaining reliable data; but as the mitochondrion became better characterized, there were increased efforts to discover the mechanism involved. At present there is no agreement about the manner in which phosphate transfer is coupled to electron transfer, but some interesting suggestions are to be found in the literature.

There are at least three reactions having a large enough free-energy change to provide for the overall transfer of  $P_i$  to the terminal position in ATP. They are<sup>\*</sup>



It is not certain that the mechanisms of oxidative phosphorylation in these reactions are identical. Assumption of identical mechanism has been widespread because inhibitors such as 2,4-dinitrophenol and oligomycin are effective in all three reactions. Repeated attempts have been made to demonstrate some type of unstable intermediate which would make comprehensible the process by which electron transfer potentiates the reaction of  $P_i$ . These attempts have met with limited success.

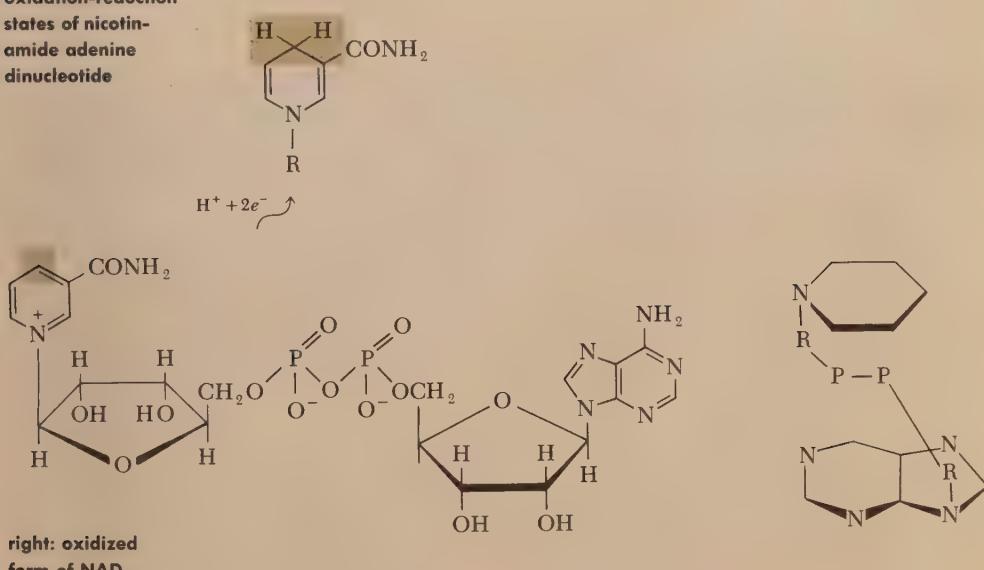
### **the electron transfer coenzymes**

Occasionally we differentiate between coenzymes, which are not difficult to dissociate from enzymes, and prosthetic groups, which are firmly bonded (REF. 13). The distinction is rather arbitrary, but it serves to emphasize the differences between NAD, flavins, and coenzyme Q in one group and the cytochromes in another. At this point in the consideration of biochemical processes it is probably better to defer a discussion of the cytochromes until some pertinent facts of protein structure have been mentioned. The electron transfer process involves a ferrous-ferric interconversion modified by the macromolecular organic matrix. In contrast, the electron transfer states of NAD and flavin are such that we can study them in the small molecules themselves.

As seen in Fig. 3.6,  $\text{NAD}^+$  is nicotinamide-ribose-(phosphate)<sub>2</sub>-ribose-

\* In this book the symbol  $:$  indicates that two systems are coupled. Usually, four small-molecule components are involved and a change in the concentration of any one necessitates an adjustment in the concentration of the others. One or more protein components may be involved.

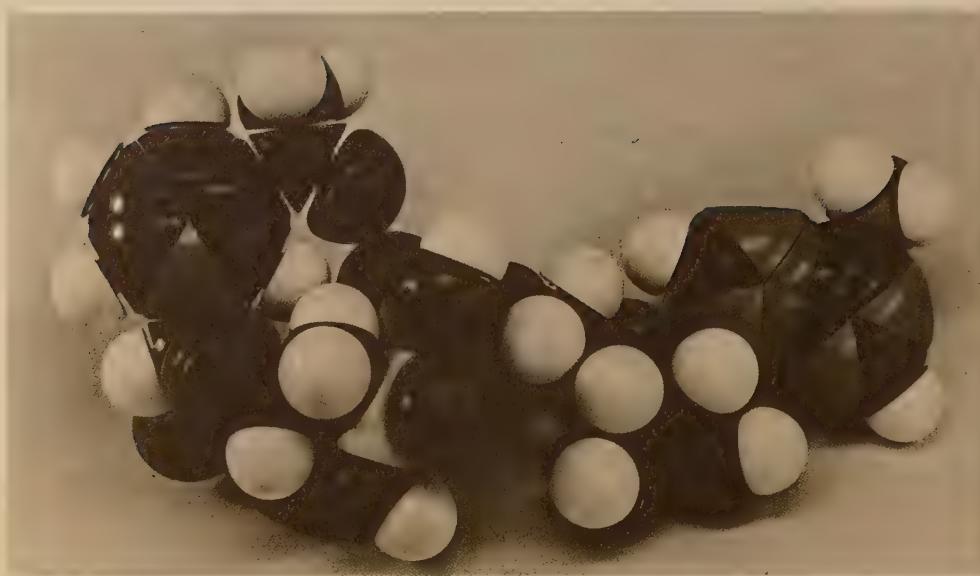
**FIGURE 3.6**  
**oxidation-reduction  
 states of nicotin-  
 amide adenine  
 dinucleotide**



right: oxidized  
 form of NAD  
 left: reduced  
 nicotinamide por-  
 tion of NADH



alternate  
view of NAD



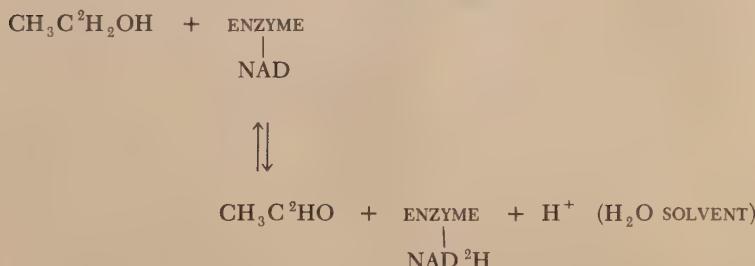
adenine.\* The reduction to NADH involves only the nicotinamide portion of the structure, and the student may well wonder why such a large molecule should be required. Only speculation can be returned in answer, but it is permissible to guess that complexity is requisite to provide opportunities for specific interactions with proteins. Several dehydrogenases requiring NAD as coenzyme have already been mentioned, and it is quite likely that the binding of coenzyme to enzyme is different in each case. Electron transfer in the cell will take place only when NAD is bound in a quite specific manner, and not when it is in free solution.

With regard to oxidation and reduction the advantages of an aromatic ring over an aliphatic structure are not readily evident. However, calculation of the uncertainty of the kinetic energy for electrons trapped in an aliphatic structure yields a value of 10 ev when the mobility is restricted to 1 to 2 Å. The uncertainty of the conjugate pair energy and time, or momentum and position, is a quantum-mechanical concept, but in qualitative terms we may view the aliphatic structure as unfavorable for controlled reactions. In contrast, an aromatic system with  $\pi$  electrons can absorb an electron with a "smearing" of charge, or a delocalization. The uncertainty in a situation analogous to that above is 0.5 ev. This energy is low enough that the electron

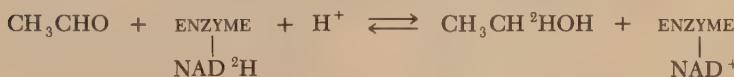
\* In the upper portion of the figure the formula for NAD is shown extended, for clarity. Recent evidence obtained from nuclear magnetic resonance studies urges the conclusion that such dinucleotides, in solution, have a conformation of "stacked" rings (sketched at the right of the figure).

can be incorporated in a reaction intermediate. In all electron transport enzymes the structure provides for just such a delocalization of charge.

Another noteworthy feature of the reduction of NAD is that hydrogen is directly and stereospecifically transferred from the substrate to the coenzyme. Vennesland and Westheimer have reported a series of elegant experiments that established this mechanism. First it was established that the hydrogen of reduced NAD (NADH) was derived from substrate during alcohol dehydrogenase (1.1.1.1) action by using ethanol labeled with deuterium ( $^2\text{H}$ ):

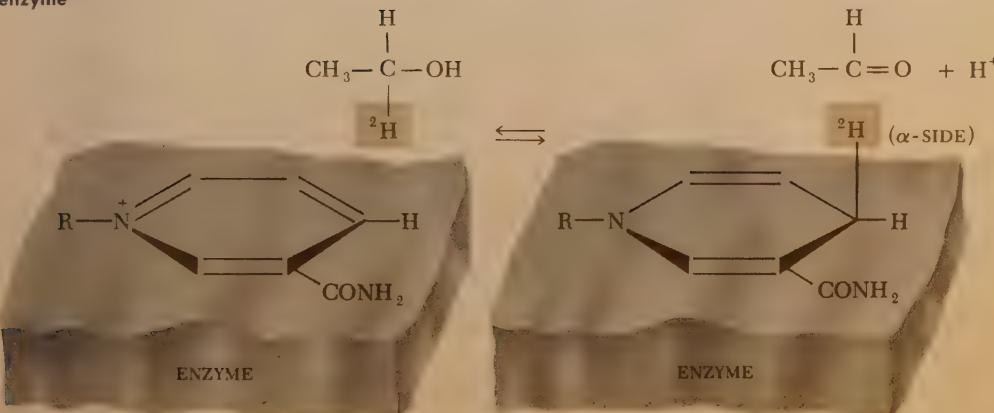


That the transfer was stereospecific was indicated by another set of experiments. It was found that all of the deuterium could be removed from the reduced labeled NAD produced in the first reaction above.



However, if the reduced NAD was produced by chemical means, by catalytic deuteration, only part of the  $^2\text{H}$  was removed from the reduced labeled NAD. This stereospecificity is detailed in Fig. 3.7. The side of the nicotinamide ring

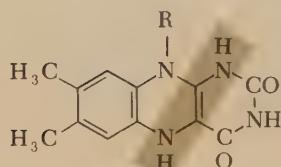
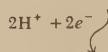
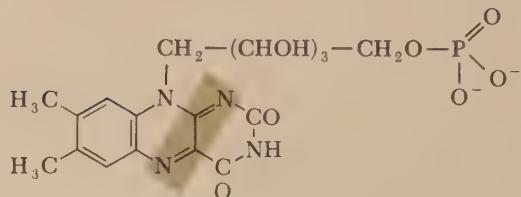
**FIGURE 3.7**  
stereospecific transfer of hydrogen from substrate to coenzyme



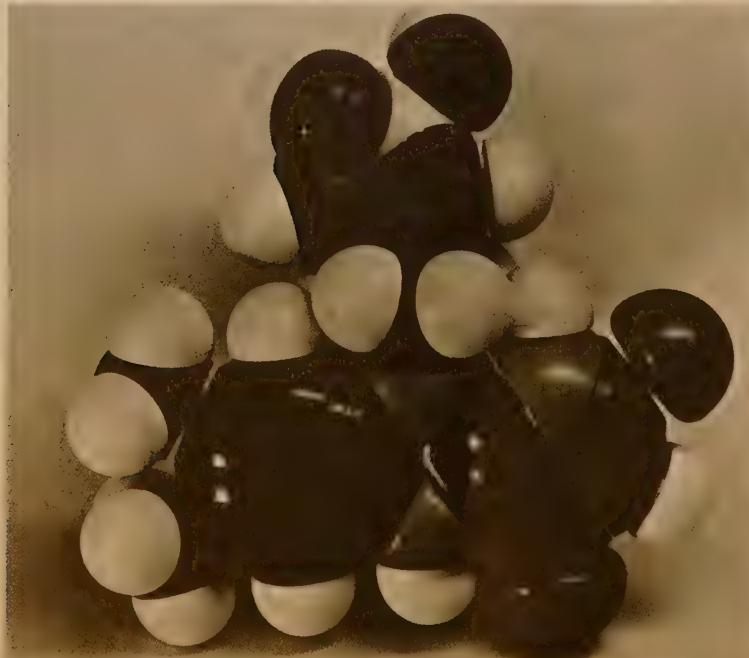
involved in hydrogen transfer in the alcohol or LACTIC DEHYDROGENASE (1.1.1.27) system has been designated, arbitrarily, the  $\alpha$  side. Other enzymes such as TRIOSE PHOSPHATE DEHYDROGENASE (1.2.1.12) bind the NAD in a different way, and the other side of the ring, the  $\beta$  side, is the acceptor.

The two types of flavin coenzyme found associated with enzymes are represented in Figs. 3.8 and 3.9. The first figure specifies the part of the iso-

**FIGURE 3.8**  
oxidation-reduction  
forms of flavin  
monophosphate



reduced form  
of FMN

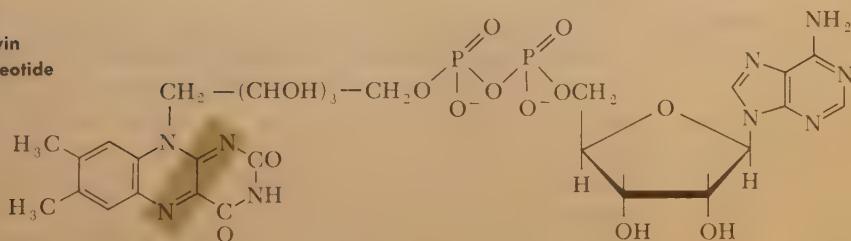


opposite side  
of preceding  
model



alloxazine ring that is involved in oxidation and reduction. It may be noted that  $2H^+$  and two electrons are involved and that no  $H^+$  is liberated as it is from the nicotinamide coenzyme. Most nucleotides contain either ribose or deoxyribose as a sugar component; here, however, ribitol, a noncyclic sugar alcohol, is present. The structural resemblances between NAD and FAD will be evident if Figs. 3.6 and 3.9 are compared.

**FIGURE 3.9**  
**formula of flavin**  
**adenine dinucleotide**



**reduced form  
of FAD**

Flavin-containing enzymes catalyze electron transfer from substrates other than succinate and NADH. In a general discussion of biological oxidation the singular case of succinate dehydrogenation is emphasized, since it is coupled to the electron transport system. In other reactions of intermediary metabolism, oxidations potentiated by flavins often result in the formation of  $H_2O_2$  and represent functions outside the present concerns.

Another noteworthy feature of the flavins is that they can be oxidized or reduced in two steps, each of which involves one electron. A semiquinone intermediate can be demonstrated in some cases by spectrophotometric means. Attempts to use electron spin resonance to obtain evidence of such intermediates have raised difficulties ascribed to charge transfer complexes. However, it is likely that the flavins are at the nexus of processes involving two electron transfers and those involving only one, as in the cytochrome system.

**comment**

Our understanding of electron transfer is really very fragmentary considering the effort expended on it. As noted above, delocalization of electrons must be involved. In nicotinamide, and in the alloxazine ring, delocalization is not too difficult to describe, but the fine detail remains to be delineated. A lucid description of delocalization in molecules as complicated as the cytochromes remains a formidable task, and it is likely that contributions from solid-state physics will be of value here. Currently, a few people are making efforts to describe formally the excited states of macromolecules

At the level where structure begins to be discernible microscopically there is also much uncertainty. Several kinds of submitochondrial particles have been obtained, each with its characteristic spectrum of chemical activities. The electron transfer system and the competence to maintain oxidative phosphorylation are localized in particles not easily solubilized. The enzymes of the citrate cycle and of fatty acid oxidation, and certain other dehydrogenases, are found in the soluble fraction and are not organized with quite the same rigidity. Part of the problem in coming to agreement about some of the structural detail is that the electron transfer system is associated with the mitochondrial membranes. From a chemical standpoint, membranes are complicated assemblages (see Sec. 27) of proteins and lipids.

At a still higher dimensional level there is a disagreement about the presence of mitochondria in bacteria. This topic will be considered again in Sec. 30. Regardless of morphology and nomenclature, in all cells there are membrane structures that contain a highly ordered electron transfer system and some mechanism for transducing the potential drop into the chemical event of ATP synthesis.

Relatively little space has been accorded a discussion of oxidative phosphorylation. The space is inversely proportional to the importance of the topic, but at the present time there are no generalizations to offer. Any attempt to simplify the discussion draws criticism. Despite this difficulty, the subject is well worth further exploration, since it is one of the truly important but unexplained biochemical mechanisms.

I noted at the beginning of the section that the relation of form to function in our knowledge of the mitochondrion has been a biochemical triumph. So it has, as continued reading will show. Yet, here too knowledge has begotten more queries. If the mitochondrion in a long-lived liver cell (150 days) has a half-life of about ten days, what governs the synthesis of new mitochondrial components? What chemical changes are occurring during the constant mitochondrial flux visible with the phase-contrast microscope? These questions, and many more, remain to be answered.

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# 4

## the mitochondrion and the citrate cycle

To recapitulate: The ETP preparation can catalyze the oxidation of NADH; the ETP<sub>H</sub> preparation can catalyze that oxidation *and* oxidative phosphorylation. What neither can do is mediate the transfer of hydrogen from a substrate molecule to NAD. For that transfer, dehydrogenases are necessary.

With this topic begins the consideration of intermediary metabolism, a subject that constituted the main concern of biochemistry for many years. The present substantial body of knowledge contains themes first set by Hans Krebs, Albert Szent-Györgyi, Fritz Lipmann, Severo Ochoa, and David Green. A complete list of contributors would include many other well-known scientists. A complex coupled set of reactions must be examined; it has been called the Krebs cycle, the citric acid cycle, and the tricarboxylic acid cycle. None of these terms is completely appropriate today. Purely arbitrarily, I shall use *citrate cycle*.

### citrate cycle

The enzymes that are involved, and include the dehydrogenases for pyruvate, malate, isocitrate, and  $\alpha$ -ketoglutarate and the decarboxylases, do not appear to be part of the isolable structure of the electron transport system. Just what the organizational relation may be is unknown. This reaction complex generates hydrogen ions and electrons to be accepted by the electron transport complex. The result is the formation of water and the conversion of ADP to ATP. Carbon dioxide is also generated; it may either be moved into the respiratory system or be utilized at some other point in metabolism. The citrate cycle also serves as a carbon source for amino acids, although the cycle itself does not provide net synthesis.

In short, the citrate cycle is a series of reactions that may furnish protons and electrons as an energy source or the actual parent compounds for syntheses. In order for such complex relations to exist, there must be a dynamic steady state. The levels of the various citrate cycle intermediates in normal rat liver are listed in Table 4.1. It has been estimated that about  $10^{-4}$  mole of each intermediate is formed per kilogram of tissue about every 10 sec. This

TABLE 4.1  
levels of citrate  
cycle intermediates  
in normal rat liver  
and acid dissociation  
constants

intermediate	molar $\times 10^4$	dissociation constants		
		$pK_1$	$pK_2$	$pK_3$
fumarate	7.3	3.03	4.44	
$\alpha$ -ketoglutarate	1.9	—	—	
succinate	1.5	4.16	5.61	
malate	1.1	3.40	5.11	
citrate	1.2	3.08	4.74	5.40
oxaloacetate	0.1	—	—	

amounts to 1 mole about every two days. These molecules have the structural relationships diagrammed in Fig. 4.1.

The initial isomerization is catalyzed by an enzyme that catalyzes the dehydration and hydration of its substrates and that has been named specifically, ACONITATE HYDRATASE (ACONITASE) (4.2.1.3). A similar reaction shown at the bottom of the scheme is catalyzed by another enzyme, FUMARATE HYDRATASE (FUMARASE) (4.2.1.2). Such reactions involve very little energy change. It will be evident that when several related forms of a compound that are, energetically, equilibrium forms exist, they will all exist in the cell. In other words, citrate and isocitrate are interconvertible in an aqueous system. An enzyme in such a case ensures only that equilibrium is attained rapidly.

The oxidation of D-isocitrate to  $\alpha$ -ketoglutarate is catalyzed by an enzyme apparently mediating two different mechanisms, dehydrogenation and decarboxylation. This is very unusual, and several attempts have been made to separate the two activities, so far without success. Nicotinamide nucleotides are required as electron acceptors in the dehydrogenation;  $Mn^{++}$  is necessary for the accompanying decarboxylation of the oxalosuccinate intermediate.

### **$\alpha$ -keto- glutarate dehydrogenase complex**

The subsequent oxidation and decarboxylation of  $\alpha$ -ketoglutarate involves another unusual enzyme system. The complex is a *soluble* organized one in which several reaction steps occur and in which the intermediates have restricted freedom of movement. It represents a cluster of reactions involving several group transfers. As illustrated in the sequence of Fig. 4.2, diphosphothiamine<sup>a</sup> and  $\alpha$ -ketoglutarate form a complex in which rearrangements occur. The  $\beta$ -imino acid decarboxylates, and a four-carbon residue is transferred to enzyme-bound lipoate.<sup>a</sup> This redistribution of atoms may be considered an oxidation to an acyl group and a reduction of the disulfide bond. The reduction of the lipoate is completed by coenzyme A.<sup>f</sup> To reoxidize the reduced lipoate enzyme complex so that it may function catalytically, an FAD-containing dehydrogenase (1.6.4.3) is present:



The +NAD/NADH system here reacts with the flavoprotein of the electron transport system. This is an unusual situation in that the reaction series is

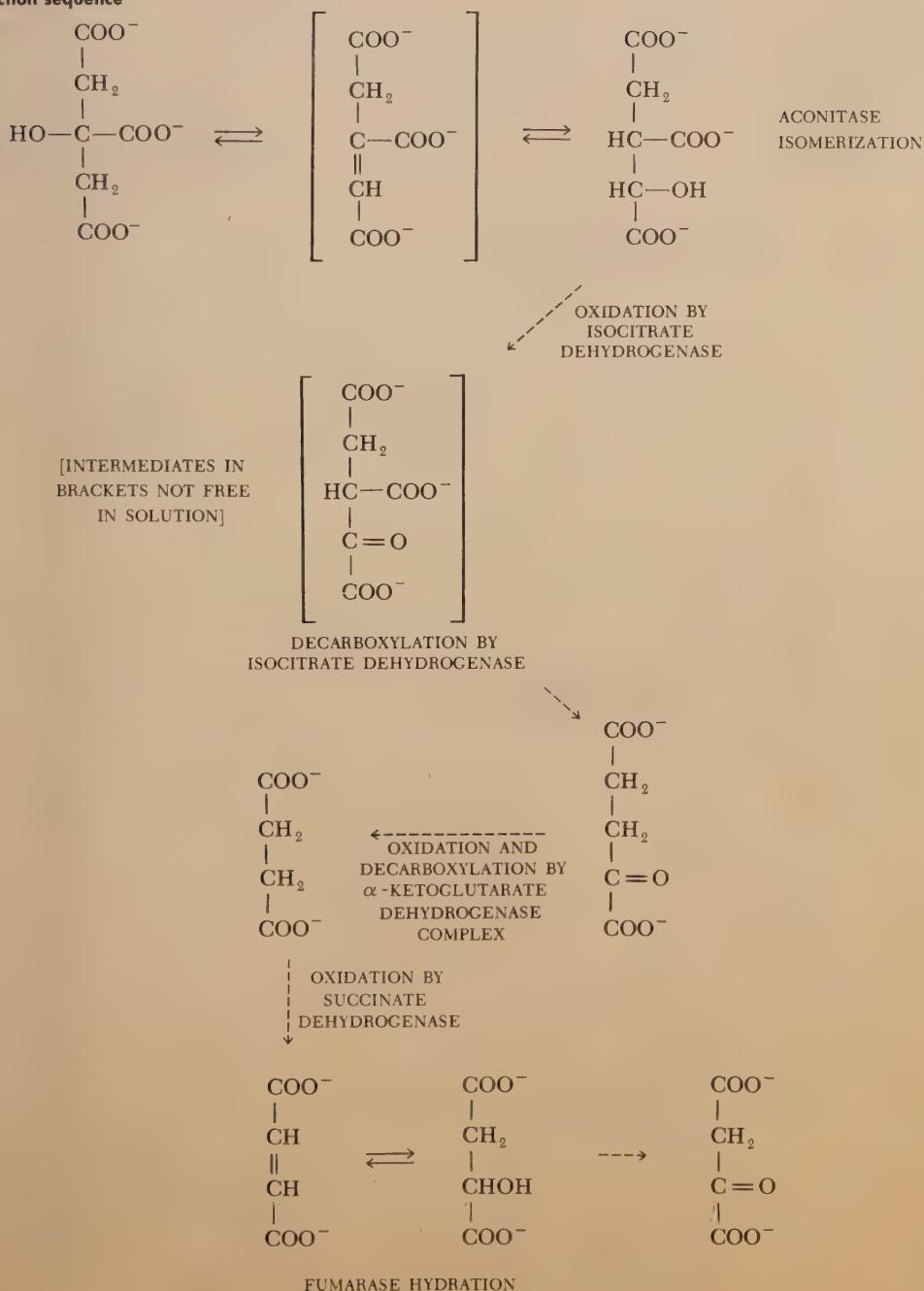


The products of the total reaction complex are succinyl CoA,  $CO_2$ , and NADH. For the seemingly simple reaction indicated in Fig. 4.1, five of the

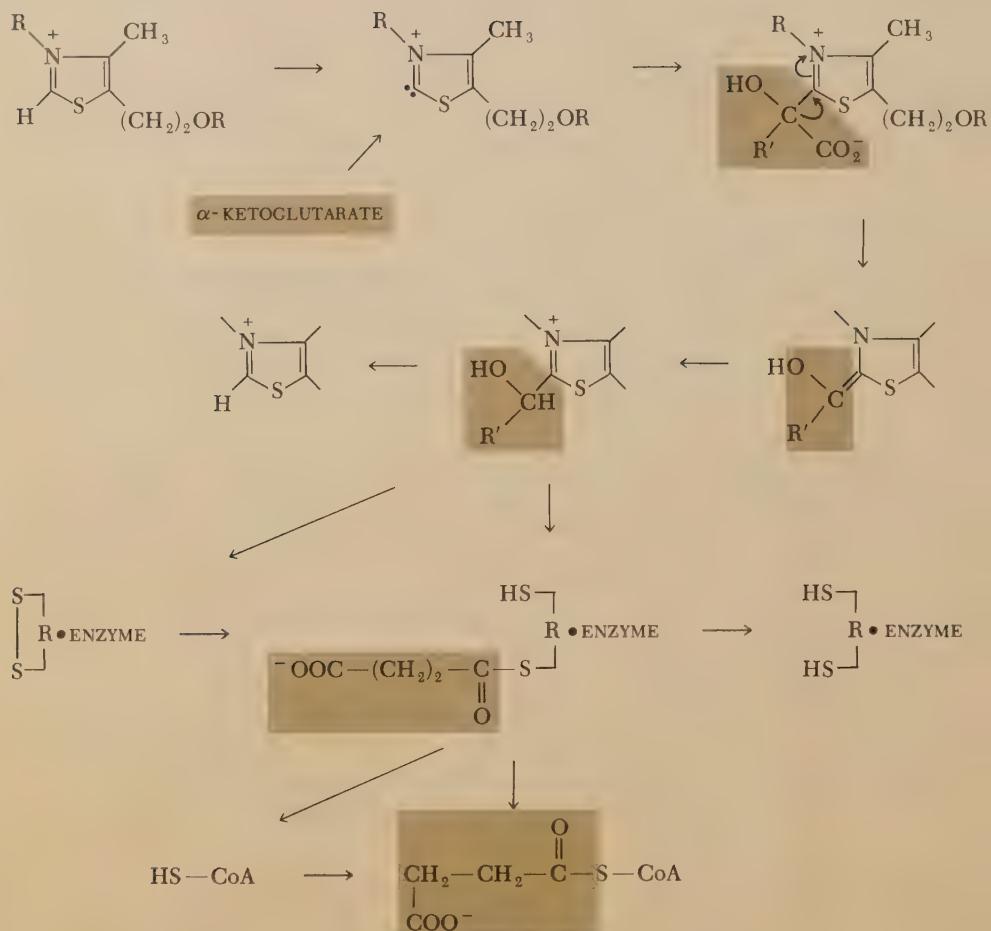
<sup>a</sup> Detailed structures of the coenzymes referred to here will be found in Sec. 29.

<sup>f</sup> *Ibid.*

**FIGURE 4.1**  
the conversion of C<sub>6</sub>  
acids to C<sub>4</sub> acids in  
the citrate cycle  
reaction sequence



**FIGURE 4.2**  
the mechanism of  
 $\alpha$ -ketoglutarate  
oxidation and  
decarboxylation

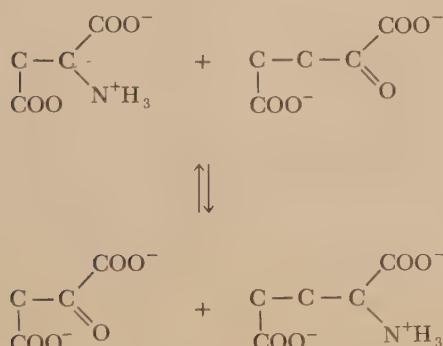


B vitamins<sup>o</sup> are needed: thiamine, lipoic acid, pantothenic acid (a component of coenzyme A), nicotinamide, and a flavin. Since the reactions being de-

<sup>o</sup> The brief descriptions of the pyruvate and  $\alpha$ -ketoglutarate dehydrogenase complexes show how the compounds of the B vitamin complex are involved in metabolism. These compounds were first sought as "factors" that could alleviate illness, but it has seldom been possible to demonstrate a causal relationship between visible pathology in tissue and the chemical behavior of a compound. There is no doubt that every cell must either synthesize, or gain, thiamine, lipoic acid, and coenzyme A. It is clear that the three compounds are involved in group transfer; but it is not clear how the visible effects of avitaminosis follow from their absence. In a practical way biochemists have triumphed in their attempts to define what is needed for good nutrition. What has not been learned is the correlation between the clinical picture and the limiting reactions that must be the cause.

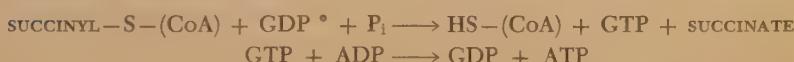
scribed occur *inside* the mitochondrion, it would not be anticipated that control would require such complexity. Because the system is complex, several alternative possibilities for reaction might be expected, and that expectation is fulfilled.

In biochemistry it has become customary to refer to "multiple pathways of metabolism." The present example is an excellent one. You have seen that the conversion of  $\alpha$ -ketoglutarate to oxaloacetate in the citrate cycle involves many steps. In contrast, the following reaction provides a one-step production of oxaloacetate:



The aspartate that interacts with  $\alpha$ -ketoglutarate is a common amino acid normally present at some small concentration. That it is not excluded from the cell may be demonstrated by incubating a tissue slice in buffer containing it. The above reaction may be observed under the proper conditions. Because the free-energy change in this reaction is very small, no substantial net "carbon flow" will occur unless one or more of the reactants or products changes markedly in concentration. In normal cell activity there is a net of reactions, and the effect of each on the other is difficult to estimate accurately. The student should begin the study of metabolism with the assumption that any reaction energetically possible will be found. Moreover, for each reaction demonstrated an enzyme may be expected. It has recently been recognized that cellular control involves not only the restrictions arising from the direct coupling of consecutive reactions but feedback phenomena as well. Further discussion of this subject is to be found in the last section of this book.

We find, then, that  $\alpha$ -ketoglutarate can participate in more than one reaction. One series of reactions is catalyzed by the  $\alpha$ -ketoglutarate dehydrogenase system, and the product containing the bulk of the carbon is succinyl coenzyme A. This product, in turn, may participate in several reactions. It may react by a mechanism that is as yet uncertain in detail and resembles oxidative phosphorylation.



- This is guanosine diphosphate, a nucleotide similar in structure to ADP except that guanine is present instead of adenine. Like adenine, guanine is present in nucleic acids (Sec. 17).

Alternatively, transacylation may occur:



In addition, succinyl-S-(CoA) may simply be hydrolyzed to coenzyme A and succinate in the presence of a HYDROLASE. In each case, succinate is formed and, as emphasized previously, this substrate has a singular electron transfer system. Other intermediates of the citrate cycle react with NAD dehydrogenases, but succinate reacts with a flavin dehydrogenase.

The product of succinate oxidation is fumarate, which is one of the two possible geometrical isomers, the other being maleate. This may be a consequence of enzyme stereospecificity, but why, it may be asked, is fumarate (the trans isomer) the result instead of maleate? The maleyl structure is not absent from tissues but is found as an intermediate in the metabolism of aromatic amino acids. Whatever the reason, fumarate is formed exclusive of any maleate and reacts further with water to form L-malate, another stereo-specified product. This reaction is catalyzed by the enzyme FUMARASE, which has been highly purified. Fumarase has been the subject of exhaustive kinetic studies and has served as an excellent experimental system for the development of certain aspects of enzyme kinetics (REF. 4).

The oxidation of malate to oxaloacetate in the presence of MALATE DEHYDROGENASE has also been carefully studied. The electron acceptor is NAD. The equilibrium of this reaction favors malate; any rise in the concentration of oxaloacetate inhibits the oxidation. Reference to the steady-state values given in Table 4.1 will verify that the level of oxaloacetate is quite low in comparison with the other citrate cycle intermediates.

Oxaloacetate is obviously a homolog of  $\alpha$ -ketoglutarate. It has been seen to be easily produced when the corresponding amino acids are present. Moreover, it is involved in a number of reactions, as is  $\alpha$ -ketoglutarate, so that it must be considered at a nexus of equilibria. In order to maintain the intermediates of the citrate cycle at a steady-state concentration, carbon compounds must enter the reaction series because carbon is continually being lost from the system as  $\text{CO}_2$  (or  $\text{HCO}_3^-$ ). The stoichiometric nature of this carbon balance led to the conception of this system as a "cycle." The immediate source of this carbon is pyruvate, which is generated from glucose by a series of reactions to be examined in detail in the next section.

### **pyruvate dehydrogenase**

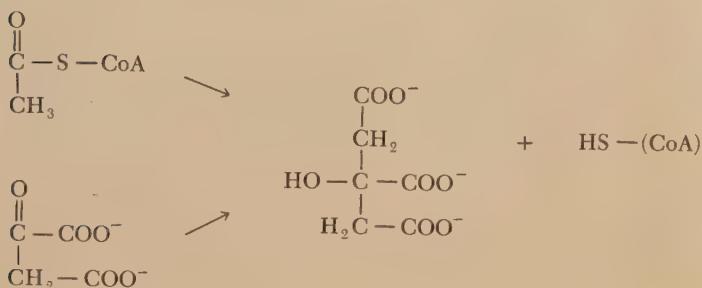
The incorporation of pyruvate and oxaloacetate atoms into citrate involves another very complex enzyme system known as PYRUVATE DEHYDROGENASE (1.2.4.1). In this complex, as in the  $\alpha$ -ketoglutarate dehydrogenase complex, there is an initial reaction of the  $\alpha$ -keto acid with diphosphothiamine. Decarboxylation ensues, and a derivative of acetaldehyde is formed. Again the aldehyde intermediate is transferred to lipoic acid. The acyl group is transferred to HS-CoA with the formation of reduced lipoic acid and acetyl-S-CoA. Reoxidation of the lipoic acid via FAD and NAD occurs as outlined previ-

ously. The pyruvate dehydrogenase complex has a "molecular weight" of nearly  $5 \times 10^6$ , and it is believed to have the composition shown in Table 4.2. Electron microscope studies indicate a polyhedral structure with a diameter of about 300 Å. The complex can be dissociated into inactive components that can reassociate to an active complex. This reassociation indicates specific interactions among the components.

**TABLE 4.2**  
components of the  
pyruvate  
dehydrogenase  
complex

component	subunit weight	aggregate weight	No. in $5 \times 10^6$
pyruvate decarboxylase		138,000	16
lipoic reductase-transacetylase	27,000		64
dihydrolipoic dehydrogenase		112,000	8

Acetyl CoA, like succinyl CoA, is a molecule that participates in many reactions. I shall limit the discussion here to the most pertinent one, the reaction with oxaloacetate catalyzed by the "CONDENSING ENZYME" (CITRATE SYNTHASE, 4.1.3.7).



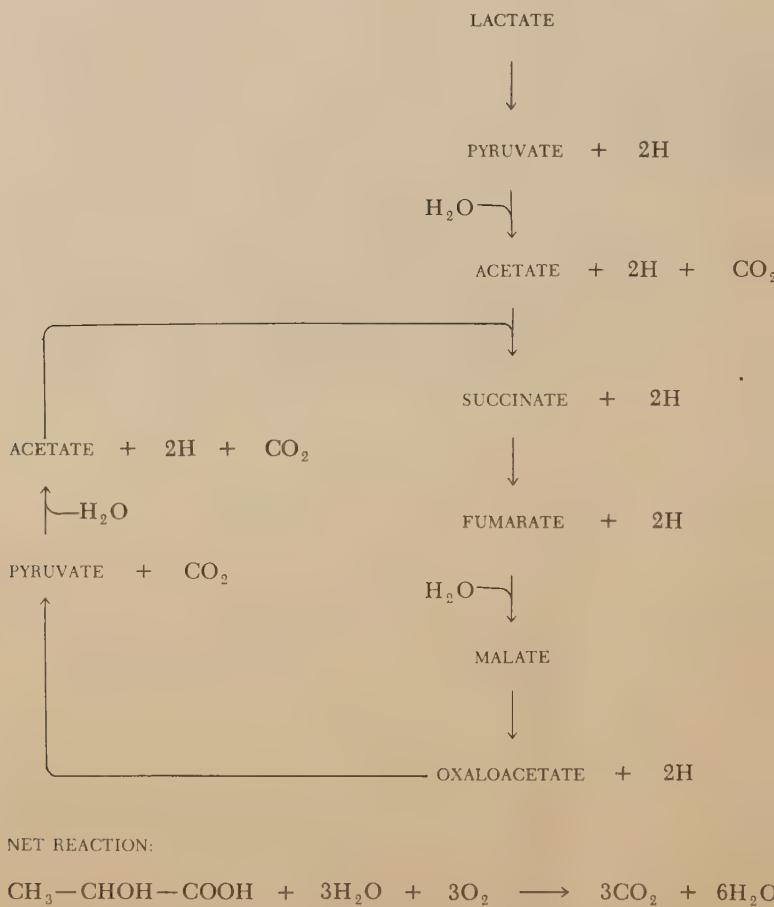
In this reaction there is no incorporation of deuterium into citrate if <sup>2</sup>H<sub>2</sub>O is used as a solvent, nor does acetyl CoA acquire deuterium if incubated with enzyme in <sup>2</sup>H<sub>2</sub>O. The observations rule out a mechanism involving dissociation of a hydrogen from the methyl group in acetyl CoA with the formation of a complex capable of exchanging H<sup>+</sup> with the solvent. It must be assumed that hydrogen transfer from the methyl group to the carbonyl group of oxaloacetate in the enzyme-substrate complex is direct. The free energy of hydrolysis of acetyl CoA is nearly that of ATP. Acetyl CoA might be expected to act as an acetylating agent, but it does not do so in this case.

### biochemical cycles

With the discussion of the condensing enzyme action and the reaction of acetyl CoA with oxaloacetate, the cyclic nature of the entire C<sub>6</sub>-C<sub>4</sub>-C<sub>6</sub> acid conversion process becomes evident. Cycles of one kind or another have become endemic in biochemistry, and others will be discussed. We have recognized several steady-state reaction sequences coupled in such a manner that a metabolically necessary net reaction is the result.

Quite early in the development of biochemistry Thunberg,<sup>°</sup> in 1920, proposed a cyclic sequence which would account for the oxidation of lactic acid in muscle (Fig. 4.3). In his scheme the acids of the cyclic reaction sequence

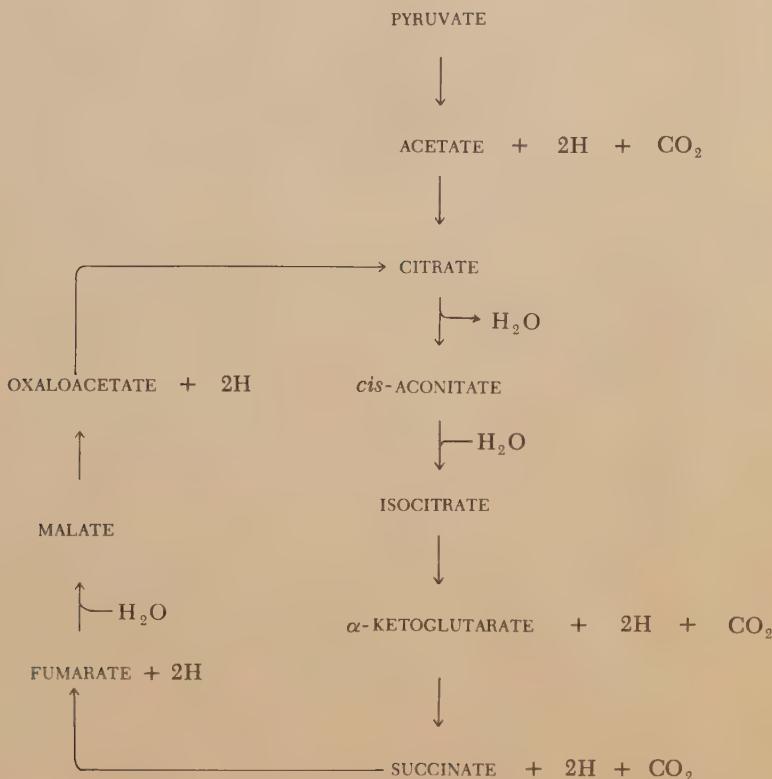
**FIGURE 4.3**  
the dicarboxylic cycle  
proposed  
by Thunberg



<sup>°</sup> The abstract of REF. 1 is quoted here for historical perspective; the paper had a considerable influence on biochemical thinking. T. studied the reactions of intermediary metabolism by observing the decolorization of methylene blue under various conditions. When striated muscle of a freshly killed frog is extd. sufficiently long with  $\text{H}_2\text{O}$  it loses its power, which it previously had, of decolorizing methylene blue in  $\text{O}_2$ -free soln. This power, however, is restored when certain substances are added to the muscle-methylene blue system, although these compds. themselves have no direct decolorizing effect. An analysis of a succinic acid-muscle-methylene blue system showed that the decolorization process consists in the transference of 2 H atoms from the succinic acid to the methylene blue through the mediation of an enzyme, a so-called "hydrogen-transportase," the reaction yielding on the one hand an acid of less H content, and on the other the leuco compd. of methylene blue. Other compds. exhibit this property of restoring the activity to the muscle tissue. Among those studied,

could be considered catalytic provided they had a steady-state concentration and also provided oxidation was strongly inhibited by their absence. Many years later, in 1936, Szent-Györgyi supplied this kind of proof by showing that the addition of dicarboxylic acids to muscle slices stimulated respiration far more than expected from their total oxidation. Then, just at the beginning of World War II, Krebs proposed the fundamental relations of a cycle including both the Thunberg dicarboxylic acids and a group of tricarboxylic acids (Fig. 4.4).

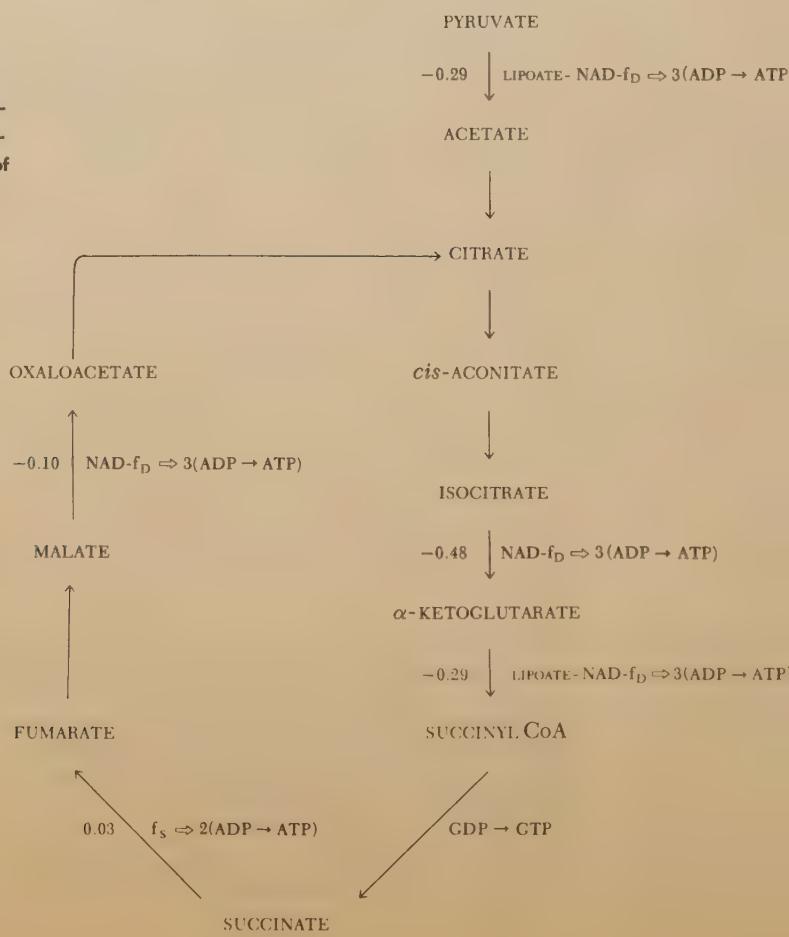
**FIGURE 4.4**  
the citric acid cycle  
proposed by Krebs



*lactic, fumaric, both  $\alpha$ - and  $\beta$ -hydroxybutyric, malic, 1-tartaric, 1- $\alpha$ -hydroxyglutaric, citric and glutamic acids all gave positive reactivating effects. As a result of these observations T. is of the opinion that the simple materials such as glucose, fat, and amino acids which are brought to the cells as nourishment, pass through a whole series of intermediate stages before final disposal. An early step in this chain of reactions is a "dehydrogenation" or indirect oxidation, combined in certain cases with hydrolysis, or  $\text{CO}_2$  splitting. A whole series of dehydrogenating or hydrogen-transferring enzymes takes part in the accomplishment of these reactions. These enzymes are generally specific for the different intermediary compds. The H that is removed from these compds. can either unite with  $\text{O}_2$  to form  $\text{H}_2\text{O}$ , or can be used to hydrate other compds. On this basis all food-stuffs are H-donors; more fundamentally H is the common fuel of the cells.* By permission of Chemical Abstracts. From F. S. Hammett's abstract in *Chem. Abstracts*, 15, 876 (1921).

At the present time we believe the primary site of oxidation to be the mitochondrion (or the cell membrane in bacteria). Part of the membrane structure, or affixed to it, is an electron transport system which is furnished with two electron ( $2H$ ) quanta by the citrate cycle. The source of the electrons for the overall irreversible and energy-yielding oxidation is, of course, pyruvate. Figure 4.5 is meant to identify those dehydrogenation steps coupled with oxidative phosphorylation. From each mole of pyruvate oxidized catalytically by the citrate cycle and the appropriate electron transfers there accrues to the cell 15 labile phosphate bonds representing about  $15 \times -7 = -105$  kcal ( $\Delta G^\circ$ ). When a mole of pyruvate is oxidized to  $CO_2$  and  $H_2O$  by combustion in molecular oxygen, the  $\Delta G^\circ = -280$  kcal. Guesses about the values of  $\Delta G$  show that about 30 to 50% of the free-energy change becomes available to the cell for its own economy. Some of the waste heat no doubt helps to keep the body temperature near  $37^\circ$ . Although the mitochondrion has been referred to as a transducer, it is not strictly that. A transducer con-

**FIGURE 4.5**  
oxidative phosphorylation coupled with citrate cycle dehydrogenations. The numbers are the values of  $E'_0$  at pH 7 and  $30^\circ$



verts one form of energy into another, but with poetic licence, one may think of the transduction of chemical potential in general into the biologically acceptable phosphate transfer energy.

The foregoing discussion is intended to be succinct and to represent the reactions to be found in the majority of cell types. The comparative biochemistry of the citrate cycle and related variations is formidable. Particularly in microorganisms, these cycles are often linked to synthetic processes (REF. 7), and species specificity is often encountered.

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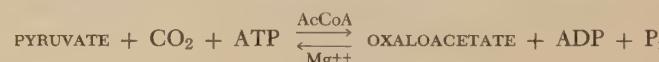
# 5

## glycolysis: function without structure

Although the general structural features of the mitochondrion have been noted, no more than a portion of mitochondrial metabolism has been considered. It is timely, then, to outline the reactions involved in moving carbon compounds into the citrate cycle via pyruvate.

### **pyruvate metabolism**

It may be worth emphasizing at the outset that pyruvate participates in many reactions both inside and outside the mitochondrion. It may even enter the citrate cycle by reactions other than the one already mentioned. For example the following reaction is catalyzed by PYRUVATE CARBOXYLASE\* (6.4.1.1).



In the presence of a TRANSAMINASE (2.6.1.2) pyruvate serves as the source of the carbon in alanine,  $\text{CH}_3\text{CH}(\text{NH}_3^+)\text{COO}^-$ . Moreover, pyruvate is a source of acetyl CoA, a metabolic unit in the synthesis of fats and other compounds. It is interesting to speculate on the factors controlling the flow of pyruvate carbon into the various reactions in which it participates. Certainly the steady state in the normal animal must represent a compromise of many factors but must be mobile enough to accommodate a wide range of physiological activity.

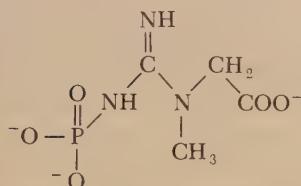
Pyruvate arises from processes that take place outside the mitochondrion. The enzymes that catalyze the reactions producing the main supply of pyruvate are soluble and are not known to be confined to any subcellular structure. They constitute a group of enzymes, the enzymes of glycolysis, that are extraparticulate and extrastructural and are often said, synonymously, to be "cytoplasmic."

In certain circumstances only small quantities of pyruvate enter mitochondrial metabolism. For example, in muscle tissue during intense mechanical activity there is but little oxygen available. Under these conditions the glycolysis reactions to be described assure an  $\text{NAD}^+/\text{NADH}$  ratio of a relatively low value. There is present an enzyme, LACTIC DEHYDROGENASE which permits NADH to reduce pyruvate to lactate ( $\text{CH}_3-\text{CHOH}-\text{COO}^-$ ). The lactate is produced in substantial quantities and may be demonstrated i

\*As noted in the preceding section, any undue rise in oxaloacetate concentration will inhibit malate dehydrogenase. Reference to Fig. 4.5 shows the  $E_0$  value to be  $-0.10$ , a value higher than that for  $+\text{NAD}/\text{NADH}$ . In order for spontaneous oxidation to occur, the ratio malate  $\cdot +\text{NAD}/\text{oxaloacetate} \cdot \text{NADH}$  must be higher than  $2 \times 10^6$ . The AcCoA specified in the equation is not directly involved in the reaction but is necessary to ensure an active conformation of the enzyme.

the blood flowing away from such tissues. As the blood moves through the liver, the lactate is converted back to pyruvate by the liver cells, which are aerobic. This conversion of pyruvate to lactate is one of the special features of muscle cells—cells specialized in function and metabolism but forming a large portion of the mammalian mass. Chemically speaking, H<sup>+</sup> and electrons are transported away from muscle cells as lactate formed from the pyruvate functioning as an acceptor in lieu of oxygen. Only a few types of cells can function normally even for short intervals in the absence of oxygen. Brain cells are among the most susceptible to anaerobiosis and quickly undergo irreversible changes.

At any instant the intact muscle cell may derive energy from two sources. The first source is the rather unusual compound, *N*-phospho-*N'*-methyl guanidino acetate, bearing the trivial name creatine phosphate:

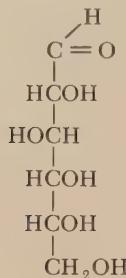


The steady-state concentration of creatine and creatine phosphate is higher than that of the ATP/ADP system. The lability of the *N*-phosphate is equivalent to that of the terminal phosphate of ATP, and there is present in muscle an enzyme that catalyzes the phosphate transfer between the two systems. During activity, the ATP concentration remains at the steady-state level but creatine phosphate decreases in concentration. It is the terminal phosphate transfer from ATP that furnishes the chemical energy transduced into mechanical energy. During periods of stress, the creatine/creatin-phosphate system acts as a buffering system. During periods of rest, the ratio is restored. Although substantial concentrations of the creatine compounds occur in muscle, and in some other tissues as well, the continuing supply of energy must derive from the alternate source, glycolysis. This is a series of reactions that occurs in nearly all cells, a series of reactions that results in the conversion of glucose to pyruvate as well as the net synthesis of ATP from P<sub>i</sub> and ADP.

The muscle cell in mammals receives from the blood flowing near it a constant supply of glucose at a steady-state concentration near 120 mg/100 ml.\* In other animals this concentration may be very different, and in some organisms it may be highly variable. Both muscle and yeast cells have been used classically in the study of glycolysis, and the two mechanisms of glycolysis are remarkably similar despite the obviously disparate environments.

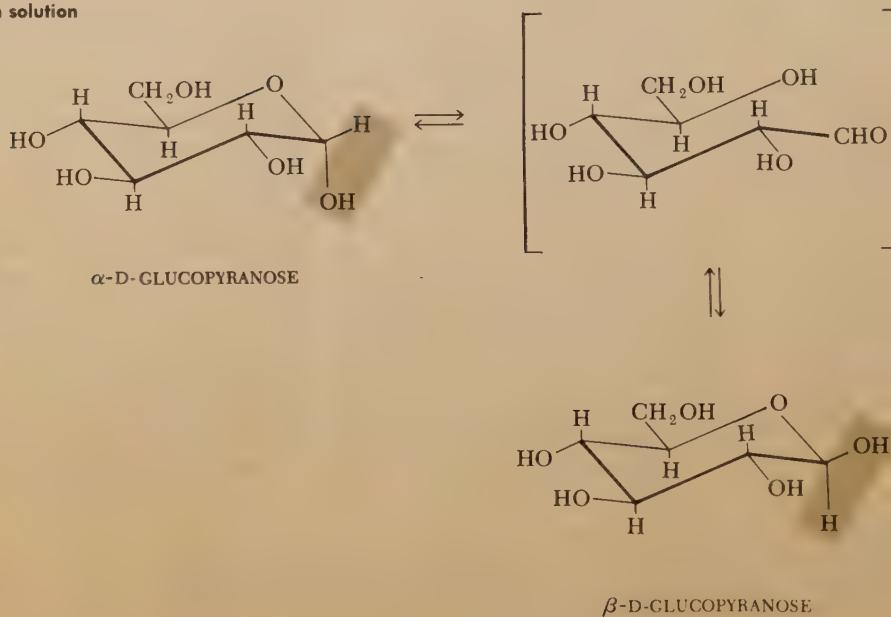
\*This is usually referred to in the biochemical literature as 120 mg %. Vol % is a unit unique to biochemistry but of obvious utility. Blood and fluids of all kinds to be analyzed are usually measured by volume, but the substance being measured is usually referred to a known weight of a standard.

**glycolysis** Emil Fischer chose to present many of his conclusions about glucose in the following formula:



It is immediately evident that a six-carbon structure could hardly hold more groups susceptible to biological dehydrogenation. Despite its utility, the structural formula presented is not consonant with all the properties of the compound. Glucose does not have a simple straight-chain structure; a free aldehyde group is not demonstrable qualitatively; and the hydroxyl groups are not on opposite sides of a plane. In solution glucose is largely present as a mixture of the  $\alpha$  and  $\beta$  anomers in the ring form depicted in Fig. 5.1. In  $\beta$ -D-glucopyranose all the hydroxyl groups, as well as the hydroxymethylene group, are equatorial. This minimizes steric interactions, so that the  $\beta$  anomer is the predominant form in solution. It has been suggested that glucose is the most thermodynamically stable of the aldohexoses, and this stability is in

**FIGURE 5.1**  
prevalent forms of  
glucose in solution



left:  $\alpha$ -glucose  
right:  $\beta$ -glucose

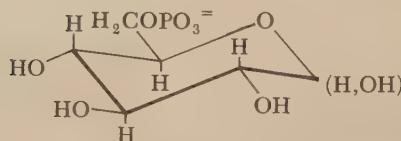


models showing  
axial hydrogens  
left:  $\alpha$ -glucose  
right:  $\beta$ -glucose

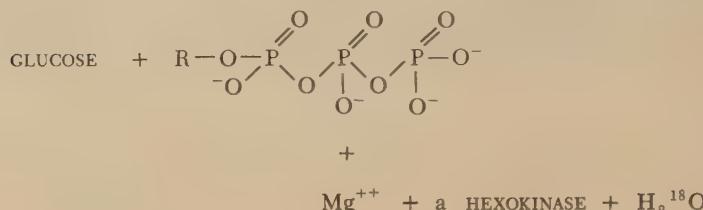


agreement with its ubiquity in biological saccharides. Ring forms other than those shown in Fig. 5.1 are present in solution but in very small amounts. The possibilities for hydrogen bonding are numerous, and such intermolecular forces influence the conformations present under various conditions. In particular, there is maximum interaction with water molecules and almost no tendency to form dimers, trimers, or oligomers except through covalent bonds. Cell membranes present no barrier to the influx of glucose molecules. Because of the many possibilities for hydrogen bonding, it is probable that other small molecules and even ions may normally be associated with glucose. Once inside the cell, glucose is converted to an ionized, and charged, derivative for

which the cell membrane is a barrier.<sup>o</sup> This conversion results in a glucose concentration gradient even when the concentration on the outside of the membrane is low. The derivative formed, glucose 6-phosphate, is the product



of glucose phosphorylation by ATP in the presence of a class of enzymes termed HEXOKINASES (2.7.1.1). In this reaction the terminal phosphate replaces the hydrogen of an alcoholic OH.

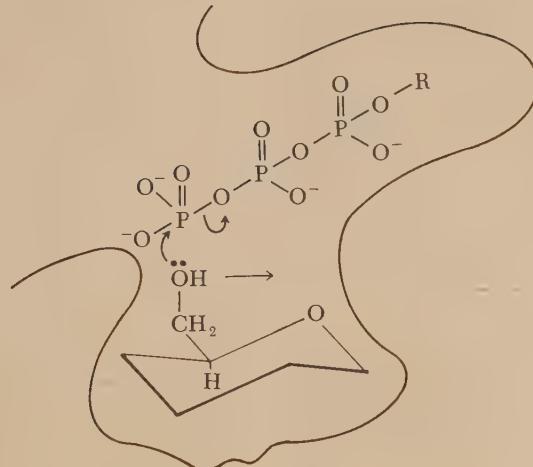


yields glucose-6-P (nonlabeled) and ADP (nonlabeled). Presumably there is a nucleophilic attack by O: within the ES complex as pictured in Fig. 5.2. Normally, glucose 6-phosphate does not accumulate; it is either oxidized (Sec. 6.1) or converted to pyruvate. If the equilibrium shift is not sufficiently great, it enters yet another series of reactions which produce a glucose polymer.

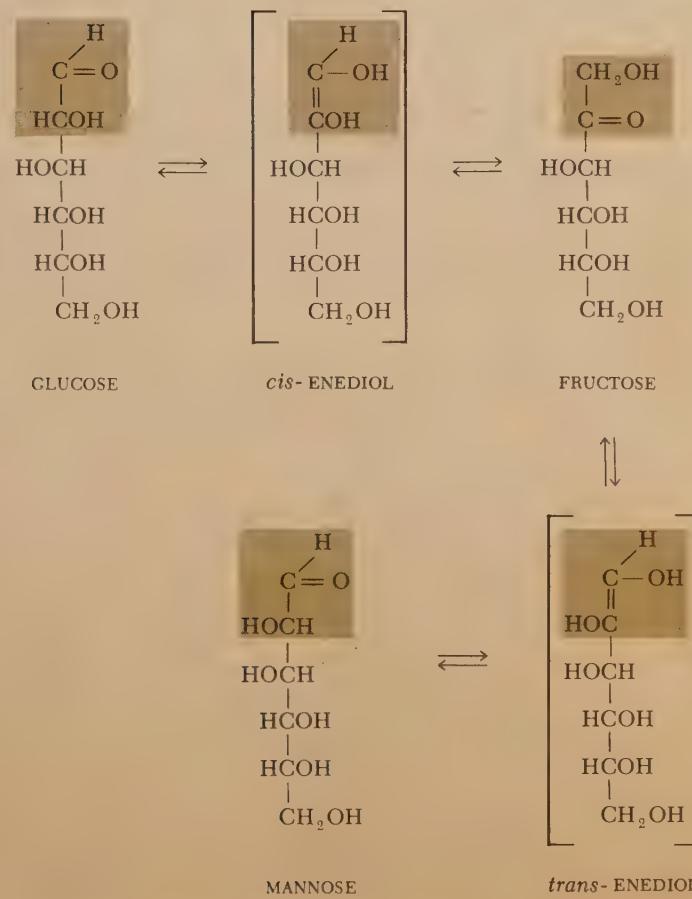
The next reaction in the glycolysis series resembles one explored during the late nineteenth century by Lobry du Bruyn and Alberda van Eckenstein. They discovered that when glucose was allowed to remain in solution at a pH near 8.5, an equilibrium system of sugars was produced. The same equilibrium could be attained by starting with any one of three sugars: glucose, fructose, or mannose. Somewhat later Nef postulated the intermediates in brackets in Fig. 5.3. The enolizations diagrammed here account for the observations, but an alternative mechanism with anionic forms instead of those pictured can be written. The essential point is that the configuration of carbon 2 is altered at a low H<sup>+</sup> concentration. If pH is raised even higher, the other carbons are affected. The straight-chain forms are pictured for convenience, but it should be remembered that the sugars are chiefly present as a mixture of ring-form anomers.

<sup>o</sup> Generally, sugar phosphates are found only inside cells and do not escape, nor do they serve as readily available carbohydrate. It has been shown recently, however, that glucose 6-phosphate (in the medium) can support the growth of an *E. coli* mutant with a deficient transport system for glucose. Similar cases are known, and it has been concluded that a special transport mechanism is involved (REF. 6).

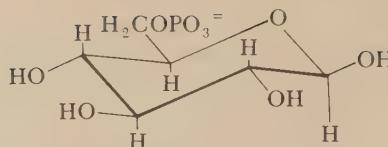
**FIGURE 5.2**  
proposed mechanism  
of glucose phosphor-  
ylation



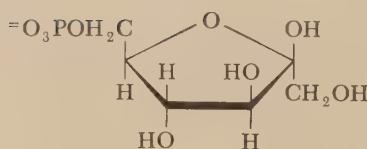
**FIGURE 5.3**  
The equilibria among glucose, fructose, and mannose in aqueous solution at pH 8.5



**FIGURE 5.4**  
hexose phosphate isomerization

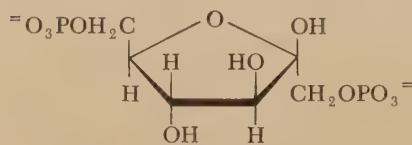


D-GLUCOSE-6-PHOSPHATE



D - FRUCTOSE-6-PHOSPHATE

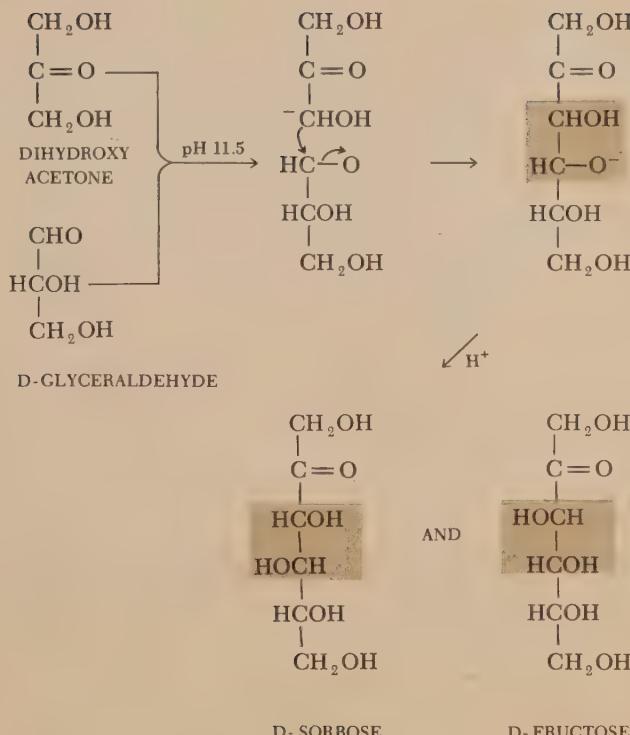
Glucose 6-phosphate likewise isomerizes, but at pH values higher than the pH in the living cell. An enzyme, GLUCOSEPHOSPHATE ISOMERASE (5.3.1.9) is present in most cells, but it catalyzes only the equilibrium shown in Fig. 5.4. There is recent evidence that the  $\beta$  anomer of the glucose 6-phosphate is the preferred substrate. Note that the fructofuranose ring is essentially planar. There is no tendency for this system to move away from equilibrium except by mass action. This shift is provided by the phosphorylation of the other primary hydroxyl in fructose 6-phosphate. Again ATP is the donor of the phosphate group transferred in the ES complex with PHOSPHOFRACTO KINASE



(2.7.1.11). Fructose 1,6-diphosphate is an historic compound because it was the first sugar phosphate to be characterized. In an era when men's names were often associated with compounds it was called the Harden-Young ester. In yeast-juice incubation mixtures it accumulates in such a concentration that it is rather easily detected. For reasons soon to be detailed, this accumulation does not occur in living cells.<sup>o</sup> Indeed, fructose 1,6-diphosphate is in a steady-state relation with triose phosphates, and the nature of the reaction may be visualized in the model reaction of Fig. 5.5. If this reaction proceeds in  ${}^2\text{H}_2\text{O}$  no isotope is bound to carbon in the hexoses. Obviously there are four pos-

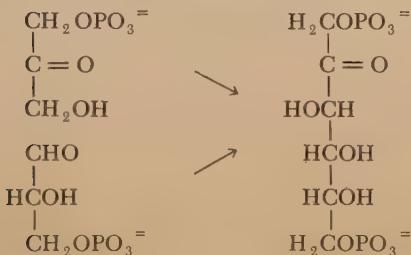
<sup>o</sup> Fructose diphosphate does accumulate in cell-free preparations because the ATP is not involved in maintaining the integrity of the cell. Under such circumstances fructose-6-P is the chief acceptor of the terminal phosphate.

**FIGURE 5.5**  
condensation products of dihydroxy acetone and glyceraldehyde at pH 11.5



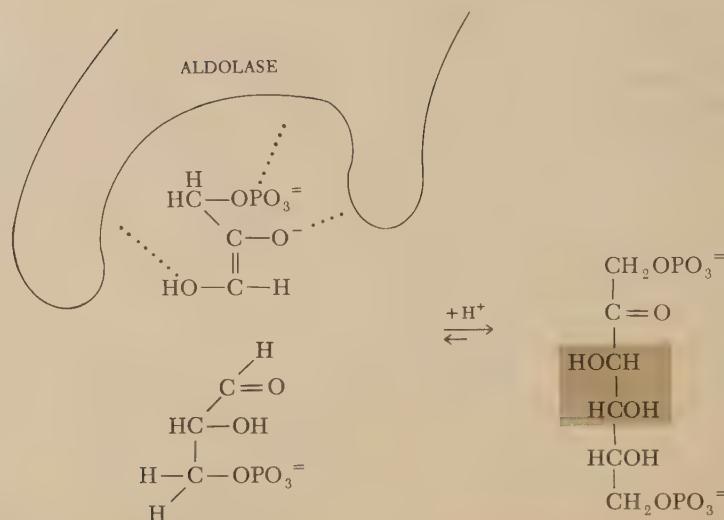
sible products, but only two have been identified. Some stereospecificity is evident, and it must be assumed that the two forms not produced are much less stable.

The analogous reaction in the cell is that catalyzed by the ALDOLASES (4.1.2.7) and (4.1.2.13), an aldol condensation of the two possible triose phosphates to fructose diphosphate.

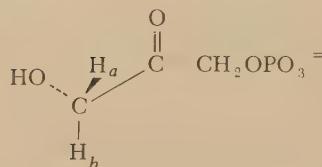


As one might anticipate from the mechanism of Fig. 5.5, dihydroxyacetone phosphate is an obligate component in this reaction, since it forms the anionic species. Several aldehydes will react as substitutes for D-glyceraldehyde-3-phosphate, although more slowly. Spectral data support the conclusion that the enzyme-substrate complex contains dihydroxyacetone phosphate. Iso-

**FIGURE 5.6**  
suggested mechanism  
of aldolase action

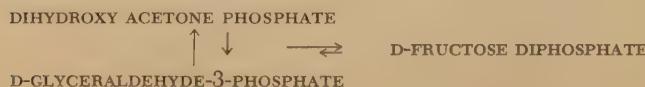


otope experiments with tritium have shown that a particular hydrogen,  $H_a$ , is mobilized from the specified substrate.  $H_a$  and  $H_b$  are not sterically equivalent in the ES complex.



In order to account for the fact that the equilibrium involves only *one* hexose isomer, D-fructose 1,6-diphosphate, the relations<sup>o</sup> in Fig. 5.6 have been suggested. The rate of the enzyme-catalyzed reaction is about  $10^3$  mole/(liter)(min) compared with about  $10^{-6}$  mole/(liter)(min) for the base-catalyzed reaction. This is an amazing disparity in rates, but by no means unique, as noted in Sec. 2.

In addition to this equilibrium there is another facilitated by TRIOSEPHOSPHATE ISOMERASE (5.3.1.1). Thus the actual equilibrium relations might more clearly be diagrammed as



When dihydroxy acetone phosphate is incubated with this isomerase in  ${}^3\text{H}_2\text{O}$  (tritium oxide), an atom of  ${}^3\text{H}$  is bound to carbon. If *this* product is incubated with the enzyme in  $\text{H}_2\text{O}$ , the  ${}^3\text{H}$  is lost. The H involved is not the one mobilized.

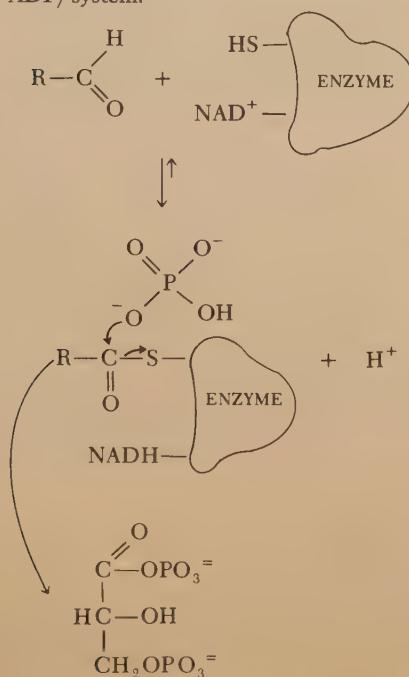
<sup>o</sup> The attribute of stereospecificity requires a three-point attachment of substrate to the enzyme as explicated in Fig. 5.6.

ized by aldolase. Presumably it is H<sub>b</sub>, in the tetrahedral diagram, that becomes labile because of the action of this isomerase. From the dissymmetry of the equilibria it follows that the concentration of glyceraldehyde phosphate will be quite low. This sugar does have a free aldehyde group, unlike most others, and it has the D configuration characteristic of the majority of biological sugars.

Even the most casual experience in the organic chemistry laboratory convinces the student that aldehydes are easily oxidized to acids. A large  $-\Delta G$  is indicated. It may be anticipated that, in the cell, oxidation of glyceraldehyde phosphate occurs. Just as in oxidative phosphorylation, there is coupling with the adenine nucleotide and nicotinamide nucleotide systems and P<sub>i</sub> is incorporated into the terminal phosphate of ATP.

The enzyme 3-PHOSPHOGLYCERALDEHYDE DEHYDROGENASE\* (1.2.1.12) contains NAD firmly bonded into the protein. The sulphydryl groups of this enzyme-must be in the reduced state (that is, as R-SH, not R-S-S-R') if catalysis is to occur, and the mechanism in Fig. 5.7 has been suggested. The product, 1,3-diphosphoglycerate, is evanescent. An enzyme, PHOSPHOGLYCERATE KINASE (2.7.2.3), is responsible for a rapid reaction with the ATP/ADP system which results in a rise in the ATP/ADP ratio and the production of 3-phosphoglycerate. Diphosphoglycerate is a mixed anhydride, has a large  $-\Delta G$  of hydrolysis, and is in equilibrium with the adenine nucleotide (ATP/ADP) system.

**FIGURE 5.7**  
proposed mechanism  
of phosphoglyceral-  
dehydronase action



\* In speaking of enzymes the singular noun is often used. This is misleading even if it is convenient. Glyceraldehyde dehydrogenases from various organisms, or even from different cells of the same organism, may be rather different in molecular weight, stability, or other properties. They are similar in that they catalyze the same reaction, but the mechanisms may not be identical.

The NADH formed (as in Fig. 5.7) during glycolysis in muscle can be re-oxidized by reaction with pyruvate in the presence of lactic dehydrogenase. Because of this, the glycolysis series of reactions provides an internal oxidation-reduction set. No immediate participation of oxygen is necessary, and a transient steady state involving two oxidation-reduction pairs may develop:

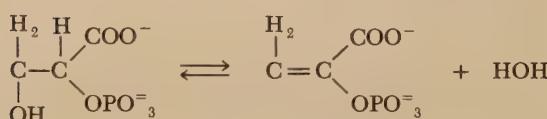


*Anaerobic* glycolysis occurs when these energy-producing reactions take place in the absence of oxygen. *Aerobic* glycolysis is similar, but pyruvate is moved into the mitochondrion, where it merges with the citrate cycle. The NADH formed cannot enter the mitochondria but is oxidized indirectly by a "shuttle" system involving the mitochondria. Some cells contain an  $\alpha$ -GLYCEROPHOSPHATE DEHYDROGENASE (1.1.1.8) that assures a rapid rate for the reaction

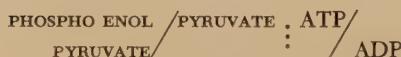


The  $\alpha$ -glycerophosphate can enter the mitochondrion, where it is reoxidized to dihydroxy acetone phosphate that can reenter the extramitochondrial reaction. Thus the extramitochondrial NADH/NAD<sup>+</sup> ratio is a partial function of the intramitochondrial respiratory electron transport. For further discussion on the control of glycolysis see Sec. 31.

Energetically, 3-phosphoglycerate differs only slightly from 2-phosphoglycerate. As might be anticipated, an enzyme, a PHOSPHO GLYCEROMUTASE (PHOSPHOGLYCERATE PHOSPHOMUTASE) (5.4.2.1), ensures rapid equilibrium. The 2-phospho derivative can be dehydrated reversibly:



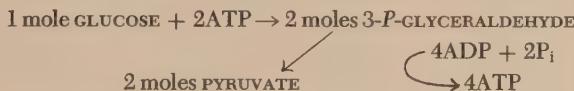
The enzyme involved, an ENOLASE (4.2.1.11) is known to be active only as a metal-protein complex<sup>a</sup> binding Mg<sup>++</sup>, Mn<sup>++</sup>, or Zn<sup>++</sup>. The enol formed, phospho enol pyruvate, has a large  $-\Delta G$  of hydrolysis. It is the phosphorylated derivative of the enol form of pyruvate and may couple with the ATP/ADP system.



This reaction is catalyzed by PYRUVATE KINASE (2.7.1.40) and requires both Mg<sup>++</sup> and K<sup>+</sup> (or NH<sub>4</sub><sup>+</sup>). The action of the metal ions is not understood.

<sup>a</sup>Mg<sup>++</sup> is often required for enzyme-catalyzed reactions involving phosphates. The manner in which Mg<sup>++</sup> participates is not yet known in most cases. It may be involved in binding the substrate to the enzyme, in forming a complex with phosphates as previously noted for ATP in Sec. 3, or in inducing a special protein conformation. Mg<sup>++</sup> is often replaceable by Mn<sup>++</sup>.

Stoichiometrically, glycolysis may be represented thus:



The reactions of glycolysis are coupled in such a way that the ATP/ADP ratio tends to be maintained even when other processes, or "energy demands," tend to decrease the ratio. The product, pyruvate, is one from which a substantial amount of energy is still to be derived provided oxygen is available.

For the yeast and certain other organisms, anaerobic glycolysis can provide enough energy (as ATP) to sustain life, but this is not to say that it will sustain normal growth rates. The fermentation of glucose by yeasts usually ceases because the concentration of ethanol interferes with some life process. In this organism pyruvate is rapidly decarboxylated to acetaldehyde that, in turn, is reduced to ethanol by the nicotinamide nucleotide system.

### ACETALDEHYDE/ETHANOL : NAD/NADH

The end product of reduction, ethanol in yeast, lactate in muscle, is moved out of the cell entirely. In the higher organism the reduction product is "retrieved" for reuse. In yeast fermentation the ethanol concentration rises, unless oxygen is admitted, until the cells become inactivated.

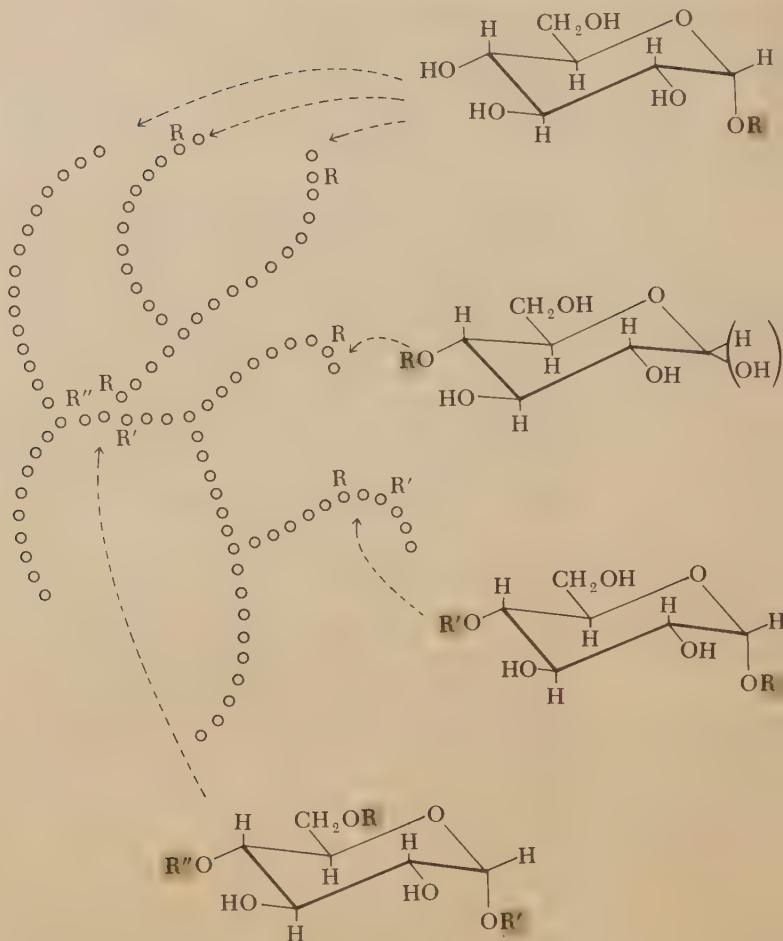
aerobic  
glycolysis

In almost all types of cells respiring in a normal oxygen supply a number of other reactions that are dependent on glycolysis can be discovered. One process, known since the distinguished and energetic research of Claude Bernard in the last century, may be demonstrated by an experiment often found in laboratory manuals. Provide yourself with two animals similar except that animal A has been fed as usual but animal B has not been fed for 24 hr. Mice, rats, or guinea pigs have often been used for this purpose. The experimental animals must be sacrificed and the livers removed. The liver sample should be minced, added to a tube containing 30% KOH, and heated to 100° for several hours.

Occasional examination will show a substantial amount of insoluble material persisting in the tube containing the tissue from animal A but very little from the tissue of animal B. If this insoluble substance is collected by centrifugation and heated briefly with dilute acid, the hydrolyzate formed can be shown to contain only glucose. Proteins and fats are solubilized and hydrolyzed in strong hot alkali solutions, but polysaccharides are much more stable.

The insoluble material from liver is indeed a polysaccharide and was given the name glycogen. It is a nonlinear polymer of glucose, each unit being joined by an  $\alpha$ -glucosidic linkage either to carbon 4 or carbon 6 of another glucose unit. Only a three-dimensional model can do justice to such a com-

**FIGURE 5.8**  
**diagrammatic sketch**  
**of glycogen structure**  
**and the glucosidic**  
**units involved**



plicated molecule, but Fig. 5.8 is a shorthand way of describing the structural characteristics. Glycogens have a bulky structure with exterior, or branch, chains of 7 to 13 glucose residues that exhibit a more rapid turnover than those of the shorter and more interior chain, i.e., the chain whose glucose residues furnish the carbon 6 position for exterior chain attachment.

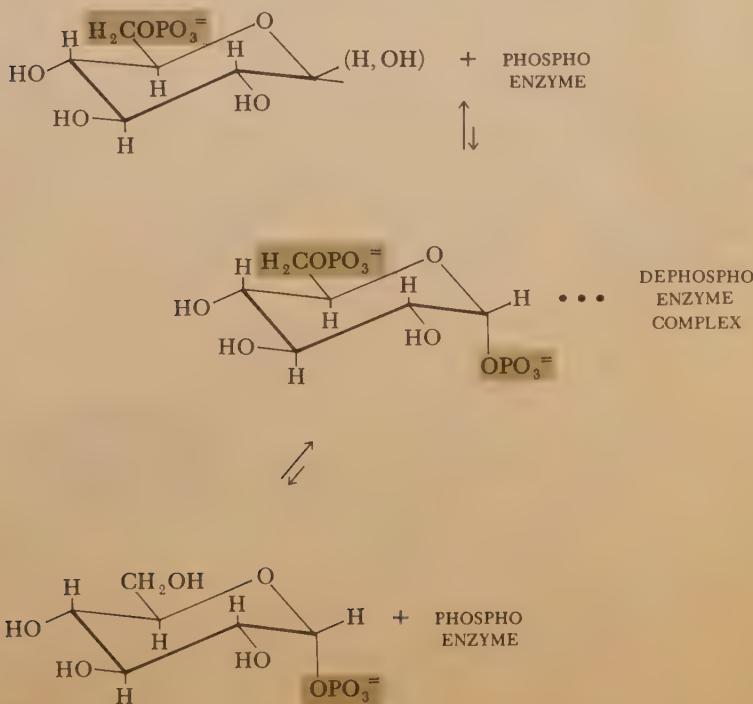
More rapid turnover means that the average life of a particular residue in an exterior chain is less than that of a particular residue in an interior chain. The use of isotopes has provided data indicating that glucose residues are removed from exterior chains without involving the interior chains. Since the chains of either type can be of varying length, and since any chain containing more than three to four glucose residues can serve as an interior chain, it seems hardly profitable to speak of molecular weight. It would be more appropriate to think of glycogen as a dynamic cross-linked polymer that occurs in aggregates up to  $10^6$  or even  $10^8$  molecular-weight units.

Investigations during the period 1935 to 1945 called attention to the reactions ancillary to glycolysis and necessary to glycogen metabolism. It was seen that in muscle, aerobically, there appears a glucose phosphate labile to acid hydrolysis. The Coris (husband-and-wife Nobel Prize winners) produced evidence that this is  $\alpha$ -glucose-1-phosphate. It was also found that this derivative is in a steady-state relation to glucose 6-phosphate and that the conversion is catalyzed by PHOSPHOGLUCOMUTASE (2.7.5.1). The mechanism of the reaction is shown in Fig. 5.9. The concentration of glucose 1,6-diphosphate is lower than that of glucose 1-phosphate, which in turn is appreciably lower than that of glucose 6-phosphate.

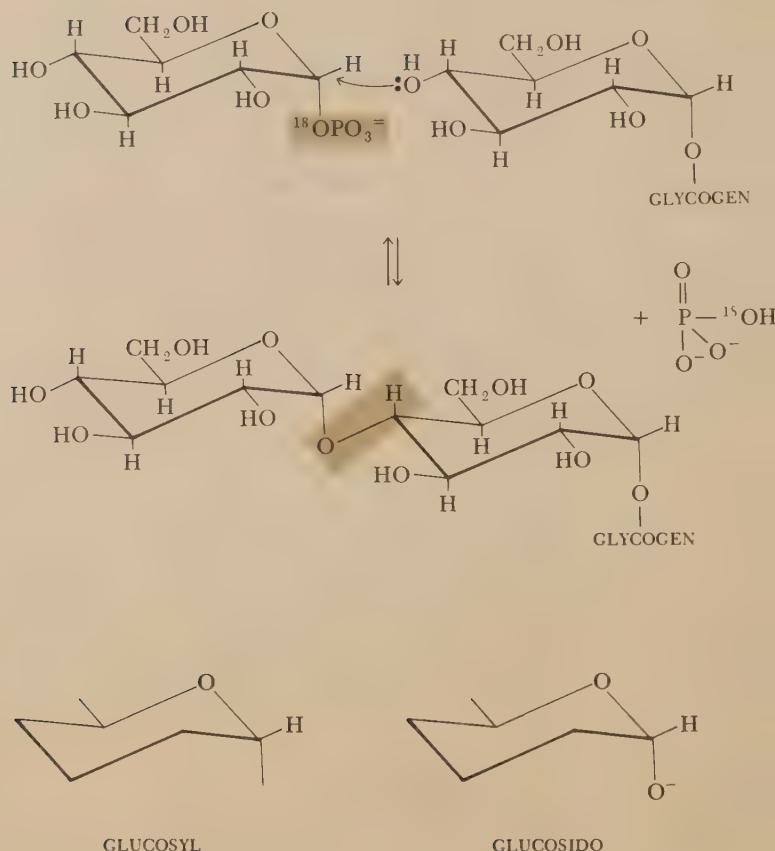
Since both glucose 1-phosphate and glycogen appeared under aerobic conditions, it seemed reasonable to seek the relation between them. The Coris were successful in isolating GLYCOGEN PHOSPHORYLASE (2.4.1.1), which catalyzes the transglucosylation shown in Fig. 5.10.

This enzyme catalyzes the making and breaking of  $\alpha$ -1,4, but not  $\alpha$ -1,6, glucosidic bonds (Fig. 5.10). Isotope data indicated *glucosyl* rather than *glucoside* transfer (Fig. 5.10) to the polymeric acceptor. It was found that the acceptor had to contain several glucose residues, but the minimum length varied with the source of the phosphorylase. Studies of the above equilibrium revealed that when the ratio  $P_i/g\text{-l-p} = 3.2$ , or greater, there was a conversion of polysaccharide to glucose 1-phosphate. Only when the ratio was less than 3.2 were the polysaccharide chains lengthened. Investigation of this ratio in tissues showed the value of the above ratio to be almost always greater than 3.2.

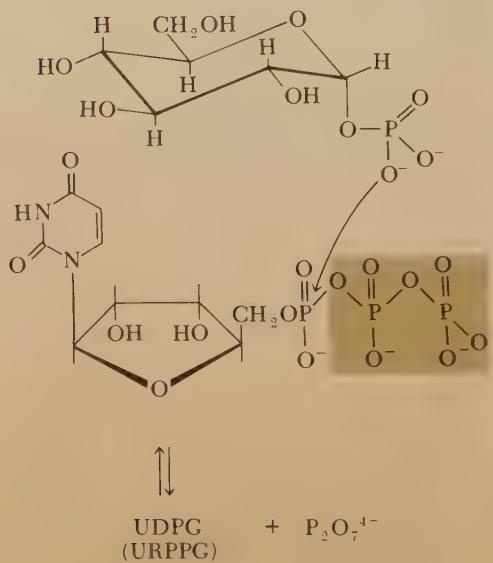
**FIGURE 5.9**  
the reaction  
catalyzed by  
phosphoglucomutase



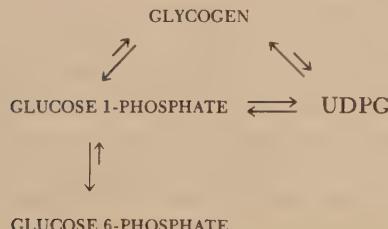
**FIGURE 5.10**  
the reaction catalyzed by glycogen phosphorylase



**FIGURE 5.11**  
the reaction catalyzed by UDPG pyrophosphorylase

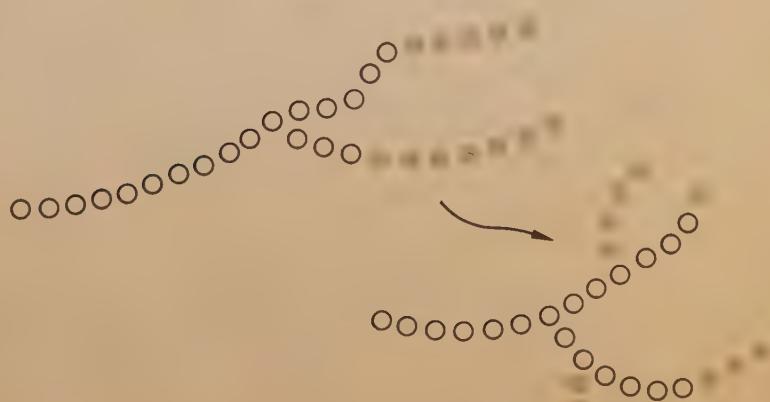


At present it is believed that polysaccharide may be commonly degraded by phosphorylase but that synthesis in vivo involves another intermediate. Glucose 1-phosphate is in a steady-state relation with uridine diphospho glucose (UDPG, Fig. 5.11). One of the reactants is uridine triphosphate (UTP), an analog of ATP that contains uracil, a pyrimidine (see Sec. 17). This reaction is catalyzed by UDPG PYROPHOSPHORYLASE (2.7.7.9). Both glucose 1-phosphate and UDPG are present in tissues in quite small concentrations because the equilibria favor glucose 6-phosphate and because UDPG is rapidly incorporated into glycogen in the presence of GLYCOGEN SYNTHETASE (2.4.1.11). The mechanism is similar to that pictured in Fig. 5.10. In this case the products are glycogen and uridine diphosphate (UDP). The latter compound is continually reconverted to UTP by reaction with ATP, and thus the UTP/UDP ratio is determined by the ATP/ADP ratio in the cell. Hence,



In the foregoing discussion of glycogen formation only the formation and hydrolysis of  $\alpha$ -1,4 glucosidic bonds have been considered. Yet  $\alpha$ -1,6 bonds do exist in glycogens, as indicated in Fig. 5.8. So-called BRANCHING ENZYMES (2.4.1.18) have been isolated from mammalian liver and other tissues and have been shown to catalyze the formation of  $\alpha$ -1,6 links. In one series of experiments it was found that labeled portions of the glycogen exterior branches were shifted intramolecularly as in Fig. 5.12. In all likelihood such intra-

**FIGURE 5.12**  
Action of branching enzyme on glycogen



molecular rearrangements occur simultaneously with the extension of the branches by transglycosylation. The hydrolysis of the  $\alpha$ -1,6 bond is catalyzed by AMYLO-1,6-GLUCOSIDASE (3.2.1.33). This enzyme is required for the complete utilization of glycogen, since phosphorylase is specific for  $\alpha$ -1,4 bonds.

The "molecular weight," or particle weight, of glycogen reflects the method of isolation. By extracting liver with buffer, it is possible to obtain a particulate glycogen which appears as clusters or rosettes 60 to 200  $\text{m}\mu$  in diameter. The more usual alkali-extracted glycogen samples have molecular weights of 1 to  $3 \times 10^6$ , about two orders of magnitude lower. The larger particles can be synthesized from glucose 1-phosphate in the presence of purified enzymes, however, and it has been concluded that only  $\alpha$ -1,4 and  $\alpha$ -1,6 glucosidic links exist between glucose residues.

The intermediates and enzymes discussed in this section have been demonstrated in many kinds of cells. Although the equilibria are dictated by the chemical nature of the intermediates, the rates of attaining equilibrium are modified by hormonal control and by competing reactions.

In liver there is an enzyme of highly restricted distribution that effects the hydrolysis of glucose 6-phosphate. The glucose resulting from the action of this enzyme, GLUCOSE 6-PHOSPHATASE (3.1.3.9), is released into the circulating blood, from which it may pass into the extrahepatic tissues. Since the blood level of glucose is maintained at a reasonably constant value in mammals, it is obvious that one or more control mechanisms must exist.

### **hormonal control**

From a purely chemical viewpoint it might seem that a feedback type of control would be adequate. Given that the blood glucose level reflects a steady state, that glucose is taken up by cells and oxidized, and that glucose 6-phosphate is the immediate hepatic source of circulating blood glucose, it follows that an increasing demand on the blood glucose, and its disappearance, will cause an increase in glucose 6-phosphate hydrolysis, thereby causing eventual depletion of glycogen. But this kind of simplistic reasoning is entirely inadequate when the object of interest is the whole animal.

We have learned that the pancreatic hormone insulin is involved in these cellular and organ interrelationships. Animals that suffer the loss of this polypeptide hormone develop the condition known as *diabetes mellitus*. The kidney loses the competence to reabsorb all of the glucose from the glomerular urine (Sec. 26); the extrahepatic cells fail to oxidize glucose at a sufficiently high rate; and the rate of glycogen formation in both liver and muscle decreases. Thus the action of insulin differs from cell to cell, and it is very unlikely that its action is due to modification of a single reaction.

Nor is insulin the only hormone whose presence affects glycogen levels. There is another pancreatic hormone, glucagon, also a polypeptide, that promotes the conversion of glycogen to glucose. The contamination of insulin samples with glucagon caused great unpredictability in biological assays until the presence of the new hormone was recognized. In addition, the medulla of the adrenal gland secretes epinephrine (adrenalin) and norepinephrine

(Sec. 15), both having hormonal actions, which promote the conversion of glycogen to glucose.

In brief, an examination of carbohydrate metabolism in multicellular creatures soon separates itself into a study of those features common to all cells and those that are cell- or species-specific. As stated earlier, the events involved in the production of pyruvate from glucose are fundamentally identical in all cells. The anaerobic equilibria involving pyruvate differ from one species to another. In yeast, ethanol is formed; in mammalian muscle, lactate is formed. Still other reactions occur in other cells. Again generally, glycogen forms in cells when the supply of glucose and of oxygen is greater than that needed to meet energy demands. But this formation is very species-specific. Plants form a polysaccharide, slightly different than glycogen, and actively growing bacteria use glucose so rapidly that glycogen formation is not possible. In animals, the complex interrelationships among cells evidently require several modifying control mechanisms.

Although the existence of hormones was recognized more than fifty years ago, no clear-cut chemical mechanism for hormone action is evident. A voluminous descriptive literature exists, and an empirical knowledge of hormone action is useful in medicine. Very recent developments (Sec. 31) encourage us in the belief that we shall soon begin to develop an understanding of hormone action in some tissues.

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## 6

# glucose 6-phosphate oxidation and ancillary reactions

Glucose 6-phosphate has been described as the first glycolysis intermediate, as the source of blood glucose in the mammalian liver, and as a compound in a steady-state relation with glycogen. In most cells, when glucose and oxygen are nonlimiting, there is a substantial direct oxidation of glucose 6-phosphate. Among the soluble enzymes of these cells is a GLUCOSE 6-PHOSPHATE DEHYDROGENASE (1.1.1.49) that catalyzes the transfer of hydrogen ions and electrons to the coenzyme nicotinamide adenine dinucleotide phosphate, NADP, also called triphosphopyridine nucleotide, TPN. The structure of NADP differs from that of NAD only in having one more phosphate group at the C-2 position of the ribose. A few enzymes are active in the presence of either NAD or NADP as acceptors but, in general, not to the same degree. Moreover, since the NADP in this instance is extramitochondrial, the reduced coenzyme, NADPH, does not mediate the transfer of electrons to the electron transfer system. It is a reducing agent in many biosyntheses. Identical spectral changes indicate that the molecular site of reduction and oxidation is the same as in NAD (Fig. 6.1). The measurement of absorption changes at 340 m $\mu$  is quite commonly used to estimate the quantity of NADH or NADPH formed in a reaction.

**FIGURE 6.1**  
absorption  
spectra of  
the oxidized  
and reduced  
forms of  
NADP and  
NAD

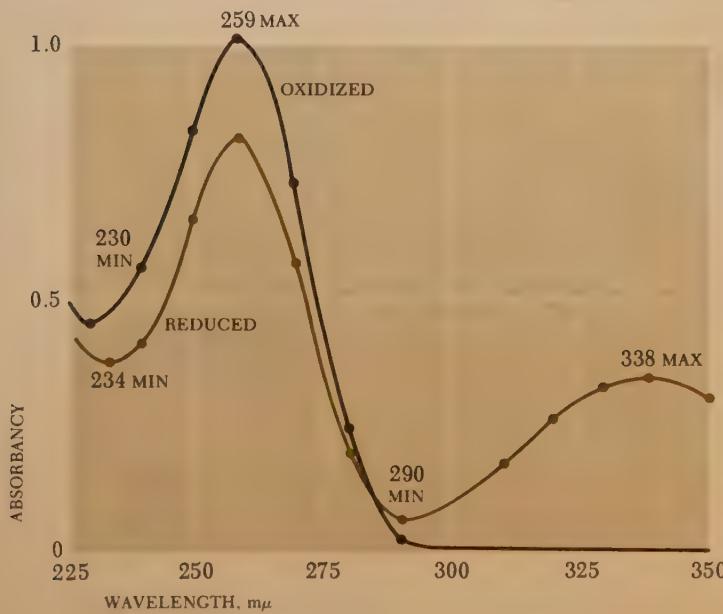
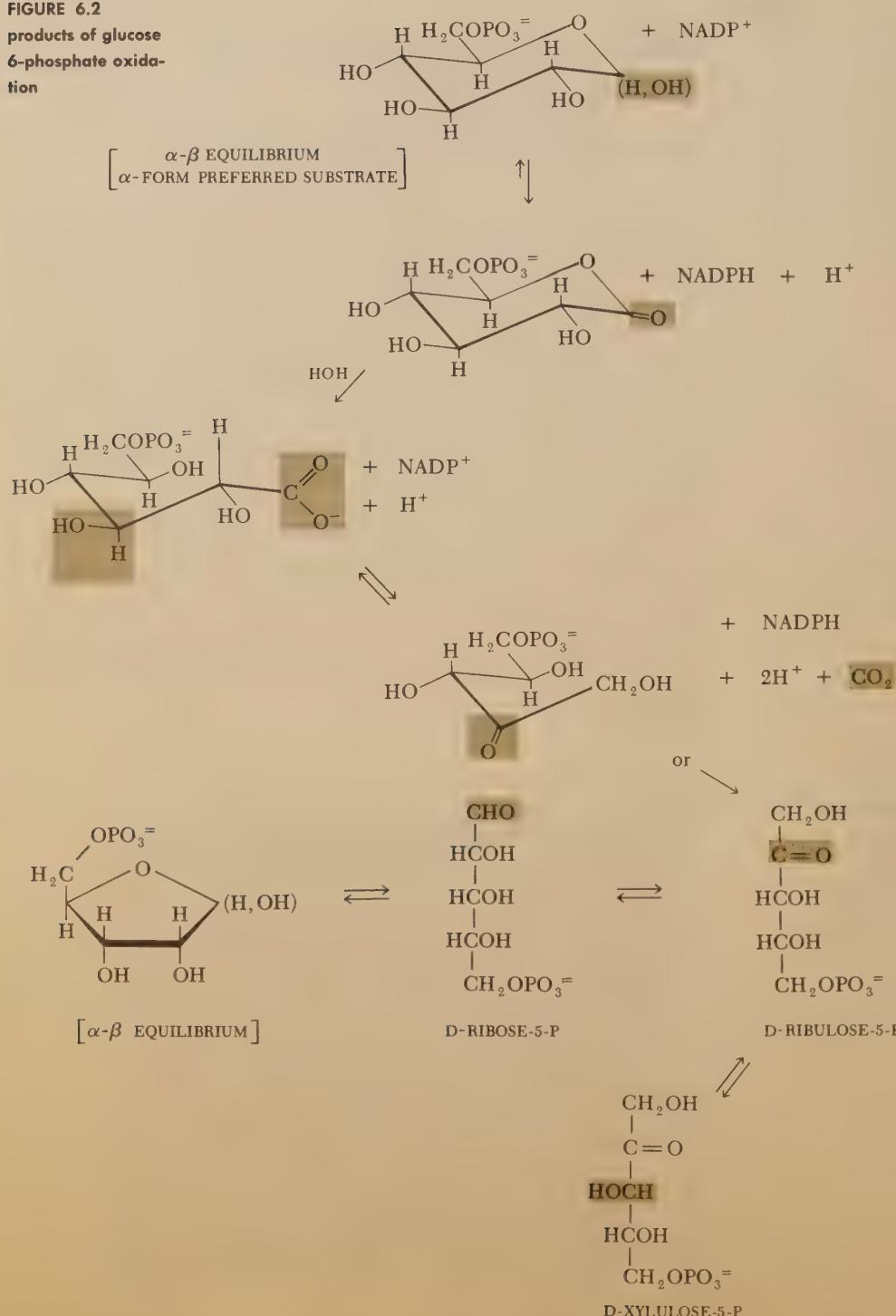


FIGURE 6.2

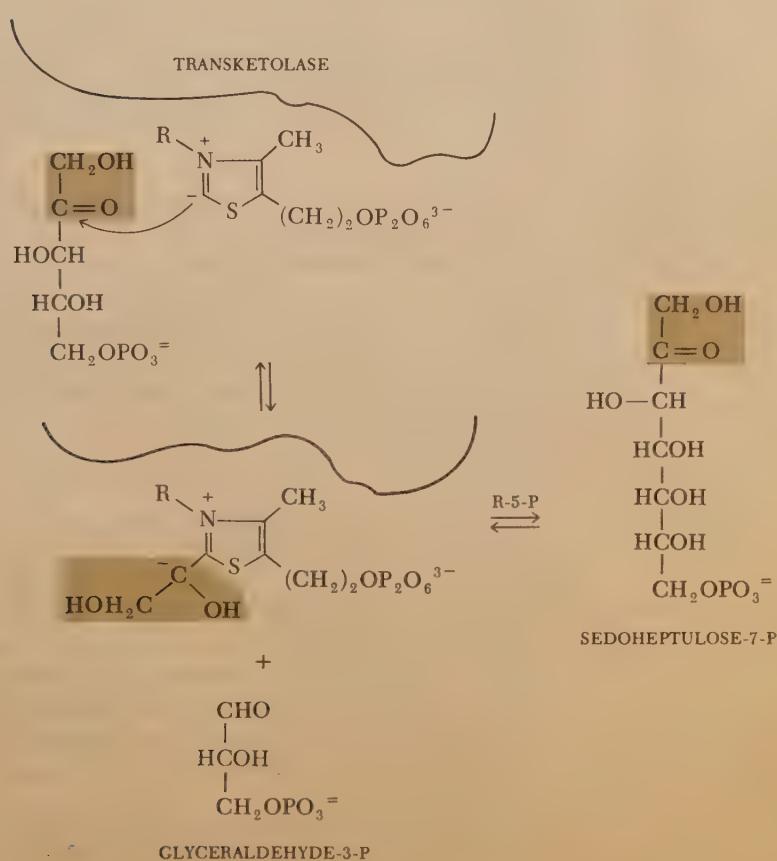
products of glucose  
6-phosphate oxida-  
tion



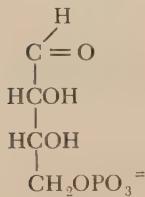
The product of the above oxidation, 6-phosphogluconate, is further oxidized by another soluble extramitochondrial dehydrogenase for which NADP is a coenzyme. The products of this second oxidation are D-ribulose-5-P and CO<sub>2</sub>, as shown in Fig. 6.2. Two enzymes catalyze the equilibria among the three D-pentose 5-phosphates shown in Fig. 6.2: ribulose-5-P, xylulose-5-P, and ribose-5-P. They are PHOSPHORIBOISOMERASE (5.3.1.6) and XYLULOSE-5-P-EPIMERASE (5.1.3.1).

Xylulose-5-P is the substrate for TRANSKETOLASE (2.2.1.1), an enzyme active only in the presence of thiamine pyrophosphate (Sec. 29). This coenzyme has already been described in Sec. 4 as a group transfer agent in the pyruvate and  $\alpha$ -ketoglutarate dehydrogenase complexes. In Fig. 6.3 is illustrated a suggested mechanism of transketolase action and involvement of thiamine pyrophosphate. In the overall reaction the ketol group of xylulose-5-P is transferred to ribose-5-P. The products are glyceraldehyde-3-P and sedoheptulose-7-P. It is not known whether this heptulose is involved in processes other than those under immediate consideration. TRANSALDOLASE (2.2.1.2) catalyzes the reaction between sedoheptulose 7-phosphate and glyceraldehyde 3-phosphate to yield fructose-6-P and erythrose-4-P. The dihydroxy

**FIGURE 6.3**  
formation of sedo-  
heptulose phosphate



acetone carbanion of the heptulose is transferred to the glyceraldehyde-3-P to produce fructose-6-P. The remainder of the heptulose has the configuration of erythrose-4-P.



The following experiments suggest that an ES complex of transaldolase<sup>\*</sup> and the dihydroxy acetone carbanion actually exists (REF. 1).

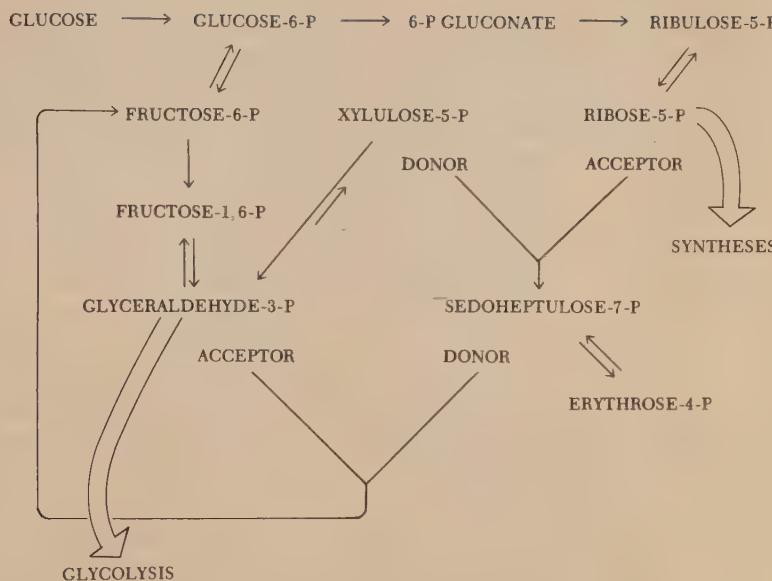
- Incubate the enzyme and fructose 6-phosphate-1-<sup>14</sup>C. Repeated precipitations show that <sup>14</sup>C is associated with the protein long after the labeled fructose-6-P has disappeared from the supernatant.
- Incubate the enzyme and fructose-6-<sup>32</sup>P. If the protein is precipitated repeatedly, no <sup>32</sup>P persists in the precipitate. Hence the preceding result is not due simply to adsorption.
- To the protein-substrate complex of the first experiment add erythrose 4-phosphate. All <sup>14</sup>C is found to leave the protein.
- Heat the protein-substrate complex of the first experiment, precipitate the protein with alcohol, and chromatograph the supernatant. It is found that a radioactive spot is formed on the chromatogram in the position of dihydroxy acetone.

The relations of the hexoses, pentoses, heptoses, trioses, and tetroses just discussed have been set forth in Fig. 6.4. The reactions involving phosphorylation by ATP, as well as the oxidation steps, are practically irreversible. Note, however, that glyceraldehyde-3-P carbon can be incorporated into fructose-6-P by a reaction that essentially reverses this portion of glycolysis. The pentose-heptose-triose-tetrose phosphates participate in reversible reactions permitting ribose-5-P formation either from oxidation or from recombinations of glyceraldehyde-3-P and erythrose-4-P. No one has yet determined how closely cell syntheses are coupled to the oxidation of glucose-6-P. If the coupling were very close, then at times there might be a deficiency of ribose, an essential component of many nucleotides and of ribonucleic acid, if the ribose were used faster than NADPH could be reoxidized. That such a deficiency does not occur is ensured by this ancillary group of reactions, integrated with glycolysis and nonoxidative in character.

The coupling of the NADP/NADPH system to synthesis has been demonstrated in several instances. However, the oxidation of NADPH is slow relative to NADH and the NADP/NADPH ratio in most tissues is less than 0.1. In anaerobic glycolysis it has been accepted that the NAD/NADH system serves to couple the oxidation of 3-phosphoglyceraldehyde to the

<sup>\*</sup> Apparently the *aldolase* ES complex is much less stable than the transaldolase complex, but reduction with borohydride allows the isolation of a derivative of the complex.

**FIGURE 6.4**  
relation of  
glycolysis  
to the "pentose  
pathway"



reduction of pyruvate. Aerobically the rate of oxidation of glucose 6-phosphate is a function of the rate of reaction of the NADP/NADPH system with which it is coupled. There is in many tissues a TRANSHYDROGENASE (1.6.1.1) that facilitates equilibration between the two pyridine nucleotide systems.

### enzym- morphology

By now the reader will have begun to appreciate the number of enzymes that must exist in the cell if all reactions are enzyme-catalyzed. Even the single process of glycolysis involves such a number that one is justified in inquiring into the *amounts* of various enzymes in tissues. For muscle tissue there is a paper of great interest (REF. 2) that describes the manner in which many enzymes of glycolysis can be isolated in a reasonably quantitative fashion. A major component of the juice that can be mechanically pressed from muscle has been termed MYOGEN. This simple extract can be used as a source of the enzymes listed in Table 6.1 which have been obtained in a state of high

**TABLE 6.1**  
enzymes  
from muscle juice

enzyme	percent of protein in expressed juice
glyceraldehyde-3-P dehydrogenase	16-23
lactate dehydrogenase	5
aldolase	7-10
enolase	12
phosphoglucomutase	2
phosphoglycerate mutase	1
triose phosphate isomerase	3
pyruvate kinase	3-4
creatine kinase	6
glycogen phosphorylase	2-4

purity. These enzymes and five others which have not been discussed account for roughly 70% of the protein in muscle juice. They compose about 25% of the total protein of white muscle and about 16% of the total protein of red muscle. Thus the one enzyme, glyceraldehyde-3-P dehydrogenase, is an impressive 5% of the total protein in white muscle. In yeast the percentage of this enzyme is nearly the same.

Not many tissues have been characterized in this fashion. As evidence accumulates, it may be supposed that the enzyme complement will be found to correspond to the specialized functions of each cell. A very useful survey of enzyme distribution has been made and reported (REF. 3).

The material of this subsection emphasizes the relation between structure and function. In a specialized tissue such as mammalian muscle one may expect a substantial fraction of the protein to be related to the specialized function, and the data bear this out. Both biochemists and physiologists were preoccupied with muscle function and energy transduction in the first several decades of the twentieth century. Muscle enzymes and the carbohydrate metabolism of muscle received constant attention, and thus, although the efforts were modest by present standards, this area of inquiry has been rather fully worked out. Fortunately, the glycolytic enzymes are not attached to structural elements, and relatively unsophisticated preparative procedures could be used.

When attention was transferred to aerobic phenomena and to other types of tissue such as liver, the preparative procedures became more complicated. By 1945 it had become evident that the separation of subcellular particles on a preparative scale by centrifugation was not only possible but virtually mandatory. The development of this technology was most important to the biochemical achievements of the ensuing years. The ability to obtain gram quantities of separated mitochondria, membranes, nuclei, or microsomes was a prerequisite to serious study of their functions. As may be seen in Tables 6.2 and 6.3, there is a very evident association of enzyme groups with subcellular structure.

**TABLE 6.2**  
subcellular  
distribution  
of enzyme  
systems

system	occurrence
electron transport	mitochondrion
oxidative phosphorylation	mitochondrion
citrate cycle	largely in mitochondria but some of the enzymes in the supernatant*
glycolysis	largely in supernatant
glycogen metabolism	supernatant
pentose phosphate interconversions	supernatant

\*Supernatant is that part of the cell material not sedimented by a centrifugal force known to sediment any subcellular structure.

The processes considered up to this point are those that occur in almost all cells; there are other processes, many of them, that are more highly restricted. They are interesting, intriguing, and important, but not the first order of business here. Our purpose is to review the common denominators of metabolism, with a few carefully chosen exceptions, and to show how they are correlated in a dynamic system.

**TABLE 6.3**  
**subcellular  
distribution  
of specific  
enzymes**

<i>specific enzymes</i>	<i>occurrence</i>
lactate dehydrogenase (in rat liver)*	one-half of the activity in supernatant; one-half of the activity in the micro-somal† fraction
malate dehydrogenase (many tissues)	some activity in supernatant; some activity in mitochondria
glucose 6-phosphate dehydrogenase (rat and rabbit liver and kidney)	supernatant only
phosphogluconate dehydrogenase	supernatant only
succinate dehydrogenase	with two exceptions, all activity associated with particles and in most cases, mitochondria
glutamate dehydrogenase (in liver)	mitochondria only
transaldolase, transketolase (in liver and kidney)	supernatant
hexokinase (several tissues)	supernatant, mitochondria, micro-somes
UDPG pyrophosphorylase (rat liver)	supernatant
glucose 6-phosphatase (liver and kidney)	microsomal membrane only
aldolase (muscle, liver, brain)	largely in supernatant; some activity in nuclei

\*Enzyme sources are in parentheses.

†Endoplasmic reticulum and monosomes or polysomes, see Sec. 20.

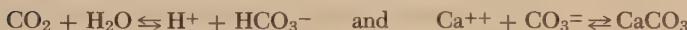
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# 7

## CO<sub>2</sub> fixation and the transport of sugars

A large number of investigations that could be grouped as *comparative biochemistry*, a legitimate field within the wider boundaries of the subject, has led to the conviction that a majority of living cells utilize glucose. The bulk of glucose synthesized arises from the process of photosynthesis in plants. The carbon source, carbon dioxide, forms a small percentage of the gaseous mixture we call air. This small percentage varies somewhat with the local balance of the processes increasing or decreasing CO<sub>2</sub> in the atmosphere. The consumption of CO<sub>2</sub> by photosynthesis is not singular; the inorganic reaction CO<sub>2</sub> + MgSiO<sub>3</sub> → MgCO<sub>3</sub> + SiO<sub>2</sub> also consumes large amounts. The respiration of living cells and the direct combustion of organic matter add CO<sub>2</sub> directly to the atmosphere. In addition, there are two equilibrium processes acting as buffers, particularly in the oceans:



An estimate of the distribution of CO<sub>2</sub> in grams per square centimeter of earth surface expressed in C as CO<sub>2</sub> is:

Atmosphere	0.46
Oceans	28
Living matter	0.012
Organic debris	2.8

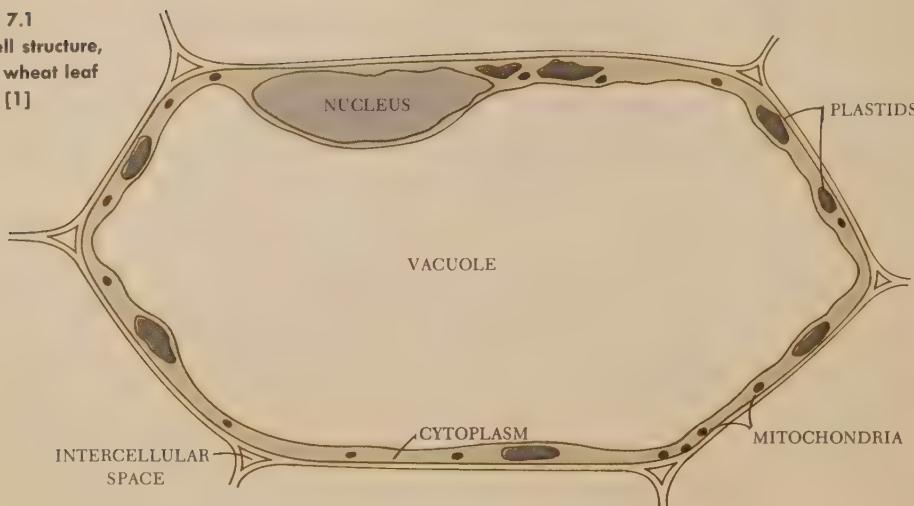
These considerations and estimates can hardly fail to emphasize that living cells are very rare and exist on a raw material which is rather scant but is well buffered by the oceans.

### photosynthesis

Photosynthesis may be a normal process in an algal cell, a bacterium, or a higher plant. This is to say that photosynthetic activity is an attribute of three large classes of organisms but is not universally found in the members of each class. For a number of practical reasons plant photosynthesis has been carefully studied, but for many purposes algae or bacteria are a far better experimental material.

In Fig. 7.1 is a stylized diagram of a plant cell. Both plastids and mitochondria are present. In the developing cell the proplastids and promitochondria are indistinguishable. They differentiate, and in the process cristae form in the mitochondria, as has already been described. The plastids have a lamellar membrane very similar to the mitochondria. With differentiation chlorophyll appears, packaged in *grana* within the chloroplast. In the chloro-

**FIGURE 7.1**  
plant cell structure,  
such as wheat leaf  
or beet [1]



plast, as in mitochondria, many reactions occur. The most characteristic are those of photosynthesis. The chloroplast has not been separated into active fragments quite as neatly as mitochondria have been. However, there is every reason to believe there is the same close relation of structure and function.

The study of the energy metabolism of many kinds of cells has revealed the existence of a reaction that can be stated in a very general form:



A range of compounds can serve as reducing agents. For example, in the green sulfur bacteria, H<sub>2</sub>S is the donor. In photosynthesis the donor is H<sub>2</sub>O and photons provide the energy to excite the electrons so that water is separated into its elements.

It has been known for many years that chloroplasts can mediate this photolysis of water, and, in the absence of CO<sub>2</sub>, a "light reaction" can be demonstrated. In order to observe the reaction something reducible, such as a dye, must be added to the chloroplast suspension. The light used must contain a 7000-A wavelength component. The use of H<sub>2</sub><sup>18</sup>O in such experiments reveals formation of <sup>18</sup>O<sub>2</sub>. The precise mechanism by which elemental O<sub>2</sub> is formed has not yet been formulated. Four electrons must be transferred from two molecules of water for each O<sub>2</sub>, and it is probable that each transfer differs from the others. The light reaction may be written



At present some investigators are working on the hypothesis that two photosynthetic pigments are involved: chlorophyll *a* and a poorly characterized "P<sub>700</sub>." It is thought that one quantum moves an electron from P<sub>700</sub> ( $E'_0 = +0.43$  volt) to pyridine nucleotides and another from +0.81 volt to about 0 volt. Thus there may be two light reactions.

If the material-balance equation for photosynthesis is written



it will not correspond to the actual mechanism but will serve for thermodynamic calculations. The free-energy change  $\Delta G^\circ$  (standard conditions) is 112 kcal/mole of  $\text{CO}_2$ . This corresponds to 4.9 ev/molecule. For 7000-A radiation there are 1.7 ev/photon, and hence it follows that three photons are required to reduce one molecule of  $\text{CO}_2$ . The mechanism of this transduction of light energy into chemical energy cannot be further described at this time and remains a challenge to inquiry.

Careful experimentation demonstrates that both "light" and "dark" reactions occur in photosynthesizing systems. Such a system can be strongly illuminated in the absence of  $\text{CO}_2$ , the light discontinued, and  $\text{CO}_2$  admitted rapidly. The  $\text{CO}_2$  will be reduced even though the "reducing power" was produced at some time before the  $\text{CO}_2$  was admitted. The dark reaction can be symbolized



### **photo-synthetic phosphorylation**

Vigorous and extended studies of the isolated chloroplast have been pursued, and good evidence has been presented for the occurrence of photosynthetic phosphorylation in this organelle.



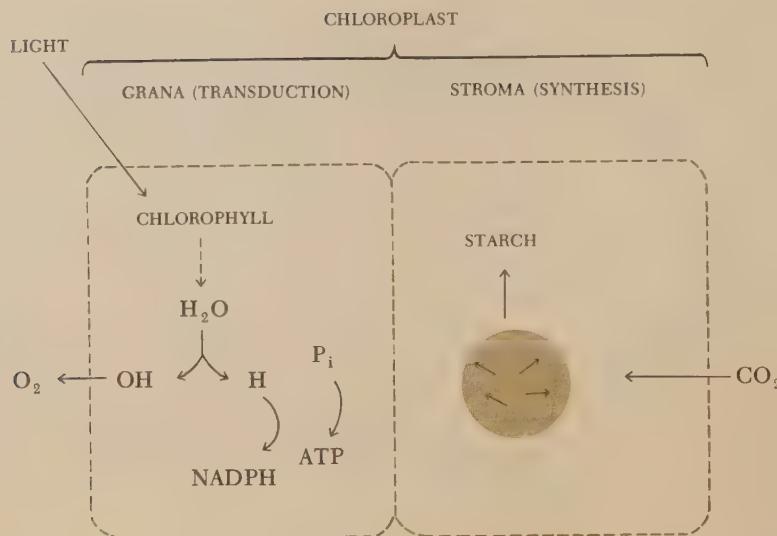
As in oxidative phosphorylation, the ATP/ADP ratio is increased with the incorporation of  $\text{P}_i$ . Oxidations occur, but since the substrate is water, the oxidized end product is  $\text{O}_2$ . Note that the NADP/NADPH ratio is lowered by this process. It is believed that in this case, as in the previous one, NADPH is the prime reductant in synthetic reactions and is not reoxidized by the electron transport system.

Again, as in oxidative phosphorylation, the details of mechanism are in question.\* For example, chloroplasts capable of ATP synthesis show very poor ability to catalyze exchange between  $\text{P}_i$  and ATP, an exchange easily demonstrated with mitochondria. Also, it has been noted that chloroplasts as well as mitochondria catalyze ADP-ATP exchange, yet this exchange is not related to photophosphorylation. This is clearly a field that will continue to demand exploration.

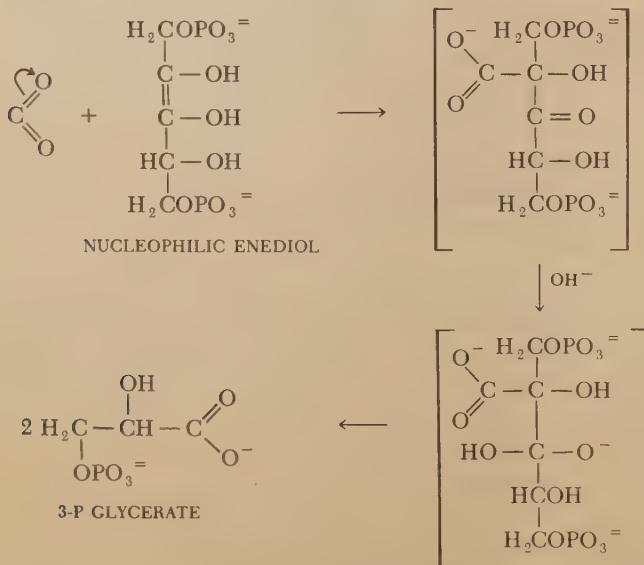
It has been proposed that the light and dark reactions of photosynthesis may be separate in the chloroplast itself (Fig. 7.2). The enzymes involved in the "fixation" of carbon dioxide, its incorporation into carbohydrate, are soluble, but the phosphorylation mechanism is associated with the grana. Although the phosphorylation is not dependent on oxygen as an electron acceptor, there is an electron transport system in the grana.

\*A recently discovered electron transfer agent, ferredoxin (Sec. 10), may be involved. It is reduced by the pigments of the chloroplast in the light and is reoxidized by NADP in the dark.

**FIGURE 7.2**  
subchloroplast structure in photosynthesis



**reduction of  $CO_2$**  The initial reaction involving  $CO_2$  as a reactant and ribulose 1,5-diphosphate as an acceptor is catalyzed by the enzyme CARBOXY DISMUTASE (4.1.1.39).



In the presence of photon-generated “reducing power,” presumably NADPH, the 3-phosphoglycerate is reduced to 3-phosphoglyceraldehyde. The presence of triose isomerase and aldolase makes possible the formation of fructose diphosphate. Since erythrose-4-P is present, sedoheptulose 1,7-diphosphate is formed. In this case a dihydroxy acetone phosphate carbanion is transferred via transaldolase to the tetrose. Phosphatases convert certain proportions of both fructose and sedoheptulose diphosphates to the monophosphate

derivatives. The presence of transketolase ensures an equilibrium with xylulose 5-phosphate as well as erythrose 4-phosphate. Ribulose 5-phosphate is in equilibrium with xylulose-5-P. PHOSPHORIBULOKINASE (2.7.1.19) facilitates the formation of the CO<sub>2</sub> acceptor.

#### RIBULOSE DI P/RIBULOSE MONO P : ATP/ADP

The sequence of reactions is thus seen to be similar to, but not identical with, those just described in Sec. 6. It may be useful to think of this as a reaction set driven reductively so that CO<sub>2</sub> becomes incorporated into triose and then hexose phosphates.

As in animals, so also in plants may be found fructose-6-P, glucose-6-P, glucose-1-P, and UDP glucose. One of the unique botanical products, sucrose,

**FIGURE 7.3(a)**  
STRUCTURE OF SUCROSE  
[2,3]

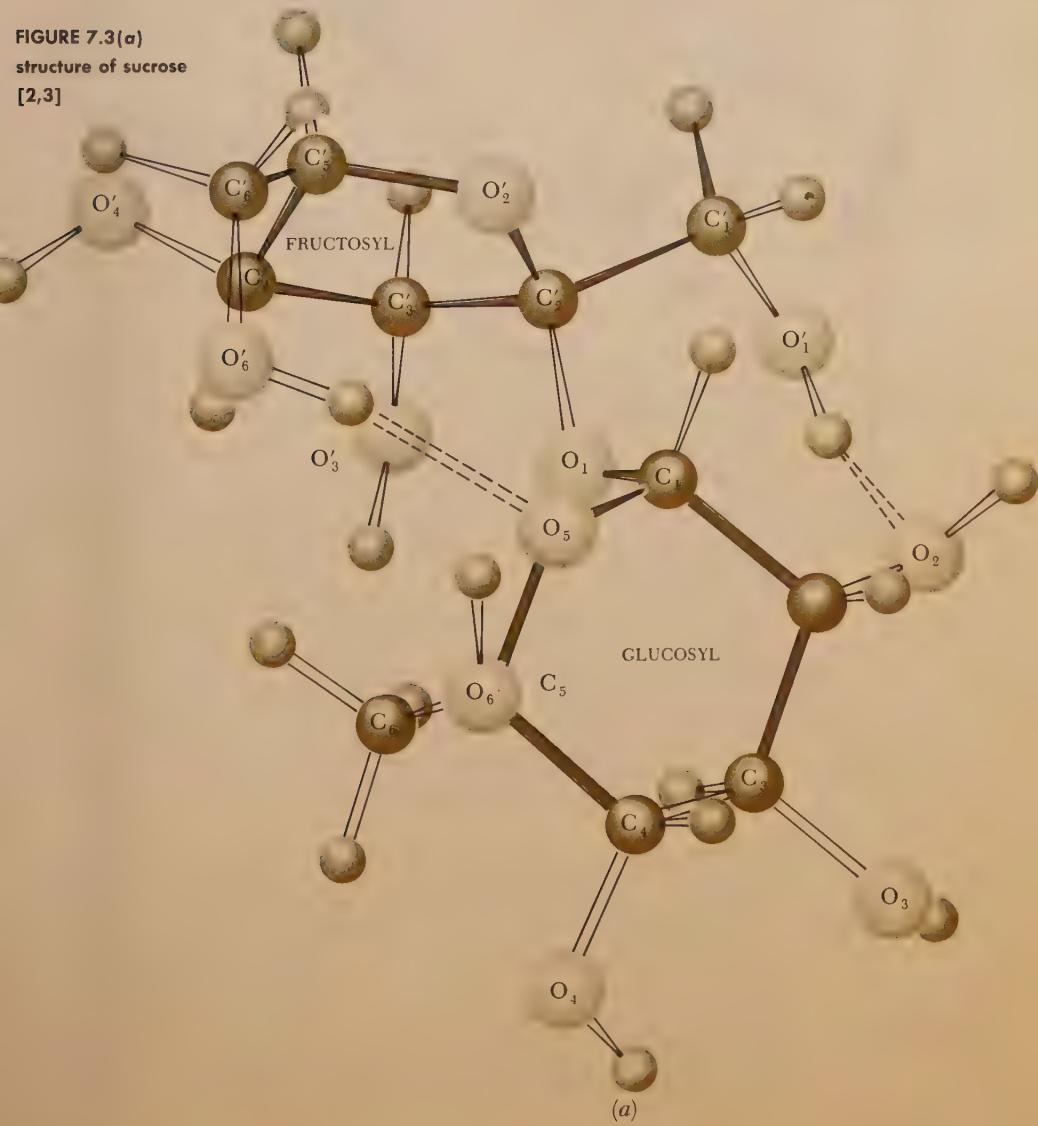
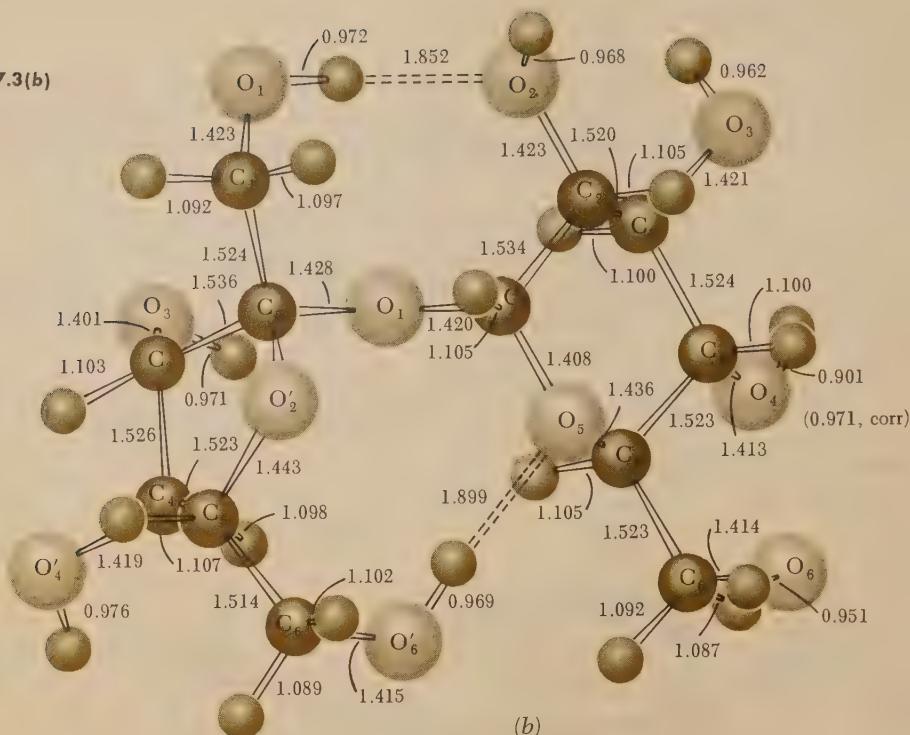


FIGURE 7.3(b)



may be the result of a reaction between fructose-6-P and UDP glucose. It is thought that sucrose phosphate is first formed and that a phosphatase is responsible for release of sucrose as such. Again, in plants as in animals, UDP glucose is a source of glucosyl groups in the formation of sparingly soluble polysaccharides. Adenosine diphosphate glucose is actually the intermediate that donates the glucosyl group but presumably is maintained at some steady-state concentration by the UDP glucose system. (See also the last portion of Sec. 30.)

During photosynthesis,  $\text{CO}_2$  furnishes the carbon for increase in sugar phosphate at a rate which is a function of  $\text{CO}_2$  concentration and light intensity. It may be presumed that steady-state controls mediate the immediate removal from solution of this net carbon increase by polysaccharide formation. Starch granules form in the chloroplast. Such granules have a layered appearance and a shape characteristic of each plant species. It has been demonstrated that granules are enlarged by new layers formed over old ones.

### starches and the amylases

*Starch* (like glycogen) is hardly a scientific term; rather, it is a generic name for a polydisperse<sup>\*</sup> polyglucose having structures varied in both chain length and branching frequency. The simplest group embraces the amyloses, which are  $\alpha$ -1,4 polyglucosides of varying degrees of polymerization. As a solid, but probably not in solution, an amylose is helical in shape. A helix

<sup>\*</sup>If all molecules of a polymer have the same molecular weight, we may speak of the polymer as monodisperse. If there is a spectrum of molecular-weight values, or a random collection, the polymer will be considered polydisperse.

is the form taken by any regular asymmetric linear array, and, as noted previously, all glucosides are asymmetric. The molecular weights of molecules of potato amylose range from  $1.5 \times 10^5$  to  $2.2 \times 10^6$  (REF. 7). Amyloses may be extracted from crude starches with hot water, but after the solution is cooled they form intractable precipitates. This precipitation has been termed *retrogradation*. It is due to the extensive hydrogen bonding that can occur. Although the stability conferred on a complex by one hydrogen bond is small, the formation of hundreds of such bonds may produce quite stable aggregates. In the present case these aggregates can be redissolved only by autoclaving or by the addition of  $\text{OH}^-$ .

Everyone is familiar with the blue color of the complex formed between amyloses and  $\text{I}_2$ . This color is believed due to the enclosure of iodine atoms in the hydrocarbon-like environment that exists inside the amylose helix. (Most of the hydroxyl groups extend outward.) Amylose structures exhibit this complexing action when the number of glucose residues exceeds 18. The blue color increases linearly until the number reaches 72. The rate of increase then diminishes, and the "blue value" of natural amyloses is reached when the chains contain about 400 residues. It is perhaps a trivial matter, but an interesting reflection upon the nature of man, that the starch-iodine color was noted within two years of the discovery of iodine by Courtois in 1812.

In addition to the linear polymers there are branched products termed amylopectins. The branching, due to  $\alpha$ -1,6 links as in glycogen, is initiated by the P and the Q enzymes. The chain sequences are longer than in glycogen and may be considered a structure intermediate between amylose and glycogen. Amylopectins are not as soluble as the amyloses but, once in solution, they are more stable and do not exhibit retrogradation. The iodine complex has a purple hue. The highly branched glycogen complex with iodine has a weak pink color. A wide variety of hues between deep blue and pink is possible depending on chain length and the degree of branching.

The starches, like the glycogens, yield glucose 1-phosphate in the presence of  $\text{P}_i$  and phosphorylase. The exact manner in which starch is used by the plant during the growing period is not a matter on which there is general agreement. It is likely that glucose 1-phosphate is formed; this in turn swells the steady-state supplies of glucose-6-P, fructose-6-P, and UDP glucose. From the UDP glucose and fructose-6-P can be formed the sucrose which is transported from the leaves to the roots and the growing tips of the plant.

Another mechanism that converts polysaccharides to smaller molecules is activated during the sprouting of seeds. This process must have been sensed by man when grains became a diet staple. When seeds, such as barley or wheat, are sprouted, the notable starch content decreases rapidly and there appears a mixture of maltose and oligosaccharides.<sup>o</sup> This mixture becomes

<sup>o</sup> An oligosaccharide contains more than two sugar residues but fewer than a polysaccharide contains. Any dividing line between oligo- and polysaccharide is arbitrary; let it be 15 residues. Maltose is the trivial name of glucosyl  $\alpha$ -1,4-glucoside and may exist as the  $\alpha$  and the  $\beta$  anomers. It is rarely found in biological materials except during the process now being considered.

more soluble, slightly sweet, and easily fermentable by microorganisms. If the sprouted seeds are heated, growth is inhibited. The result of this *malting process* is a mixture of sugars which can be fermented at will by inoculation with a selected strain of yeast. These processes are elementary to brewing, and they result in the dilute solution of alcohol called beer. The enzyme that catalyzes hydrolysis of starch to maltose is  $\beta$ -AMYLASE (3.2.1.2). ( $\beta$  because the anomer of maltose formed is  $\beta$ .) In the plant, or the seed, the action of  $\beta$ -amylase on the starch granule is slow. It is now recognized that the outer layers of the granule contain amylopectin that is hydrolyzed, one maltose unit at a time, from the nonreducing ends. This is a process similar to that described for glycogen.  $\beta$ -amylase action ceases when the bond to be hydrolyzed is at a branch point. If the starch is extracted and brought into solution, the hydrolytic action is much more rapid and the amylose fraction is completely converted to the disaccharide, maltose.

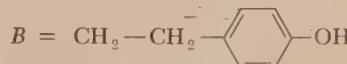
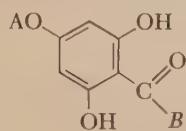
The solubilization of starches by animals involves hydrolase action by the quite distinct enzyme  $\alpha$ -AMYLASE (3.2.1.1). When this enzyme is added to a starch solution, the viscosity drops precipitately. The *liquefaction* or *dextrinization* is due to random hydrolytic scission. The  $\alpha$  anomer of maltose is formed (indicating a mechanism different from that of  $\beta$ -amylase), as well as glucose and oligosaccharides. It is possible to separate the oligosaccharides on charcoal-celite columns and to obtain maltotetraose (four glucose residues), maltopentaose, and so on. These may also serve as substrates for  $\alpha$ -amylase, although the rate of hydrolysis is much lower. The even-numbered oligosaccharides yield only maltose; the odd-numbered ones yield glucose and maltose. Amyloses are thus completely hydrolyzed, but there is no hydrolysis of the  $\alpha$ -1,6 bonds in the branched molecules.

The amylase of saliva is also used in primitive cookery but is of more immediate concern in relation to digestion. In actuality it seems of little import, since enzyme action is nearly abolished when the food is acidified in the stomach. Most of the starch digestion occurs in the upper intestine and is due to an amylase that, together with other hydrolases, is synthesized in the pancreas and carried by the pancreatic juice into the intestine. A MALTASE (3.2.1.20) also present in intestinal juice ensures that the end product of starch digestion will be glucose.

The study of the digestive process in animals has attracted some imaginative and ingenious experimentation in the past. Nearly 200 years ago Spallanzani became so curious about digestion that he fed an animal pieces of meat on a string and then pulled the partly digested masses out from time to time to examine them. This heartily direct approach was matched by the determined studies of Beaumont in the early nineteenth century. Young Doctor Beaumont discovered a French Canadian with a gastric fistula—a bullet wound that did not heal—through which he obtained samples from the stomach during digestion. The attention of chemists was eventually drawn to the subject, and a considerable amount of the basic information was at hand before 1900.

**phlorizin  
action and  
sugar absorption**

One of the curious phenomena discovered during these early investigations is that the drug phlorizin has an unexplainable effect. Phlorizin is a glucoside, one of a large number that occur in plants (REF. 12). It is found in the root bark of apple, pear, cherry, and plum trees and was first isolated in 1835. The aglycon—the nonsugar moiety—is phloretin:



Large amounts of glucose appear in the urine of animals after phlorizin has been ingested. It was also found that absorption of glucose from the intestine is inhibited. Phloretin alone was found to be inactive; the entire glucoside is required for drug action. The two biological effects seem unrelated, but, in fact, blood glucose continually escapes via the glomerular urine in the kidney and is continually reabsorbed in the tubule (see Sec. 26). Hence it can be concluded that phlorizin inhibits the absorption of glucose or the transport across cell membranes. It was further found that this drug inhibits some phosphorylation reactions, and consequently the belief grew that the movement of glucose across cell membranes—at least in liver and kidney—required phosphorylation. This hypothesis was never fully validated. Moreover, it has been demonstrated that the movement of the common monosaccharides across the membrane of the red blood cell in man and primates is inhibited by phloretin but unaffected by phlorizin. Phlorizin also has been found to inhibit biological oxidation in mitochondria and to cause swelling in this organelle.

In a slightly different context the movements of sugars across membranes were studied by the Coris about thirty years ago. They measured the rates at which various sugars were absorbed from the rat intestine and found that there was no correlation with utilization. For example, galactose was absorbed more rapidly than glucose, yet this sugar is metabolized so slowly that it passes from the blood to the urine in large measure and is a very inefficient source of energy or carbon.

The process by which glucose is moved across cell membranes is more than simple diffusion; it requires an energy exchange, and it may be assumed to be coupled with mitochondrial activity. The coupling probably involves as an intermediate some molecule other than ATP. Sugars known not to occur in biological tissues, such as 1-deoxy- or 6-deoxyglucose or 3-methyl glucose, are not phosphorylated and are not oxidized, yet are accumulated against a gradient. Stranger still, 3-methyl glucose accumulation is heightened by insulin. Clearly, justice cannot be done this topic by generalizations. We have not yet learned to ask the right questions, much less answer them. The movement of glucose across membranes must be one of the most common biological events known, but the mechanism is obscure.

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# 8

## fats, lipids, aliphatic compounds, and acyl groups

Except for plants, most organisms are heterotrophic with respect to glucose and ultimately depend on plants for a source of glucose. Most creatures need no other carbohydrate in their diet, and most of the other carbohydrates are, or can be, synthesized from intermediates deriving from glucose. There is a curious exception: men and guinea pigs require ascorbic acid in their diet. It is for them a vitamin, a substance that cannot be synthesized rapidly enough if at all. Presumably this lack of synthetic ability is due to a mutational loss.

It is obvious from casual observation not only that carbohydrate yields energy but that it can be converted readily to fat. Even when carbohydrate is not fed in excess, conversion to fat occurs. In the normal rat about 30% of the carbohydrate intake is converted to fat. Sooner or later the question arises whether such fat is metabolically mobile or whether it tends to be inert. In short, what is its biological half-life? How long does it take to replace half the fat in a tissue? Studies performed with rats exposed to  $^{3}\text{H}_2\text{O}$  for six months (REF. 1) allowed the following conclusions:

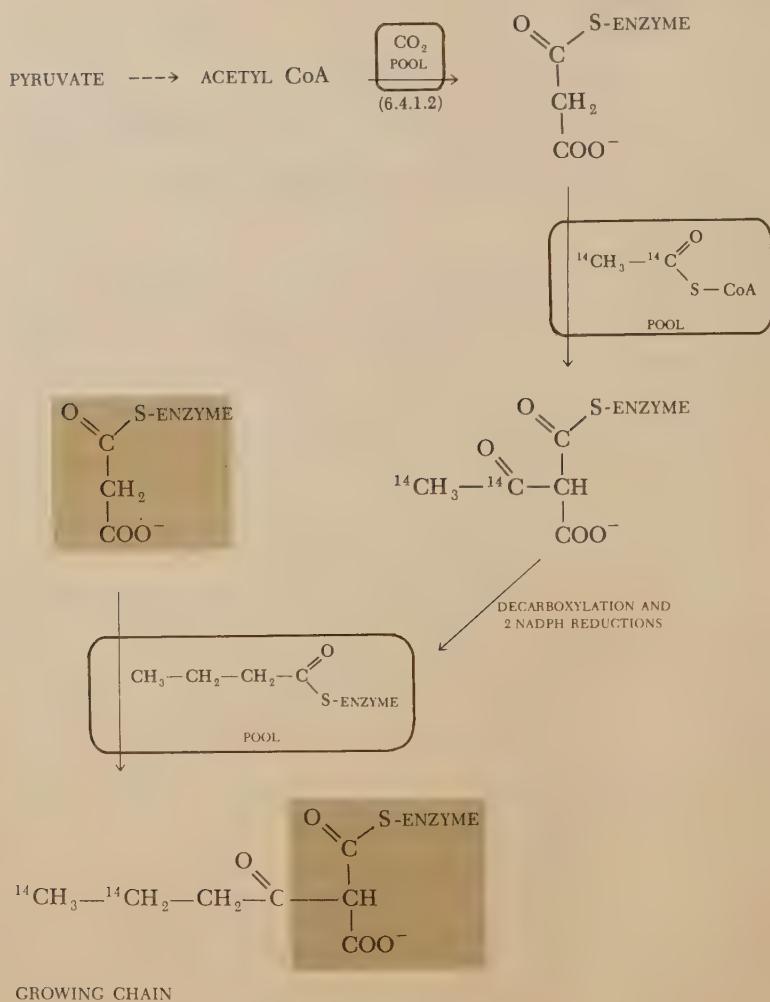
- Dynamic components with half-lives of a few days constitute a very small proportion of the whole animal.
- About half of the organic material of the rat has a half-life greater than 100 days.
- The majority of rat lipids have half-lives greater than 70 days.
- From 20 to 30% of the hydrogen of most compounds in tissues is derived from body water.

Although the glycogen content of tissues, or the starch content, may fluctuate rapidly, the same rate of flux is not characteristic of the bulk of fat in the so-called fat depots.

Reviews of twenty years ago declared that carbohydrate was *the* energy source utilized during muscle movement. This view has been modified, since it has been shown that metabolites deriving from fat can be oxidized by muscle *aerobically*. Only during anaerobic vigorous activity is the muscle unable to derive energy from molecules other than carbohydrate.

Despite our superficial impressions that fat is restricted to adipose tissue, there is ample evidence that it is a normal component of every cell. This is true not only for "fat," used in the generic sense and hence equivalent to the term "lipid," but also in the more specific sense of a triglyceride ester of aliphatic acids. Fats considered in the latter sense are comparatively dehydrated, water-insoluble substances constituting the most condensed source of biologically available hydrogen and electrons. Since they are highly re-

**FIGURE 8.1**  
**fatty acid synthesis**



duced compounds (with respect to the aliphatic chains), it should follow, and does, that they provide a high energy yield per gram. The value of  $\Delta H_{293}^\circ$  for the combustion of starch is  $-4.18 \text{ kcal/g}$ , whereas the value for triglyceride is  $-9.46$ .

The chemical analytical work on fats and on aliphatic acids during the nineteenth and early twentieth centuries demonstrated that most of these acids contain even numbers of carbon atoms per molecule. Of the aliphatic acids isolated from fats, both saturated and unsaturated, by far the most predominant were oleic (18C, unsaturated), palmitic (16C, saturated), and stearic (18C, saturated) acids.

**fatty acid  
synthesis**

The metabolic reason for the dominance of even-numbered acids was suggested by the work of the 1946 to 1950 period, when it was demonstrated that acetate is the precursor of aliphatic acids. The use of deuterium by

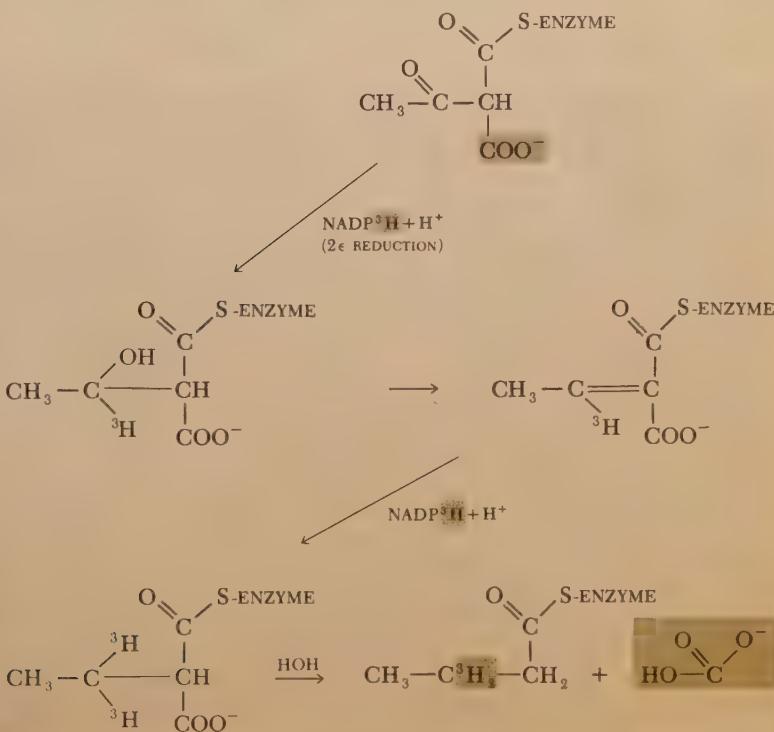
Schoenheimer (REF. 2) and many others led to the very fruitful concept of dynamic equilibrium in living creatures. This concept abolished the notion, prevalent at that time, that adipose tissue is inactive and slow to change. But this effect was almost incidental—the greater import of the concept markedly changed the thinking of most biochemists. It was realized that proximate analyses of tissues could be of questionable value. If analyses were made of samples taken from a dynamic system continually in flux, then samples had to be taken under many kinds of conditions and in a manner that would reveal fluctuations with time.

The origin of the acetate, shown by tracer experiments to be the precursor of the fats, was eagerly sought and found to be pyruvate. In Sec. 4 it was shown that acetyl CoA is one product of pyruvate dehydrogenase. The formation of fats from this precursor is an alternative to its oxidation in the citrate cycle.

There are two demonstrable systems for fatty acid syntheses. One is mediated by soluble enzymes, the other by mitochondria. It is uncertain how each of these should be rated in importance and therefore each will be described.

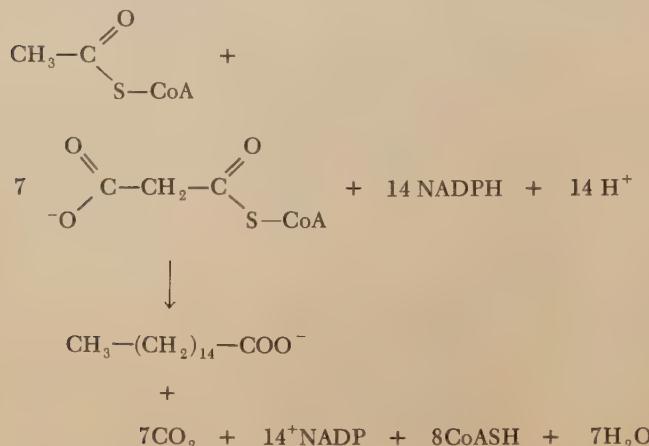
Fatty acid syntheses in cell-free systems require as minimal components: acetyl CoA, ATP, NADPH, Mg<sup>++</sup> or Mn<sup>++</sup>, HCO<sub>3</sub><sup>-</sup>, and the appropriate cell preparation. Experimenteres were baffled by the finding that the compounds synthesized were not labeled if H<sup>14</sup>CO<sub>3</sub><sup>-</sup> was added. It was dis-

**FIGURE 8.2**  
detail of reductive  
decarboxylation. This  
detail is to illustrate  
the number of steps  
known to occur and  
to illustrate how  
alternate carbons can  
be labeled by  
NADP<sup>3</sup>H. The exact  
mechanism of decar-  
boxylation may differ



covered that the first reaction is indeed a  $\text{CO}_2$  fixation, a carboxylation of acetyl CoA to malonyl CoA, but that the  $\text{CO}_2$  is not incorporated in the final product (Fig. 8.2). The precise mechanism has not yet been agreed upon, but there is evidence that biotin is involved. This vitamin, another of the B group (Sec. 29), received much attention some time ago because of a deficiency that can be caused by adding raw egg white to the diet of animals. This led to the unexpected discovery of a protein (avidin) in egg white that combines very firmly with biotin. It has been shown that biotin is present in the enzyme preparations (ACETYL COA CARBOXYLASE) (6.4.1.2) which mediate malonyl CoA synthesis and that fatty acid synthesis is inhibited by avidin. Perhaps, it has been suggested, ATP is necessary for the carboxylation of biotin and the biotin- $\text{CO}_2$  complex is the carboxyl donor.

An enzyme preparation that catalyzes fatty acid synthesis has been purified from the supernatant fraction of rat liver and of rat brain, from plants, and from microorganisms. The primary product is palmitic acid, and the reactions can be summed up as follows:



If  $\text{NADP}^3\text{H}$  is used, it is found that direct hydrogen transfer occurs and that, beginning with the  $\beta$ -C of palmitate, alternate carbon atoms are tagged with tritium. It has been concluded that the condensed assembly of acetyl CoA and malonyl CoA is reductively decarboxylated.

The sequence of events is sketched in Figs. 8.1 and 8.2. Note that the acetyl group participates as a coenzyme-A derivative but that other acyl groups are present as —S— linked enzyme complexes.\* This scheme predicts that even-numbered aliphatic acyl groups are formed with a variety of chain lengths, but only in special cases does the free acid appear. Until the chains become 16 carbons in length, the acyl CoA derivatives are the only ones present. Experiments performed with labeled acetyl CoA and nonlabeled

\* It has been discovered very recently that a protein-like "coenzyme" (molecular weight 9,100) containing the phosphopantetheine portion of coenzyme A bears the —SH group that accepts the acyl group. This is called the *acyl carrier protein*. It seems to have no catalytic function but exists in a protein complex with the enzymes that mediate the reactions of Figs. 8.1 and 8.2.

malonyl CoA result in palmitate labeled only in carbons 15 and 16. Evidently the carbonyl end of the chain is the site of addition of new two-carbon units.

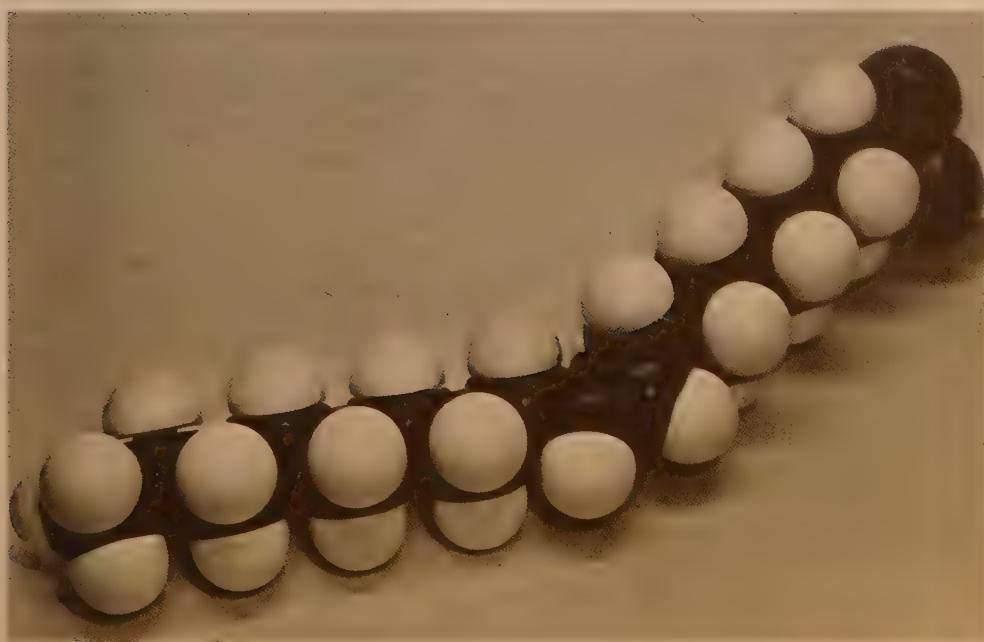
Since acetyl CoA presumably arises inside the mitochondrion, it is obvious that transfer into the extramitochondrial space must occur. It is doubtful that acetyl CoA as such is transferred, and therefore it is assumed that some other intermediate, possibly citrate, is involved.

There is reason to suspect that the mitochondrion is responsible for the variety of aliphatic acids formed. An "elongation system" that eases the addition of acetyl CoA to various acyl CoA derivatives has been described. This system does not require ATP or  $\text{HCO}_3^-$  but does require NADH and NADPH. There is also a mitochondrial system comparable to that which produces palmitic and stearic acids in the supernatant. Lastly, there is a mitochondrial system for desaturating aliphatic acids. According to recent speculation, most of the aliphatic acids synthesized by the mitochondrial apparatus are incorporated into phospholipids.

The unsaturated acids such as oleic (*cis*-octadeca-9-enoic), linoleic (*cis*-octadeca-9,12-dienoic), and linolenic (*cis*-octadeca-9,12,15-trienoic), and others are likely produced by more than one mechanism. The latter two seem to be synthesized at a rate less than optimal in animals, and they have a quasi-vitamin status. The mitochondria that have been isolated from the livers of rats deficient in these dietary factors are not normal. There is evidence that some morphological differences are reflected in the capacity for oxidative phosphorylation, but this is not to imply that there is a single effect of such a deficiency.

In the rat, in molds, algae, and *Mycobacterium phlei*, oleic and palmitoleic

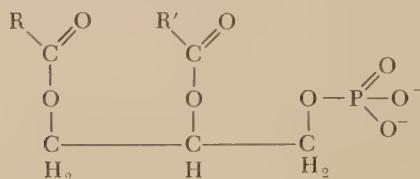
oleic acid



acids arise from stearic and palmitic acids oxidatively in reactions involving molecular O<sub>2</sub> and NADPH. This aerobic dehydrogenation is localized in microsomes (Sec. 16) or similar small particles. In *E. coli*, *Lactobacilli*, *Clostridia*, and several others, fatty acids with only one double bond are a result of β, γ dehydration (of β-hydroxy fatty acids) which may be anaerobic and is catalyzed by a soluble enzyme. However, in most *Eubacteriales* this reaction does not occur.

### triglyceride synthesis

Some details of the formation of triglycerides from fatty acids remain obscure. It is clear that there is in many cells an L-α-GLYCEROLPHOSPHATE DEHYDROGENASE (1.1.99.5) that aids in the reduction of dihydroxyacetone phosphate by NADH. In mammalian liver there is an enzyme (2.3.1.15) that catalyzes the reaction of glycerolphosphate with two moles of acyl CoA, resulting in the formation of a phosphatidic acid:

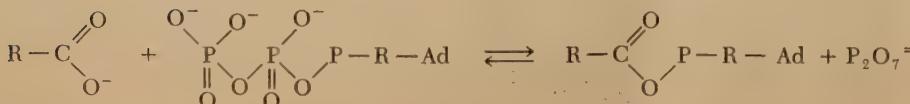


The phosphatidic acids hydrolyze (3.1.3.4) to diglycerides which, in turn, react (2.3.1.20) with further acyl CoA to produce triglycerides.

### fatty acid oxidation

One of the exciting periods in the investigation of fat metabolism followed closely the end of World War II. The isolation and characterization of coenzyme A by Lipmann and his coworkers (REF. 5) gave experimentation a marked impetus. In 1951 Lynen and Reichert identified "active acetate" as acetyl CoA and ended a long search for the "active two-carbon fragment" postulated from numerous tracer studies. Moreover, Green, Lehninger, and others demonstrated that mitochondria could catalyze the oxidation of aliphatic acids to CO<sub>2</sub>. This oxidation was shown to involve the reactions of the citrate cycle. Acetone powder\* prepared from mitochondria was found to provide a model system for study.

The addition of an aliphatic acid to such systems of enzymes was without effect unless ATP and coenzyme A were added. It was eventually demonstrated that they react in the following way. First an adenylyl acylate is formed:



\* Acetone powders have been used as an enzyme source for many years. Tissue is ground into cold acetone and further dehydrated by several acetone washings, and the residue is dried in the cold. In such preparations some enzymes remain active for long periods.

The evidence for this reaction is twofold. If  $^{32}\text{P}$ -labeled pyrophosphate is added to the system, there is no exchange of  $^{32}\text{P}$  with ATP except in the presence of an aliphatic acid. Alternatively, incubation of the enzyme system with synthetic adenylyl acylate and inorganic pyrophosphate results in the formation of ATP. The enzymes involved, called THIOKINASES, possess group specificities: one is specific for acetate; one for C<sub>4</sub> to C<sub>12</sub> acids; and one for C<sub>10</sub> to C<sub>18</sub> acids.

The adenylyl acylate participates in a group transfer mechanism with enzyme A, resulting in adenylic acid (AMP) and acyl CoA ( $\text{R}-\text{C}(=\text{O})-\text{S}-\text{CoA}$ ) as products. The adenylyl acylates are nonaccumulating transient intermediates.

The oxidation of aliphatic acids provides a veritable tapestry of reactions. The initial reaction involving acyl CoA as a substrate is complex. Hydrogen is transferred only to a flavoprotein termed the ELECTRON TRANSFERRING FLAVOPROTEIN (or ETF), which interacts with a system not yet fully characterized but involving the cytochrome c system as an acceptor. The dehydrogenases involved are also flavoproteins containing flavin adenine dinucleotide (FAD) as the coenzyme. One, often referred to as BUTYRYL DEHYDROGENASE (1.3.99.2), is effective for C<sub>4</sub> and C<sub>6</sub> acids, another catalyzes the dehydrogenation of C<sub>4</sub> to C<sub>16</sub> acids but is more effective for the longer chains. The dehydrogenation product is a trans  $\alpha$ - $\beta$  ethylenic acyl CoA. The ES complex in this reaction involves the bonding of both the acyl and CoA moieties so that no dynamic interchange of either group can occur. The  $K_m$  values have been estimated at  $10^{-6} \text{ M}$ , and it is obvious that no acyl CoA, except *acetyl* CoA, can exist in mitochondria in a measurable concentration.\*

There ensues a hydration reaction similar to that seen in the citrate cycle. The ethylenic bond is hydrated, and the intermediate formed is oxidized. The reversible hydration is catalyzed by ENOYL HYDRASE (4.2.1.17) (occasionally called CROTONASE). Only a single enzyme is involved despite the varying chain lengths of the substrates. Normally only the trans form of the acyl CoA is hydrated, but model studies have shown hydration of the cis form is possible.

The  $\beta$ -hydroxy acyl CoA intermediates that result are dehydrogenated to the corresponding  $\beta$ -keto acyl CoA analogs. One or more  $\beta$ -HYDROXYACYL DEHYDROGENASES (1.1.1.35) mediate transfer of protons and electrons to the NAD/NADH system.

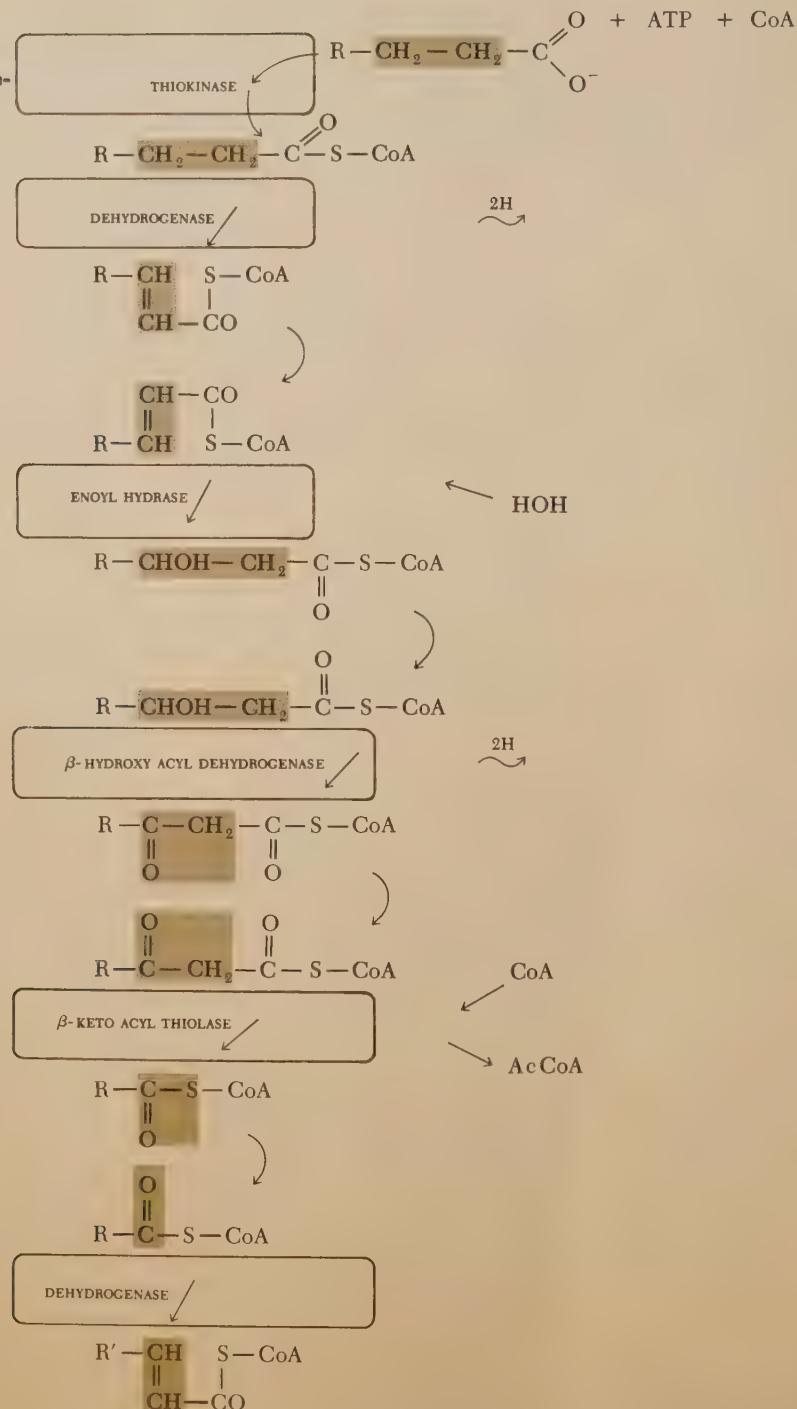
The final step requires additional coenzyme A. In the presence of a  $\beta$ -KETOACYL CoA THIOLASE (2.3.1.16) there is a transfer of two carbons (as acetyl) to CoA, and the new acyl CoA formed, now two carbons shorter, is substrate for the above series of reactions in repetition (Fig. 8.3).

The thiolase group transfer reaction may be related in principle to a Claisen condensation as seen in Fig. 8.4. It has been suggested that the thiolase contains sulfhydryl groups and that the acetyl group is first transferred (from the

\* Figure 8.3 is drawn to emphasize this.

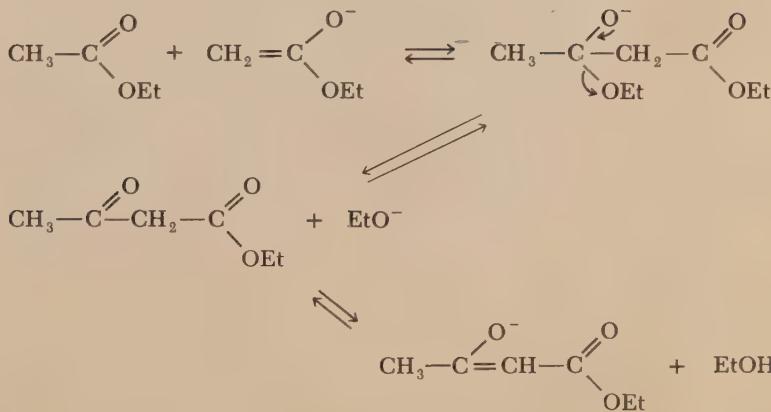
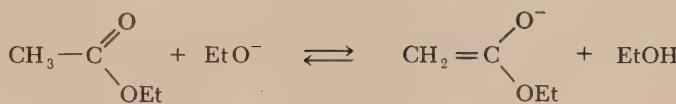
FIGURE 8.3

oxidation of fatty acids in the mitochondrion

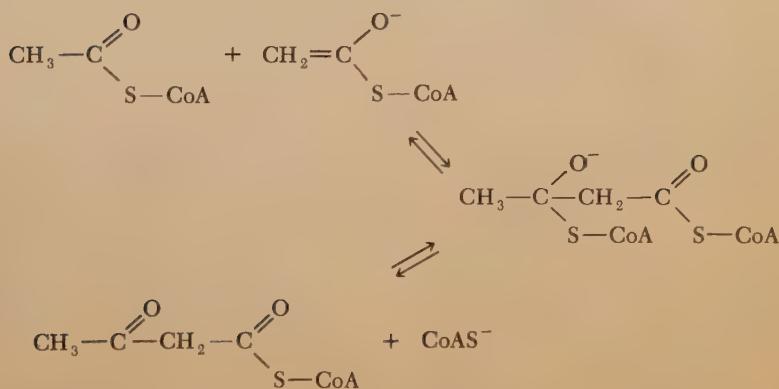


**FIGURE 8.4**                           **TYPE REACTION**

### **comparison of thiolase reaction to Claisen condensation**



**COMPARE WITH**

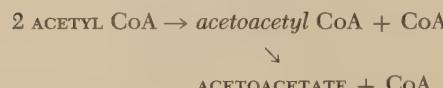


chain to be shortened) to the enzyme and in a subsequent step transferred from the enzyme to coenzyme A.

The acetyl CoA molecules resulting from this process merge with those arising from other sources such as pyruvate. Thus oxidation is completed by condensation with oxaloacetate and by the other reactions of the citrate cycle.

Each of the enzymes necessary to the  $\beta$ -oxidation-scission process appears to be incorporated in the structure of the mitochondrion. Complete reduction of the ETF is prevented by the cytochrome system, which involves  $O_2$  as an ultimate electron acceptor. Undue decrease of the NAD/NADH ratio during the oxidation of the  $\beta$ -hydroxy acyl esters is prevented by the coupling with the electron transport system that is also coupled to the citrate cycle.

Early testing of fatty acid oxidation by mitochondria showed the necessity for a "spark," one of the compounds of the citrate cycle, to be present. The reason is now evident. Unless oxidation occurs, neither ATP nor CoA will be available in sufficient concentration. However, in the cells of the liver, but not of other mammalian tissues, the following reactions can occur:



Thus, in liver, CoA tends to be nonlimiting but a need for ATP still exists.

The reactions involved in fatty acid oxidation are reversible in theory, and there was speculation initially that synthesis was simply a reversal of oxidation. This now seems to be unlikely, yet it is still possible that the "elongation system" of mitochondria may be a reversal of the oxidation mechanism.

### **fat transport and synthesis**

Our common experiences show that all monocarboxylic aliphatic acids except those of lowest molecular weight are water-insoluble, as are the triglycerides. Studies of model systems have shown that many lipids form micelles, droplets that vary in size but are small enough that they tend to be aligned in characteristic patterns. The arrangement is such that there is a concentration of nonionic residues with a maximum exposure of carboxyl or other polar groups to the surrounding aqueous phase. In Secs. 27 and 30 we shall consider the

**TABLE 8.1**  
**lipid composition of**  
**rat liver cell**  
**fractions\***

fraction	percent total lipid			
	total lipid, % dry weight	phospho- lipid	choles- terol	tri- glyceride
nuclei	16	93	4.5	2.5
mitochondria	21	93	5.5	1.4
microsomes	32	94	5.8	0
supernatant	7	28	3.9	68

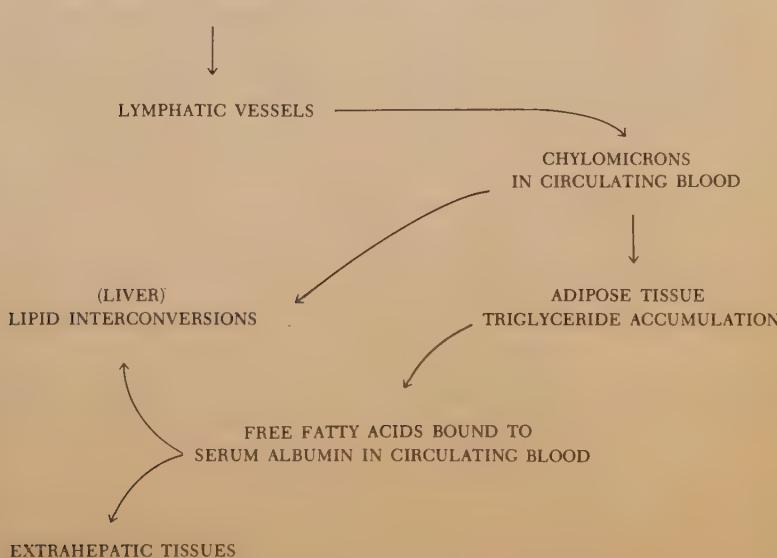
\*From M. J. Spiro and J. M. McKibbin, *J. Biol. Chem.*, **219**, 643 (1956). By permission of *The Journal of Biological Chemistry*.

structure of cell membranes in which lipid is associated with protein. Much of the structural lipid is not a triglyceride but rather a phospholipid or other derived compound. It already has been noted that lipids are part of the functional fabric of mitochondria and chloroplasts. A glance at Table 8.1 will show that liver cells contain relatively little triglyceride in terms of total lipid and that most of it is to be found in the supernatant fraction. This is the case in the average cell, if there is such a thing, whether bacterial, plant, or animal. Animals possess a specialized tissue, adipose tissue, in which triglyceride synthesis and accumulation is marked. As shown in Fig. 8.5, dietary fat may contribute to the triglyceride of adipose tissue; but even when fats are absent from the diet, triglyceride is formed from carbohydrate. The chylomicrons referred to in Fig. 8.5 are similar to micelles, but they are surrounded by a protein film. Again, the bulk of fatty acid transfer occurs as a loose complex of fatty acid and a protein in the blood. The concentration of free fatty acid (FFA) in the blood is quite low, but the turnover is rapid and the total flux is of considerable consequence metabolically.

In brief, the triglycerides are analogous, in a certain sense, to glycogen. They tend to accumulate under certain conditions but are in constant interchange with the free fatty acids which are continually being oxidized after incorporation into the components of the citrate cycle. Triglycerides may be considered as limited heteropolymers that form a separate phase. In each case depolymerization must occur before oxidation can take place. As suggested above, most lipids occur in association with protein. The manner of association of lipids with protein is slowly being clarified. Both lipoproteins and lipopolysaccharides will be mentioned in later sections.

**FIGURE 8.5**  
triglyceride distribution in animals

FAT EMULSION OF INTESTINAL CONTENTS



**regulation  
of lipogenesis**

The regulation of lipogenesis remains a challenge both theoretically and practically. It is an all too common experience that the production of fat may be difficult to stem. It is agreed that the carboxylation of acetyl CoA to form malonate is a prime candidate as a limiting step in fatty acid synthesis. Nevertheless, neither the supply of acetyl CoA nor the supply of carboxylase seems to be the deciding factor. If the CO<sub>2</sub> supply is limiting, then it is not clear what switching reaction is operative. Free fatty acids are effective inhibitors of fatty acid synthesis. It has been suggested that the supply of L- $\alpha$ -glycerophosphate deriving from carbohydrate metabolism may be a controlling factor, since it is necessary for esterification. Although it has been long recognized that the livers of patients with *Diabetes mellitus* are unable to synthesize fatty acids, no adequate explanation is at hand. It is also known that insulin stimulates fatty acid synthesis both in adipose tissue and lactating mammary gland. Studies on the latter, a highly specialized tissue, have been extensive, since it exhibits large fluctuations in lipogenesis and hence seems to be a focus of control mechanisms.

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# 9

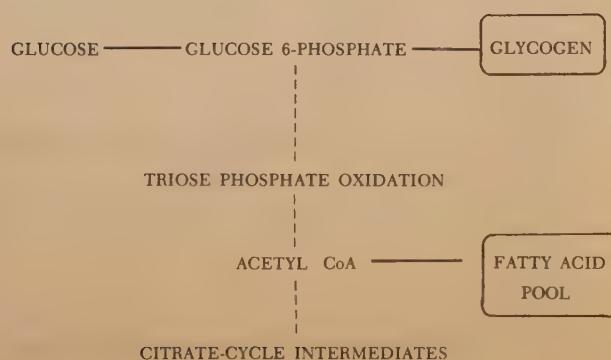
## some schematic generalizations

The discussion of the preceding sections has been restricted to those processes common to most cells and directly involved in furnishing energy for biological functions. The compounds which must be taken into the cell in quantity contain only carbon, hydrogen, and oxygen. So far, no nitrogen-containing compounds have been involved, save the enzymes and coenzymes. A succinct summation of energy-yielding compounds is presented in Fig. 9.1. It is valid for the majority of cell types, but because it is succinct, it does not permit far-reaching inferences.

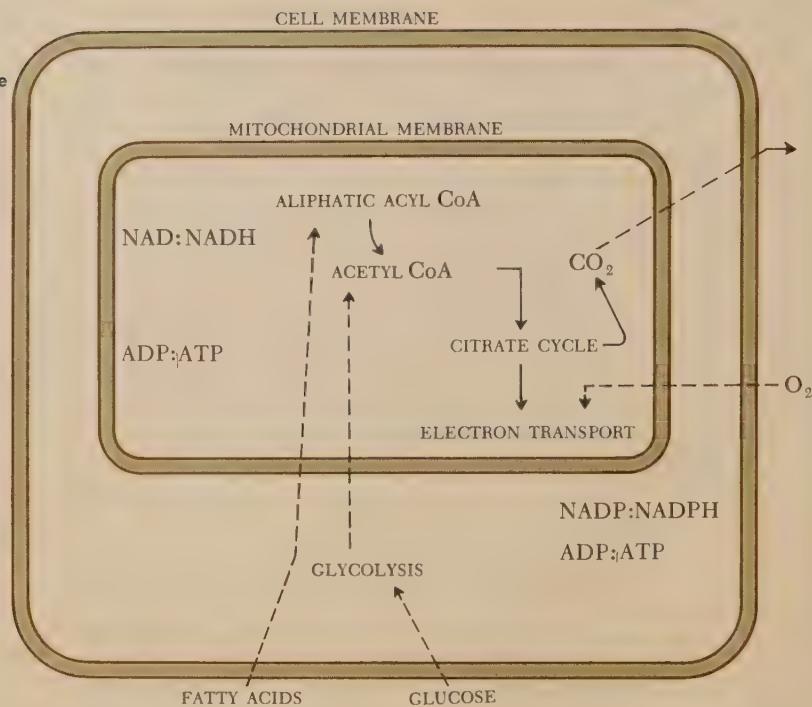
For example, a diet of fatty acids would seem to be adequate for energy purposes or, for that matter, so would a diet of citrate cycle intermediates. For some cells this is actually so, but the majority of multicellular organisms cannot exist on highly simplified diets. Nevertheless, it is useful to think of the sketch below as the lowest common denominator of carbon compound relationships among those substances from which energy is derivable. It can be seen that a fat-free diet, or a carbohydrate-free diet, can be tolerated as far as energy requirements are concerned. Difficulties which arise on such diets are related to other processes. It remains to examine how amino acids and proteins are related to the intermediates of these processes. A diet which is exclusively protein is almost satisfactory for many animals, since the above oxidizable compounds derive from protein.

Figure 9.2 is intended to emphasize the restriction of the citrate cycle and electron transport to the mitochondrion, and the NADP outside, but this is only pedagogic license. Both systems are found both inside and out but in very different concentrations. The mitochondrial membrane is rather impermeable to the NAD and NADP systems and to citrate cycle intermediates and, surprisingly, is relatively impermeable to adenine nucleotides and in-

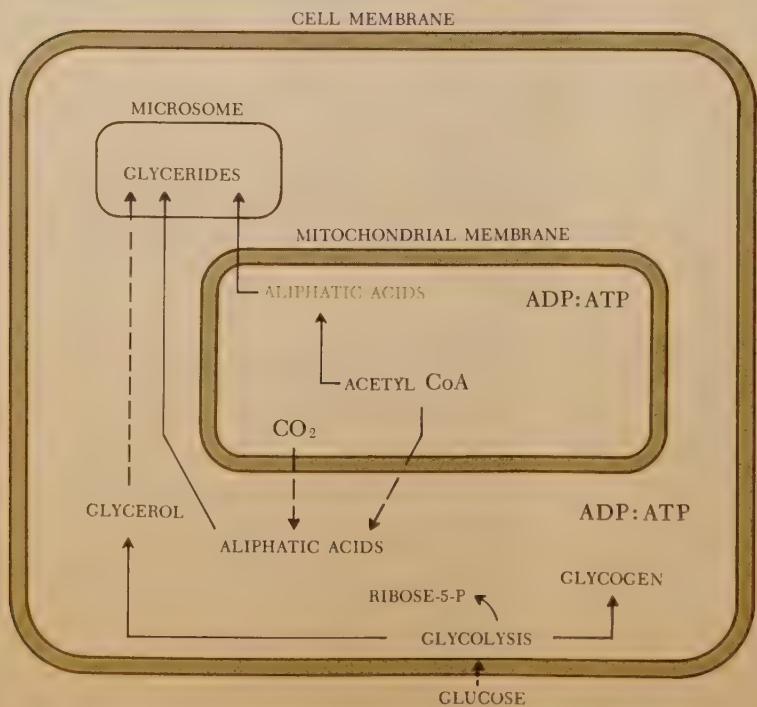
**FIGURE 9.1**  
biological energy  
sources



**FIGURE 9.2**  
energy-yielding  
reactions and where  
they occur

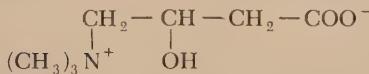


**FIGURE 9.3**  
some syntheses and  
where they occur



organic phosphate. Thus the idea that ATP is the "product" of biological oxidation is entirely too naive. Biological oxidation does raise the ATP/ADP ratio, but there is no direct transfer of ATP to the extramitochondrial space. By a mechanism as yet unspecified, the increase in chemical potential is transferred to the extramitochondrial ATP/ADP system.

Figure 9.3 is intended as a summation of the syntheses that occur in the extramitochondrial space and that are coupled either to biological oxidation or to glycolysis. The NADPH necessary for some syntheses probably comes mainly from glucose 6-phosphate oxidation outside the mitochondrion and perhaps in part from NADPH that is slowly leaked out of the mitochondrion. The ATP needed for syntheses derives indirectly from processes inside the mitochondrion. The synthesis of fatty acids requires acetyl CoA produced inside the mitochondrion, but, as noted in Sec. 8, it is not clear how it is transferred out of the mitochondrion. The reverse process, the movement of free fatty acid into the mitochondrion, seems to be potentiated by carnitine,



in the presence of a TRANSFERASE (2.3.1.7) that catalyzes the reaction



Since the oxidation of long-chain fatty acids by mitochondria is stimulated by carnitine, the suggestion has been made that the above reaction is involved in acyl transfer across the mitochondrial membrane, possibly in both directions.

Even this cursory and fragmentary examination of coupled metabolic processes dwells on the importance of biological structure. In the forthcoming sections many more relations between structure and function will be seen.

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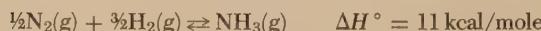
# 10

## nitrogen fixation

The bulk of the food substances utilized by organisms, the raw material for metabolic comings and goings, is composed of carbon compounds with hydrogen and oxygen. As noted before, life depends on a constant turnover of carbon compounds in a dynamic equilibrium with CO<sub>2</sub>, a minor component of the atmosphere. But the compounds unique to living things also contain nitrogen, which, in its elemental form, comprises the bulk of the gaseous atmosphere. Here, in contrast, no sort of dynamic equilibrium exists. There is a "nitrogen cycle" involving several life forms, but the facility for incorporating diatomic nitrogen into organic compounds is by no means widely scattered, nor indeed, is the ability to convert organic nitrogenous compounds into inorganic forms of nitrogen.

Man, biologically, is a seeker of nitrogen compounds, and his welfare is limited by the availability of organic nitrogen. It is well known that the bulk of the population of Asia and Africa suffer a mere subsistence level of nitrogenous foods. In the United States there is a plentiful supply of ammonia from petroleum production operations, and in Europe nitrogen-fixation technology has assured access to the ultimate reserve of the atmosphere.

Nitrogen is an extraordinarily stable molecule (Table 10.1) with maximum bonding. Through a process as yet imperfectly understood, the diatomic molecule is converted to the trigonal-pyramidal molecule, NH<sub>3</sub>. The overall process is not energetically prohibitive, since



However, for each molecule of N<sub>2</sub> at least three reduction steps are necessary, and the hypothetical intermediates have never been detected in biological systems. It is notable that N<sub>2</sub>, unlike H<sub>2</sub> or O<sub>2</sub>, does not form a half cell, and hence no direct electrode data are available.

With the exception of the root-nodule bacteria (*Rhizobium* sp.), only free-living microorganisms fix N<sub>2</sub>. These include photosynthetic algae (*Anabaena*, *Nostoc*), all photosynthetic bacteria (*Rhodospirillum rubrum* and others), and heterotrophic bacteria (*Clostridium pasteurianum*, *Bacillus polymyxa*, *Azotobacter vinelandii*, *Chromatium* species). This is certainly not an extensive list, and even among this group it is difficult to demonstrate active nitrogen fixation. The term fixation is used because it has not yet been proved that reduction only is involved or, indeed, that NH<sub>3</sub> is the only product of fixation.

In 1960 there was reported a method for obtaining cell extracts capable of fixing nitrogen, a method that yielded reproducible results, and nitrogen fixation has now been demonstrated in extracts of *Clostridium*, *Azotobacter*, *Rhodospirillum*, *B. polymyxa*, and *Chromatium*.

The extracts of *C. pasteurianum*, *R. rubrum*, and *B. polymyxa* require the

addition of pyruvate before N<sub>2</sub> reduction can be demonstrated. Since the first isolable product is NH<sub>3</sub>, which represents a six-electron transfer, it must be presumed that there are at least two intermediates that do not dissociate from the enzyme. Dialysis-fixation systems have been devised; in them substantial rates of fixation are observed but no dialyzable intermediates are discoverable.

**TABLE 10.1**  
**comparison of**  
**chemical bonds**

<i>bond</i>	<i>type</i>	<i>length, Å</i>	<i>bond-dissociation<sup>a</sup> energy, kcal/mole 0°K</i>
H—H			
diatomic H <sub>2</sub>	1σ	0.74	103
N≡N			
diatomic N <sub>2</sub>	1σ, 2π	1.10	225
O=O			
diatomic O <sub>2</sub>	1σ, 1π	1.21	118
C—H			
in C <sub>2</sub> H <sub>4</sub>	σ	1.08	99
C—C			
in C <sub>2</sub> H <sub>6</sub>	σ	1.54	83
C=C			
in C <sub>2</sub> H <sub>4</sub>	2π	1.35	125
C≡C			
in C <sub>2</sub> H <sub>2</sub>	1σ, 2π	1.21	230
C=O			
in HCHO	1σ, 1π	1.21	166
C—N		1.47	73
C=N			147
C≡N			
in CH <sub>3</sub> CN	1σ, 2π	1.14	213
N—H			
in NH <sub>3</sub>	3σ	1.014	93
N—N			
in hydrazine			38
N=N			
in azobenzene			98

<sup>a</sup>The standard bond-dissociation energy is that required to break the bond, giving isolated ground-state atoms.

Extracts of blue-green algae such as *Nostoc* or *Anabaena* show much lower capacity for nitrogen reduction, but no additions are required. As yet, net fixation has not been observed in cell-free preparations of root nodules (*Rhizobium*).

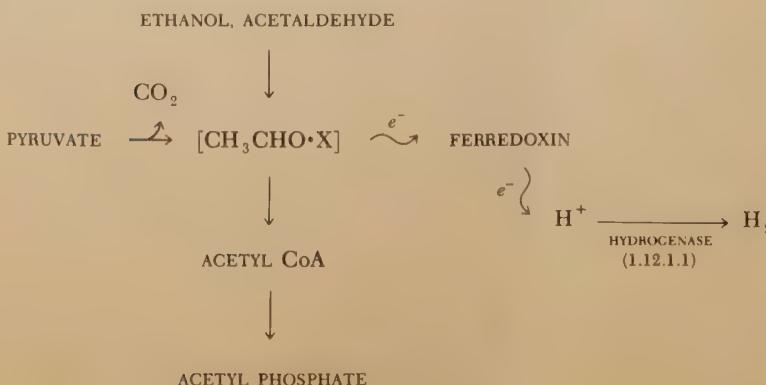
A search for the function of pyruvate has been continued. It can be replaced by a mixture of acetyl phosphate and potassium borohydride. Alternatively, creatine phosphate and  $\text{KBH}_4$  can be used. In part, the labile phosphates serve to maintain the ATP/ADP ratio at a proper value. Since ATP additions alone are insufficient, this explanation is only partial. The  $\text{KBH}_4$  acts as a source of  $\text{H}_2$  which, in such organisms, can be reduced because a HYDROGENASE (1.12.1.1) is present. It is not possible to report anything more specific at this time about the details of  $\text{N}_2$  reduction. Pyruvate both generates ATP (via acetyl phosphate) and participates in some fashion in the actual reduction process. Coenzyme A and  $\text{Mg}^{++}$  are also required.

The reduction process involves ferredoxin, a substance discovered during research on this problem. This electron-transferring protein contains iron, 10 atoms per molecular weight 12,000 (but not in the usual heme complex); it contains *inorganic* sulfur; and it exhibits  $E'_0$  (pH 7) =  $-0.41$  volt [ $\text{H}_2$  electrode,  $-0.42$  volt]. It is possible to adsorb this protein from an extract active in fixing  $\text{N}_2$  and to show that removal inhibits fixation. When ferredoxin is restored, activity returns.

Research on ferredoxin has resulted in recognition of a new type of  $\text{CO}_2$  addition to acetyl CoA when  $\text{H}_2$  and ferredoxin are present. What this may mean is that organisms which reduce  $\text{N}_2$  to ammonia derivatives possess a network of reactions in which  $\text{CO}_2$  fixation is a mandatory component. The use of  $\text{H}_2$  here is an experimental device, of course, and it is likely that the actual coenzymes involved in the intact organism are pyridine nucleotides.

The relation between  $\text{H}_2$  formation and  $\text{N}_2$  fixation in bacteria has intrigued investigators for many years. In *Clostridium pasteurianum* there is clearly competitive inhibition of fixation by  $\text{H}_2$ . Conversely,  $\text{H}_2$  evolution by photosynthetic purple bacteria is inhibited by  $\text{N}_2$  serving as the sole nitrogen source for photosynthetic growth of these bacteria.

**FIGURE 10.1**  
formation of  $\text{H}_2$   
in heterotrophic  
anaerobes



In the *Clostridia* and other heterotrophic anaerobes the evolution of hydrogen is thought to result from the sequence shown in Fig. 10.1. In all likelihood N<sub>2</sub> is a successful competitor for electrons in the presence of a postulated "nitrogenase." The control mechanisms that select H<sup>+</sup> or N<sub>2</sub> as the prime electron acceptor have not yet been described.

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# 11

## amino acid metabolism, part I

Most cells can use ammonium ion as a source of nitrogen. For some specialized cells, nerve cells in particular, ammonium ions are injurious and prevent normal function (Sec. 13). A few microorganisms thrive on nitrites and nitrates. Simple organic nitrogen compounds such as urea serve as excellent nitrogen sources for many organisms. In general, plants and wild-type microorganisms need only an adequate source of nitrogen for growth and are able to synthesize the necessary amino acids. Animals are distinctly different in that certain carbon chain configurations cannot be synthesized. All animals require for normal growth and maintenance a continuous supply of certain amino acids to be specified later. Moreover, nitrogen compounds do not accumulate, necessitating a steady intake of reduced nitrogen. The animal can withstand deprivation of energy sources, fats, and carbohydrates, for days or weeks with relative impunity. To the contrary, deprivation of nitrogen compounds and of water, both escaping continually from the metabolic pool, is a serious matter, and irreversible changes occur in a much shorter time.

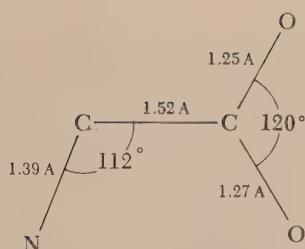
The metabolic relations of the amino acids differ from those of carbohydrates and fats in exhibiting more variability, more marked species differences, more evidences of mutational loss, and more types of chemical structures which must be furnished the organism.

It may not be taken for granted that all the types of amino acids occurring in living cells are to be found in protein. More than 40 amino acids have been isolated, and some of them have never been found as part of the protein structure. Only 19 amino acids commonly occur in the polypeptide chains of proteins; these are  $\alpha$ -amino acids<sup>o</sup> of the L-configuration. Each will be examined as part of the network of compounds forming the patterns of metabolism.

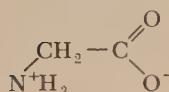
The three-dimensional models of amino acids have been carefully constructed for several reasons. Since amino acids are components of proteins, which are conveniently studied by physical methods, it is useful to have working models. Since amino acids are compounds bearing both positive and negative charges, several modes of interaction are possible, and models help to define the limits of interaction. And, as will be seen, the physical properties of such molecules are not readily predictable from a knowledge of the structure in only two dimensions.

**glycine** Glycine (Gly) has a melting point of  $232^\circ$ , a high value for a compound with such a simple constitution. The following relations have been deduced from X-ray diffraction patterns.

<sup>o</sup> In the strictest sense, proline and hydroxy proline are not  $\alpha$ -amino acids but derivatives thereof.



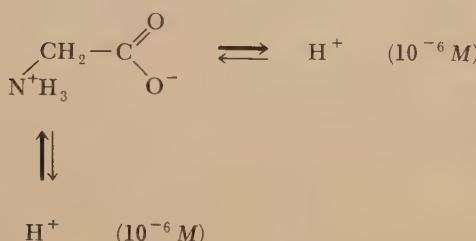
These atoms are essentially coplanar. Since the C—N bond in aliphatic amines usually has a length of 1.47 Å and in many other amino acids has a length of 1.50 Å, it is obvious that in glycine this bond has some double-bond character. In aqueous solution and in the solid state glycine is a doubly charged ion.



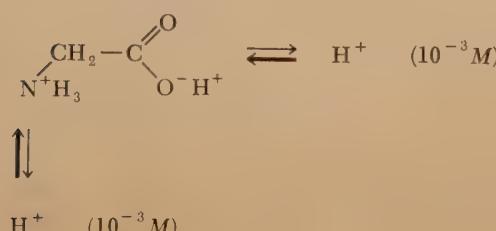
In the crystal, hydrogen bonds hold the glycine molecules together in coplanar double layers and adjacent double layers are probably maintained in contact by van der Waals forces.

In media less polar than water, for solvents of sufficiently low dielectric constant, the nonionic form predominates, with a tendency for dimers to form.

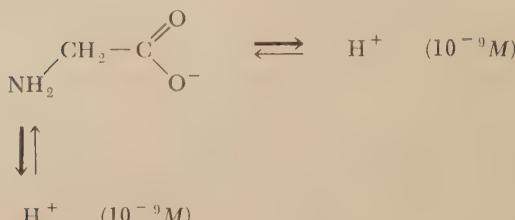
In the biological environment, glycine is an amphoteric molecule, is always ionic, and bears a net charge except at one hydrogen-ion concentration. At pH 6.1 the following equilibria exist.



The nitrogen atoms bear a positive charge and the carboxyl groups a negative charge, so that the net charge on the molecule is zero. If an electric field is imposed on a solution of glycine at pH 6.1, the glycine molecules will not migrate. Thus pH 6.1 is the isoelectric point (pI). If the hydrogen-ion concentration is increased to  $10^{-3} M$ , the situation may be diagrammed as follows:



The nitrogen atom bears a positive charge as before, but only a small percentage of carboxyl groups are dissociated, and there is a net positive charge. The situation at pH 9, then, is



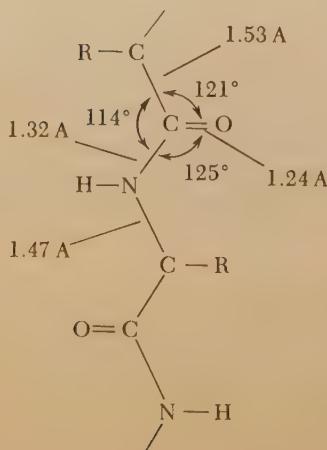
Only an occasional nitrogen atom is charged; all carboxyl groups dissociate; and the net charge is negative.

In sum, except at pI and at very high or low concentrations of hydrogen ion, there exists a mixture of ionic forms; and no succinct notation now in use is adequate to represent this. In the animal the pH of many fluids and tissues is regulated and maintained at a value of about 7.3. Here glycine exists as a population of molecules with a small net negative charge. Some of the molecules in the population bear a positively charged nitrogen. Any particular molecule has a chance of being negative which is greater than its chance of having no net charge and very much greater than its chance of being positively charged.

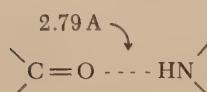
All  $\alpha$ -amino acids are both acceptors and donors of  $\text{H}^+$  and therefore enter into the equilibria of living systems in response to changes in  $\text{H}^+$  concentration. Needless to say, when amino acids are added to tissue preparations, good pH control requires that the ratio of ionic forms be carefully adjusted.

When amino acids form polypeptide chains, the ionic charges just discussed are abolished and there appears a much greater probability of hydrogen bonding.

**FIGURE 11.1**  
dimensions of the  
peptide bond



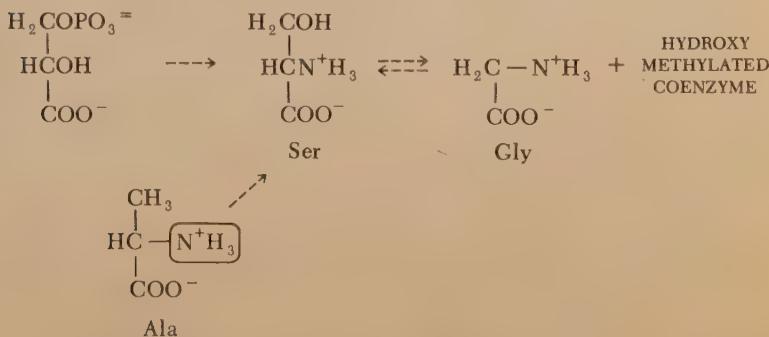
## peptide bond



The repeating unit in the polypeptide chain and some of its characteristics are shown in Fig. 11.1.

The peptide bond is a planar, rigid amide bond. The C—N bond is estimated to have 40% double-bond character, the C—O bond 60%. The consequences of these attributes will be discussed in the section on protein structure.

Glycine appears to derive from serine (Ser) in many cell types: mammalian liver, bacteria, higher plants. The carbon for serine derives from glycerate and the nitrogen from alanine (Ala) by transamination.

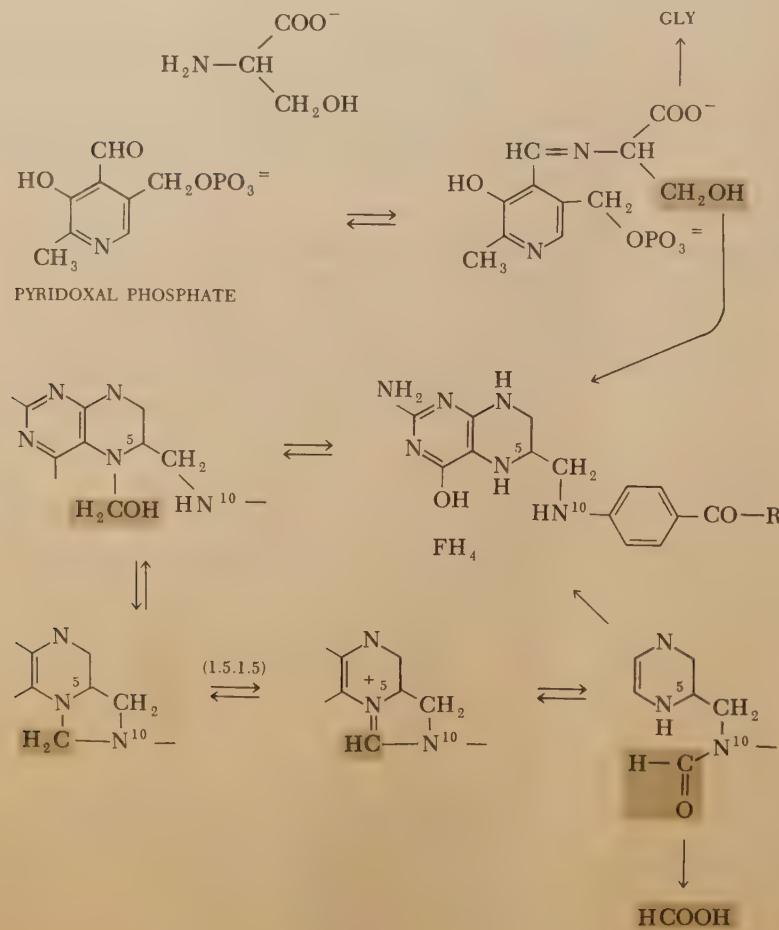


The glycine-serine interconversion is catalyzed by an enzyme variously termed SERINE HYDROXY METHYLASE (2.1.2.1), SERINE TRANS HYDROXY-METHYLASE, and SERINE ALDOLASE. The enzyme system includes two co-enzymes, pyridoxal phosphate and tetrahydro folic acid ( $\text{FH}_4$ ) (Sec. 29), which

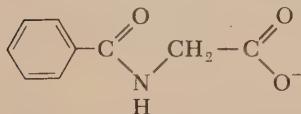
act as donor-acceptors as shown in Fig. 11.2. In the enzyme complex, serine reacts with pyridoxal phosphate to form the imino derivative shown. The alpha carbon becomes nucleophilic, and electron rearrangement occurs to effect a carbon-carbon scission. The hydroxymethyl group is transferred to tetrahydro folate ( $\text{FH}_4$ ) (Sec. 29), and a hydroxymethyl derivative is formed. The unstable complex remaining is hydrolyzed to glycine and pyridoxal phosphate. Hydroxymethyl tetrahydrofolate\* exists in equilibrium with a dehydrated intermediate which may be oxidized. The dehydrogenase (1.5.1.5) involved couples this reaction to the NADP/NADPH system. The 5,10-methenyl tetrahydrofolate formed exists in equilibrium with 10-formyl tetrahydrofolate, a reaction catalyzed by a HYDROLASE (3.5.4.9).  $\text{FH}_4$  is regenerated from the formyl derivative by a DEFORMYLASE (3.5.1.10) with the liberation of formate. Further consideration of the metabolism of this and other one-carbon compounds will be found in later sections.

\*This intermediate may also be formed nonenzymically from  $\text{FH}_4$  and  $\text{HCHO}$ .

**FIGURE 11.2**  
mechanism of the  
formation of glycine  
from serine



Since glycine is not a necessary dietary component for mammals, it may be presumed that the above synthesis, complex though it may be, is adequate for growth and maintenance. A method for estimating the synthesis rate was found long ago. If animals are fed aromatic acids, very little oxidation occurs. Reaction with glycine occurs in the liver, and the reaction product is excreted in the urine as a substituted amide. Benzoic acid, for example, is converted to hippuric acid.



The rate of excretion of this compound indicates that glycine can be formed in human liver in quantities greater than 0.5 g/hr. This reaction has been studied in cell-free systems in order to determine the mechanism of formation

**serine: pyridoxal  
phosphate complex**

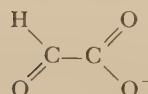


of the peptide bond. Both ATP and CoA were found necessary, and it was shown that benzoyl CoA is an intermediate. The mechanism is not similar to that in protein synthesis but does resemble that of sulfonamide acetylation.<sup>6</sup>

Schoenheimer also took advantage of hippuric acid synthesis to demonstrate dynamic equilibrium. Glycine-<sup>15</sup>N was fed to an animal together with benzoic acid. The hippuric acid isolated was found to contain a much smaller amount of <sup>15</sup>N than would have been the case if only fed glycine had reacted. This correlates with other evidence that an amino acid derived from the diet enters a "pool" and that the fate of any *particular* molecule is a function of many variables.

### **amino-keto intercon- version**

In all cases save two (lysine and threonine), an amino acid and its keto analog are essentially equivalent metabolically. Most tissues contain oxidases (1.4.3.2) that catalyze conversion of the amino form to the keto form. For glycine there is a *specific oxidase*, a flavoprotein containing FAD. The products of this reversible reaction are NH<sub>4</sub><sup>+</sup> and glyoxylic acid:<sup>†</sup>



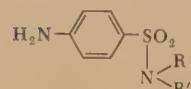
There are also TRANSAMINASES, or AMINOTRANSFERASES, that mediate the interconversion of amino and keto acids of different carbon chain configurations. For example,



Such reactions have low activation energies and occur, although more slowly even in the absence of enzymes.

Thus, although certain amino acids must be present in the diet of some organisms, it is usually the special configuration of the carbon chain that is needed. The nitrogen may be added after ingestion and may derive from a variety of sources including ammonium ion.<sup>‡</sup>

<sup>6</sup> Many sulfonamides are excreted as the N4-acetyl derivatives. Acetyl CoA reacts with the free amino group in the presence of an enzyme (2.3.1.5) with wide specificity.



<sup>†</sup> In microorganisms and in plant seedlings, but not in mammals, glyoxalate is an intermediate in a variant of the citrate cycle. Isocitrate is cleaved by an ISOCITRATE LYASE (4.1.3.1) to succinate and glyoxalate. The succinate is metabolized via fumaric acid; the glyoxalate is condensed with acetyl CoA in the presence of a SYNTHASE (4.1.3.2) to yield malate.

<sup>‡</sup> The ammonium ion is readily incorporated into organic compounds by plants and by microorganisms. Animals, possessing a nervous system, can tolerate only low concentrations of NH<sub>4</sub><sup>+</sup>. At the proper levels NH<sub>4</sub><sup>+</sup> can be incorporated into protein in feeding experiments (Sec. 24). It is likely that most of the nitrogen which moves from one organic form to another is transferred as a group and does not form an NH<sub>4</sub><sup>+</sup> pool.

**nitrogen balance**

The comparative biochemistry of nitrogen metabolism is rich in variation, but among all animals, as already noted, the phenomenon of "nitrogen balance" occurs. Nitrogen compounds are continually excreted in the urine and to a much smaller extent in perspiration. There is also a continued loss of nitrogen due to the growth of hair, nails, and skin. In the adult only enough nitrogen need be ingested to balance these losses. Any excess nitrogen is excreted in the urine. It is not obvious why there is no mechanism for accumulating nitrogen. Of course a growing animal, plant, or microorganism does accumulate nitrogen in its structure, and a growing organism is considered to be in positive balance.

In microorganisms there seems to be no steady "turnover" of protein as in the animal and hence there is no nitrogen balance in the same sense. In plants, a still different situation obtains. Amino acids are synthesized during photosynthesis, and it seems certain that the uptake of nitrogen is regulated by a different set of reactions.

**optical activity**

Alanine is the simplest optically active acid found in protein. L-( $-$ )-alanine, the predominant biological species, is configurationally related to L-( $+$ )-lactic acid. In neither the amino acid series nor the carbohydrate series are the D- and L-designations correlated to ( $+$ ) or ( $-$ ). The first pair of symbols refers to configuration, the second to the direction of observed optical rotation. The optical rotation of amino acids varies substantially with changes in the pH of the solution. If the rotation is measured initially at the isoelectric point and the hydrogen-ion concentration is then increased, it is found that the rotation of the L-form becomes more positive (which can also mean less negative). Precisely the opposite behavior is observed for the D-isomer.

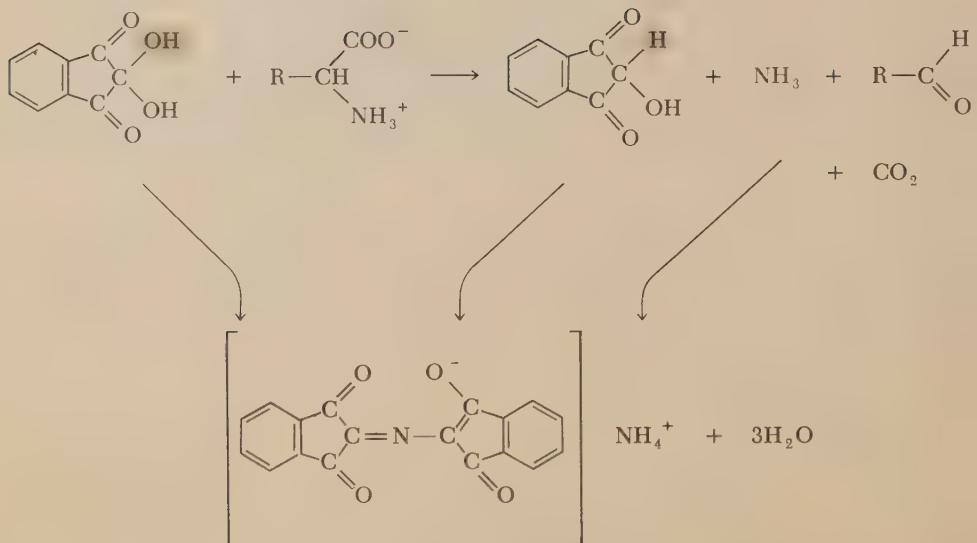
**alanine**

Alanine is one of a small group of amino acids possessing remarkable stability. These amino acids have been detected in fossil bones, in fossil shells over  $10^6$  years old, and in anthracite whose age has been estimated at  $250 \times 10^6$  years (REF. 1). The demonstration or detection of amino acids in very small quantities is facilitated by reaction with ninhydrin. As may be seen in Fig. 11.3, one of the final products is a resonance hybrid that is colored, the exact tint varying with the amino acid.

Alanine and glutamic (Glu) and aspartic (Asp) acids are formed by transamination from the corresponding ubiquitous keto acids: pyruvate,  $\alpha$ -ketoglutarate, and oxaloacetate. The transaminases involved are to be found in almost every cell, both in the supernatant fraction and in organelles. Those purified, and hence well studied, are GLUTAMIC-OXALOACETIC TRANSAMINASE (2.6.1.1) and GLUTAMIC-PYRUVIC TRANSAMINASE (2.6.1.2). Both require pyridoxal phosphate as a coenzyme. (For mechanism studies see references 3 and 4.) Several transaminases in addition to these have been found but are less widely distributed.

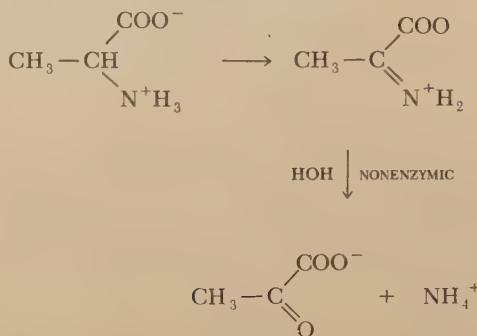
When inorganic nitrogen is incorporated into amino acids in plants, it is these same amino acids—alanine, glutamate, and aspartate—which are formed first. They are at the nexus of a large number of reactions.

**FIGURE 11.3**  
reaction of ninhydrin  
with amino acids



**oxidative deamination**

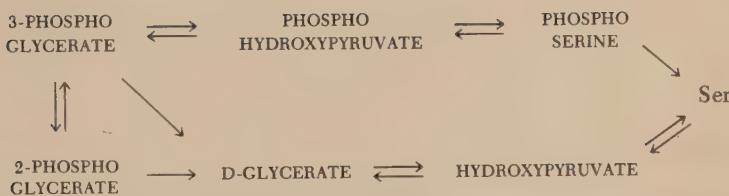
In many cells an enzyme (1.4.3.2) that catalyzes the oxidative deamination of L-amino acids can be demonstrated. The mechanism of this reaction is



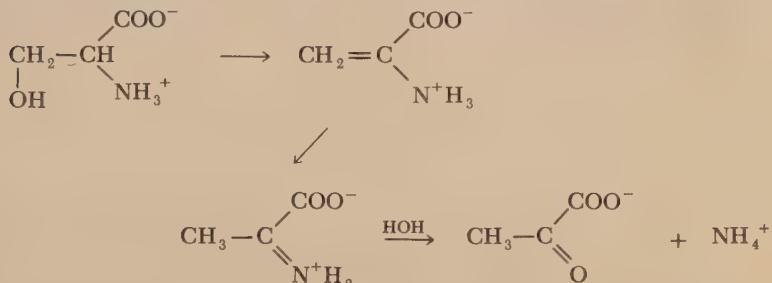
The catalysts are flavoproteins that directly activate oxygen and hence are termed **OXIDASES**, although they also act as dehydrogenases. The reaction rates are not high, and it has not been determined whether this reaction is indispensable in metabolism. It should be noted that "oxidative deamination" can occur either by dehydrogenation or by transamination. It is likely that the latter process predominates and is the first to occur after an amino acid enters a cell unless it is incorporated into some structure *in toto*.

**serine**

In considering the synthesis of glycine it was stated that L-serine derives from glycerate. The reaction sequence varies from one organism to another and various possibilities are realized.

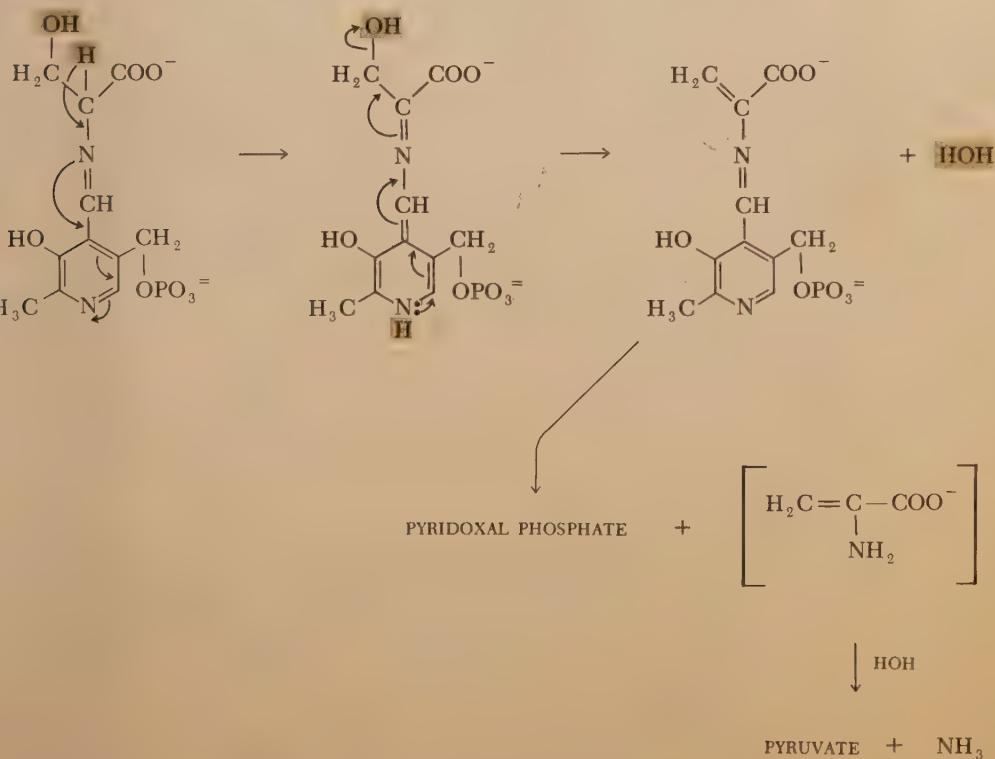


A specific enzyme, SERINE DEAMINASE (dehydrolase) (4.2.1.13) ensures a facile relation with pyruvate. In this reaction an apparent oxidation is the result of dehydration.



**FIGURE 11.4**  
mechanism of serine  
dehydration

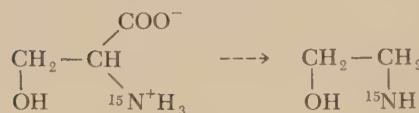
The coenzyme participating in this chemical change is pyridoxal phosphate. A postulated mechanism is outlined in Fig. 11.4.



This  $\alpha$ ,  $\beta$ -elimination reaction involves a complex identical with that in Fig. 11.2. It may be assumed that the reaction path differs because of the enzyme associated in the complex.

### decarboxylation

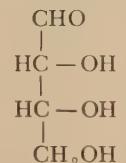
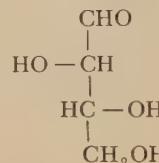
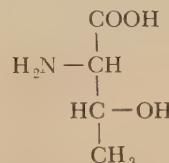
Another general reaction of amino acids in various tissues is that of decarboxylation. DECARBOXYLASES are particularly abundant in microorganisms, but these enzymes also occur in plants and in animals. It is to be noted, with interest or perhaps surprise, that pyridoxal phosphate again is the coenzyme and that intermediates somewhat similar to those above are likely formed. In this regard, however, serine is an exception and no serine decarboxylase is known. However, the following reaction has been demonstrated.



This decarboxylation product, ethanolamine, is a normal component of phospholipids, and it is believed that the mechanism of Fig. 11.5 is responsible for its formation.

### threonine

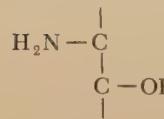
L-Threonine (Thr) is related configurationally to the two possible tetroses as shown here.



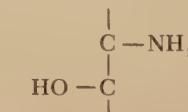
D-THREOSE

D-ERYTHROSE

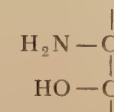
The L- designation is assigned because of the configuration about the  $\alpha$  carbon. Four isomers are possible.



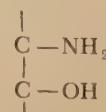
L-



D-



L-allo

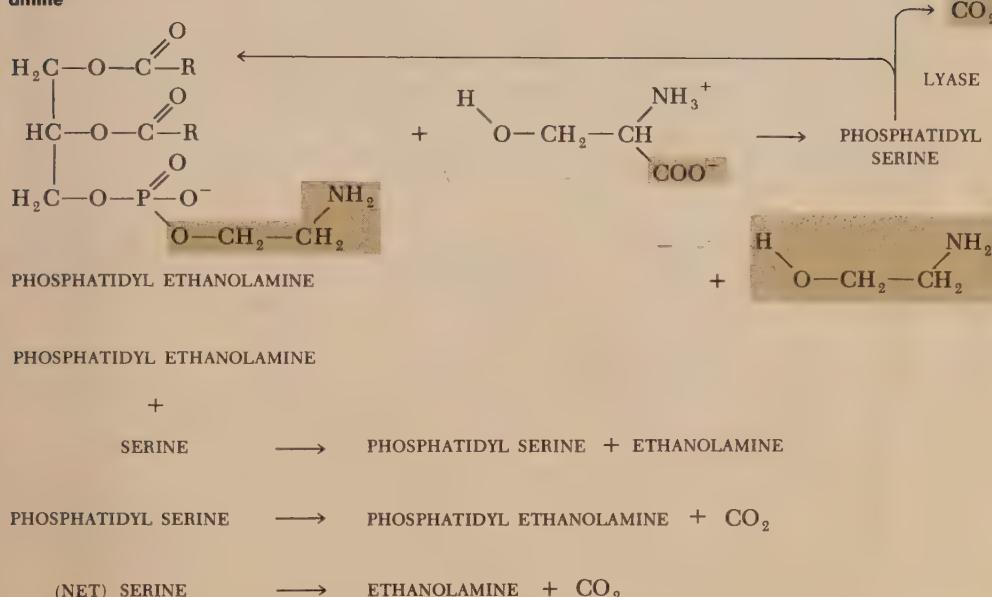


D-allo

### essential amino acids

Threonine was one of the last amino acids normally found in protein to be discovered. Its recognition is credited to Rose, who published his findings in 1935. This discovery was a product of a type of research which formed an important part of biochemistry in the first part of the twentieth century. The idea that certain essential amino acids were mandatory components of a mammalian diet was strongly supported by the work of Osborne and Mendel (REF. 5). Extensive nutritional studies eventually led to an agreement on the

**FIGURE 11.5**  
formation of ethanol-  
amine



qualitative and quantitative amino acid requirements. The data for humans are listed in Table 11.1.

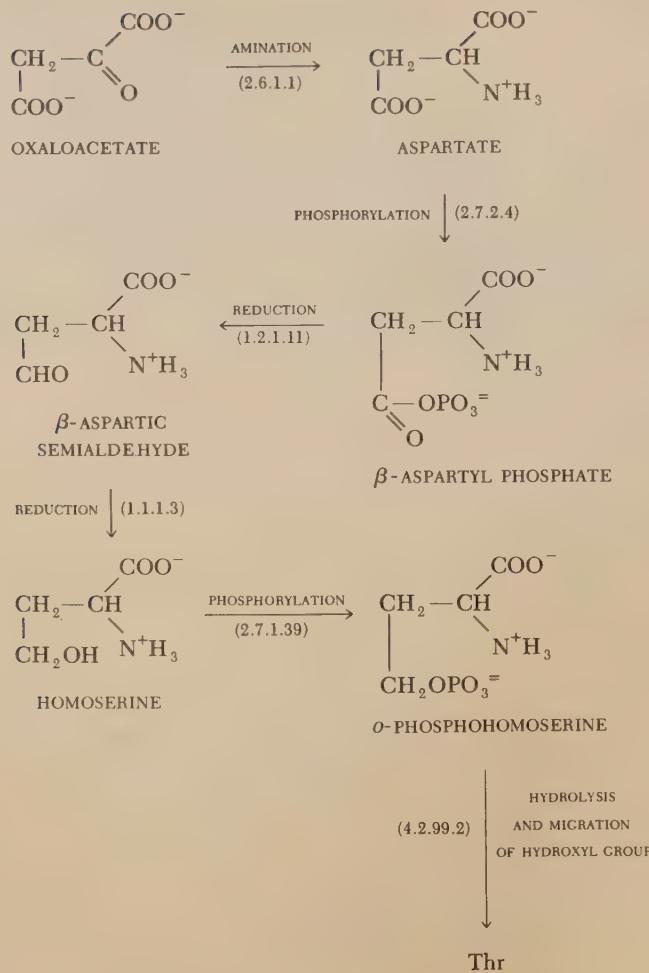
The biosynthesis of threonine can be demonstrated in yeasts, fungi (*Neurospora*), bacteria (*E. coli*), and higher plants. It is likely that the sequence of reactions in each is represented by that in Fig. 11.6. The conversion of oxaloacetate to aspartate occurring in most organisms is followed by two reduction steps resulting in homoserine. Homoserine will later be seen to participate in serine formation as well as the synthesis of threonine.

**TABLE 11.1**  
the daily  
requirements for  
essential amino acids

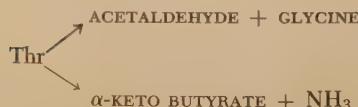
amino acid	minimal	recommended
L-tryptophan	0.15	0.5
L-phenylalanine	0.8–1.1	2.2
L-valine	0.8	1.4
L-leucine	0.5–1.1	1.4
L-isoleucine	0.7	1.4
D- or L-methionine	1.1	2.2
L-lysine*	0.4–0.8	1.6
L-threonine*	0.5	1.0

\*These amino acids, unlike the others, cannot be replaced by the keto analog. Both histidine and arginine are required by children but not by the adult male.

**FIGURE 11.6**  
the biosynthesis of  
threonine



In the liver and the kidney of several animals have been found an aldolase-like enzyme (4.1.2.5) and a deaminase (dehydrase) (4.2.1.16) that catalyze the following reactions:



It is probable that some threonine is metabolized in this way, but it is unlikely that these reactions serve as a source of threonine. The α-ketobutyrate serves as a precursor for a portion of the carbon configuration of isoleucine (Fig. 13.1). It is not surprising, therefore, that ketobutyrate is an inefficient substitute for threonine as the α-keto analog. Yet isoleucine is itself an essential amino acid and, therefore, it may be concluded that the deamination of threonine is irreversible biologically.

**comment** Even the briefest discussion of amino acid metabolism calls for the consideration of transamination, oxidative deamination, reductive amination, and decarboxylation. In order to secure a wide view of biochemistry, it is necessary to consider the processes of synthesis as well as those of disposition, use, or metabolism. Since some amino acids are not synthesized at all by mammals, it is necessary to consider other forms of life. Such considerations have often revealed unsuspected relations that exist even in the animal but are not readily evident. The number of interrelations, the network of reactions, is complex.

A glance over this section shows contributions by organic chemists (Fig. 11.4), physical chemists (Fig. 11.1), animal and plant physiologists, nutritionists (Table 11.1), and microbiologists (Fig. 11.6), as well as biochemists. This section is to a large extent descriptive and lacks a great predictive value. Yet out of this melange of observations have arisen generalizations which have become working hypotheses of biochemistry. The enormous detail of amino acid metabolism can be oppressive to the neophyte and hence the treatment here is minimal but, I hope, adequate. To the specialist it is a continuing challenge to describe more and more narrowly the obvious diversities that exist.

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# 12

## amino acid metabolism, part II

### cysteine

In so far as interesting, and occasionally baffling, chemistry is concerned, there is no amino acid to match cysteine (CysH). The structure of the sulfur atom permits a variety of reactions quite beyond that usually seen. For example, cysteine, like other thiols, reacts with molecular oxygen in air, particularly at a pH greater than 7.



But in the presence of other disulfides the following type of disulfide interchange may be observed.



The importance of this is that proteins and peptides contain —S—S— linkages formed by the oxidation of the sulphydryl groups of peptide-linked cysteine. Thus the addition of small-molecular-weight thiols to proteins and peptides can result in a mixture of products difficult to separate and capable of further interaction.

The common designation of disulfides as R-S-S-R tends to imprint the notion that they are linear assemblies of atoms. They are not. Two possible conformations may be diagrammed as in Fig. 12.1.

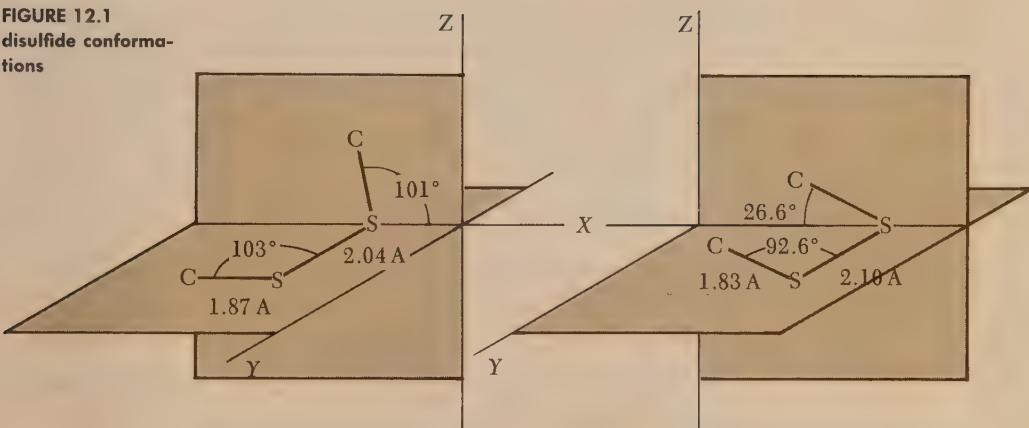
Since there is a barrier to rotation, the formation of disulfide links is influenced by steric factors, particularly when the sulphydryl precursor groups are attached to macromolecules.

That the sulfur atom has some unique attributes may have been suggested to you by some of the mechanisms considered in preceding sections. You may recall the function of the sulphydryl group in transfer reactions involving

### cysteine



**FIGURE 12.1**  
disulfide conformations



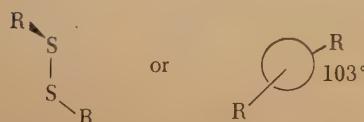
coenzyme A (fatty acid oxidation) and lipoate ( $\alpha$ -ketoglutarate dehydrogenase).

### sulfur orbitals

The complex and extensive organic chemistry of sulfur compounds has called for a detailed account of the atomic architecture of sulfur, and it is rewarding to compare it with that of carbon. Carbon has two electrons in the 1s orbital. In the ground state two electrons occupy the 2s orbital and there are two lone electrons ("electrons celibataires") in the  $2p_x$  and  $2p_y$  orbitals. But tetravalent carbon must have *four* equivalent orbitals, and therefore it is postulated that there exist four  $sp^3$  hybrid orbitals that can accommodate the eight electrons that potentially can fill  $2s + 2p_x + 2p_y + 2p_z$ . Alternatively, there may be three  $sp^2$  orbitals at  $120^\circ$  to each other. Both sigma and pi bonds are involved in carbon bonding. Comparing sulfur, it is to be noted that the 1s, 2s,  $2p_x$ ,  $2p_y$ , and  $2p_z$  orbitals are filled with paired electrons. The six valence electrons are in the 3s, 3p, and 3d orbitals. In total, nine orbitals ( $18\epsilon$ ) are available.



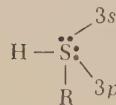
Various hybridizations are possible. The 3p orbitals form the  $\sigma$  bonds and the angle is near  $90^\circ$ , since 3p orbitals are essentially at right angles to each other. A case can be made that 2p-3p  $\pi$  bonds (C—S) are less stable than 2p-2p  $\pi$  bonds. Also, bonding overlap may occur between 2p and 3d orbitals, but the latter, for sulfur, have no electrons and the result is probably conjugative  $\pi$  bonding. The azimuthal angle in several disulfides has been found to be



$103^\circ$  and is the resultant of  $p^2$  covalent bonding and repulsion between the unshared 3p electrons on the two atoms. The dissociation energy for the

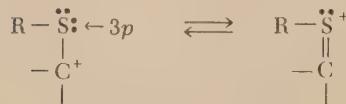
S—S bond is relatively high, about 64 kcal/mole as contrasted with that for O—O or N—N (Table 10.1), which is less than 40. This points to some species of double bonding that has not yet been defined. Alternatively, the repulsion between the two nuclei may be less than anticipated. The barrier to rotation has been evaluated for thioctic acid (lipoic acid) at about 10 kcal/mole.

In the compound



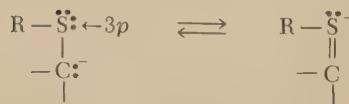
two  $3p$  orbitals are used for the  $\sigma$  bonds to H— and R—. The two unshared pairs are assigned to  $3s$  and  $3p$ .

In the case of the carbonium ion,

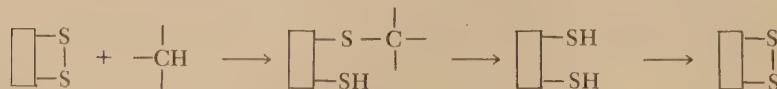


there may be the formation of a  $2p\text{-}3p \pi$  bond of intermediate stability.

For the carbanion also,



a  $2p\text{-}3p$  bond may form, but there is, in addition, accommodation of unshared electrons in a hitherto empty  $3d$  orbital. Therefore, the successive reactions



representing pyruvate oxidation, for example, are seen as a system,  $2-\text{SH} \rightarrow -\text{S}-\text{S}-$ . There is only a small difference in potential across the arrow, but the  $-\text{S}-\text{S}-$  form can stabilize a carbanion at the right energy level. But the advantage of the sulphydryl-disulfide system for protein structure is not obvious at this time.

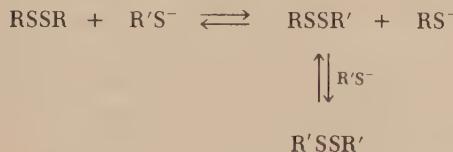
### sulphydryl-disulfide relations

It is surprising that the mechanism of the reaction



is not known in detail. This reaction is slow in the complete absence of metals and quite rapid in the presence of  $\text{Cu}^{++}$  or  $\text{Fe}^{3+}$  ions. The effect of pH on the rate is variable with R. For example, for thioglycolate the maximum rate occurs in the pH range 5 to 6. For cysteine the maximum is in the pH range 7.4 to 9.

Reactions between thiols and disulfides are not at all anomalous and apparently involve the mercaptide ion as intermediate.

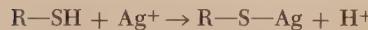


Such reactions are thought to occur continually in cells, but it is not known how they are catalyzed or controlled. Disulfide interchange reactions also occur, and these are



also catalyzed by traces of mercaptide.

The determination of sulfhydryl groups has challenged analytical ingenuity for many years. Simple thiols react as follows:

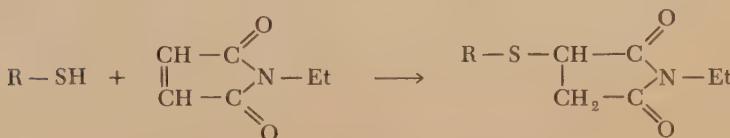


Since the silver mercaptide is undissociated, this reaction provides the basis for an amperometric titration.

A compound often used is *p*-chloro mercuribenzoate (PMB), which, in solution, is actually a hydroxide. Reaction is accompanied by a change in the absorption spectrum, which can be measured conveniently.



*N*-ethyl maleimide (NEM) or iodoacetate is often employed as a "blocking" agent because the derivatives are reasonably stable.



Such blocking agents are frequently used in studies of protein structure when it is useful to remove the characteristic reactivity of the sulfhydryl group.

Cysteine can exist as an oxidation-reduction pair:



Since the metabolizing cell has a reducing potential, it is to be expected that most sulfhydryl groups will be reduced but some —S—S— groups will occur,

as has already been noted for thioctic acid. A reaction useful for determining the presence of the oxidized form employs sulfite.



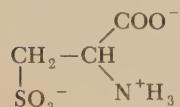
In the presence of  $\text{Cu}^{++}$ ,  $\text{RS}^-$  is reoxidized to  $\text{R—S—S—R}$  and the result is complete conversion to  $\text{R—S—SO}_3^-$ . The course of this reaction and its stoichiometry may be followed by amperometric titration.

Disulfides may also be cleaved with performic acid under properly controlled conditions so that

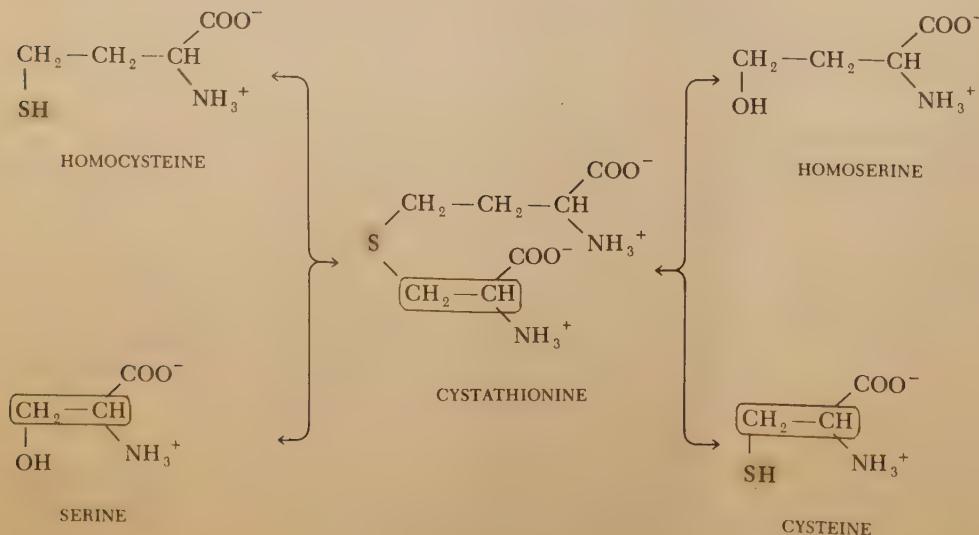


### cysteine metabolism

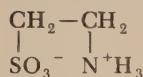
The biosynthesis of cysteine in microorganisms has been found to involve the compounds and relations in Fig. 12.2. Cysteine seems to be important as a determinant in protein structure; it forms a part of catalytic assemblies in enzymes; it is a constituent of both coenzyme A and glutathione; but it is not known to form part of an extensive metabolic network. It is converted by unknown mechanisms in some cells to cysteic acid,



**FIGURE 12.2**  
the biosynthesis of  
cysteine

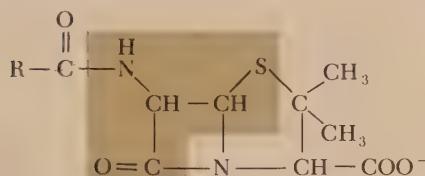


(or a derivative), which can be further converted to taurine,

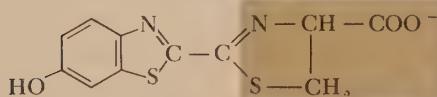


by decarboxylation. Other reactions are involved, since, in mammals, the end product is  $\text{HSO}_4^-$ , excreted continuously in urine. The carbon portion of the cysteine becomes part of the pyruvate pool.

There are two noteworthy compounds containing the elements of cysteine: penicillin,



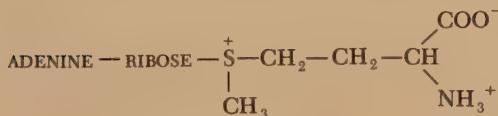
into which L-cysteine is incorporated as such, and D-luciferin,



which contains the elements of the D isomer.

### **methionine**

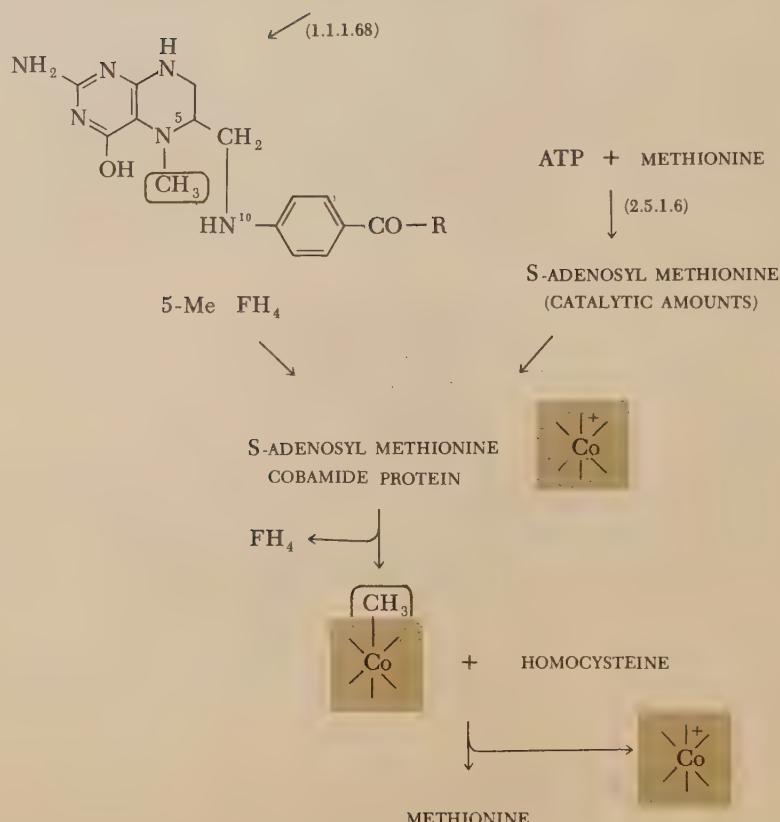
The amino acid homocysteine (Fig. 12.2) that entered the discussion a few paragraphs back is one not found as a protein component. However, the S-methyl derivative does form part of protein structure and bears the trivial name of methionine (Met). Like threonine, it was discovered because it is a dietary component necessary for growth but, in this case, necessary for the growth of a microorganism. It was later found that the methyl substituent is very mobile in the cell and participates in the formation of other needed metabolites in higher animals as well. This system forms part of what some are pleased to call "one-carbon metabolism." (We have already encountered a member of this metabolic reaction group in considering the synthesis of glycine from serine.) Studies of transmethylation have revealed that the metabolically active form of methionine is



(S-adenosyl methionine) formed from methionine and ATP. This adenosyl derivative acts catalytically as a component of an enzyme system incorporat-

**FIGURE 12.3**  
methionine synthesis

5,10-METHYLENE TETRAHYDROFOLATE



ing cobamide (Sec. 29) as a coenzyme. Figure 12.3 presents a current view of the mechanisms and components involved in the methylation of homocysteine. The exact participation of adenosyl methionine in this synthesis cannot yet be described. Aside from this, however, S-adenosyl methionine is a very active methylating agent and the methyl group is transferred from this compound to many different acceptors in the presence of methyl transferases (2.1.1). The adenosyl homocysteine formed is hydrolyzed (3.3.1.1) to adenosine and homocysteine.

Since methionine is a component of proteins, the question that might arise is whether the methyl group can be transferred to other compounds. Apparently not, either because the methyl group is "buried" in the structure or because it cannot be labilized by adenylation. That is to say, the enzyme specificity does not permit the reaction to occur.

**amino acid  
pools**

In this elementary review of amino acid metabolism it is not possible, or even desirable, to consider every metabolic interrelation. If the discussion is to avoid superficiality, certain generalizations must emerge, and to this end

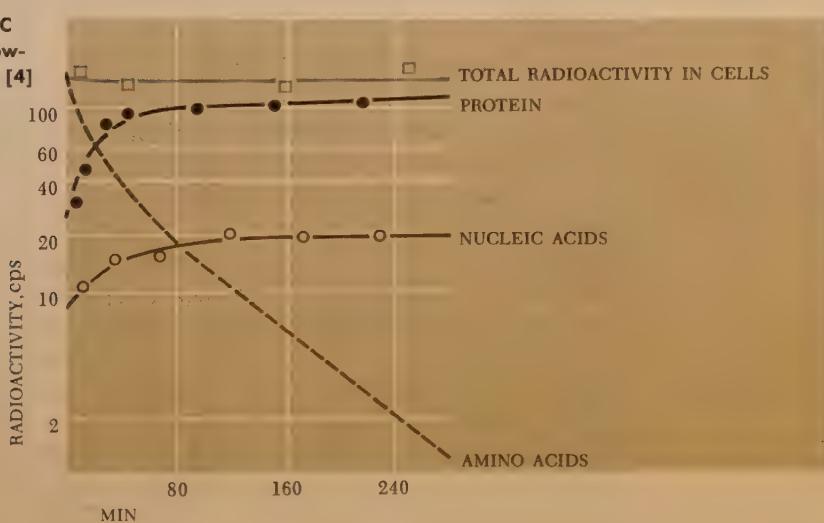
some general questions must be posed. In each case it is desirable to inquire whether an amino acid is incorporated into protein and to list or to note other "small-molecule" reaction sequences involved. If the amino acid is a necessary component of the diet, it is desirable to ascertain which reactions would be embarrassed if the amino acid were in short supply. A large percentage of the reactions are near-equilibrium reactions involving low activation barriers, and such a circumstance requires control mechanisms. Both mass action and feedback mechanisms are operative. It is assumed that enzyme specificity ensures that the reactions of free amino acids are not exhibited by the peptide-bonded amino acids in the assembled protein. During growth, or during steady-state turnover of protein, a balance is established among those processes using up free amino acids for protein synthesis and those converting amino acids into other products.

An exploration of this latter consideration has been reported by Cowie (REF. 7), and some of the experiments will be referred to briefly in the following discussion.

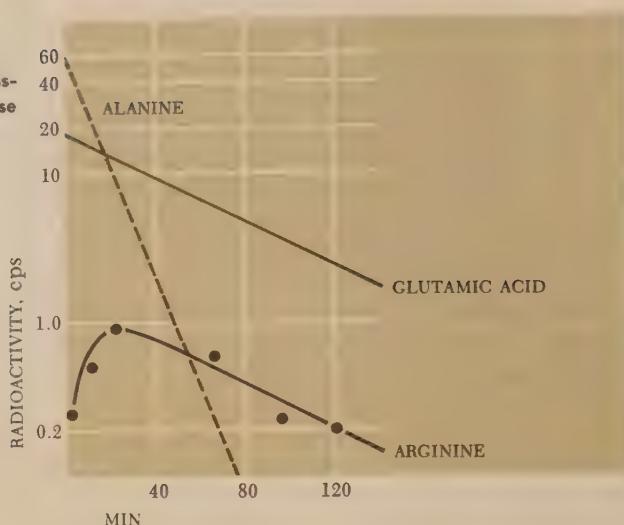
A yeast, *Candida utilis*, was grown for several generations on fructose-<sup>14</sup>C. In the steady state, 50% of the carbon was recovered as protein, 8 to 10% as nucleic acids, 10% as lipids, and 12 to 14% as free amino acids. An inoculum, or an aliquot, of these labeled cells was then added to nonlabeled medium, and cell proliferation was allowed to continue. Changes in the distribution of <sup>14</sup>C are shown in Fig. 12.4. According to these results, very little <sup>14</sup>C was lost, the expected increase in both protein and nucleic acid labeled with <sup>14</sup>C was observed, and there was a corresponding decrease in the <sup>14</sup>C-labeled amino acids in the "free" state.

The time course of <sup>14</sup>C incorporation was determined by performing what is known in the trade as a "pulse experiment." A rapidly growing culture of the yeast was exposed to fructose-<sup>14</sup>C for a few minutes and then transferred to nonlabeled medium. The changes in <sup>14</sup>C content of specific amino acids

**FIGURE 12.4**  
semilog plot of <sup>14</sup>C  
distribution in grow-  
ing *Candida utilis* [4]



**FIGURE 12.5**  
semilog plot of  
changes in  $^{14}\text{C}$  dis-  
tribution in a pulse  
experiment [4]



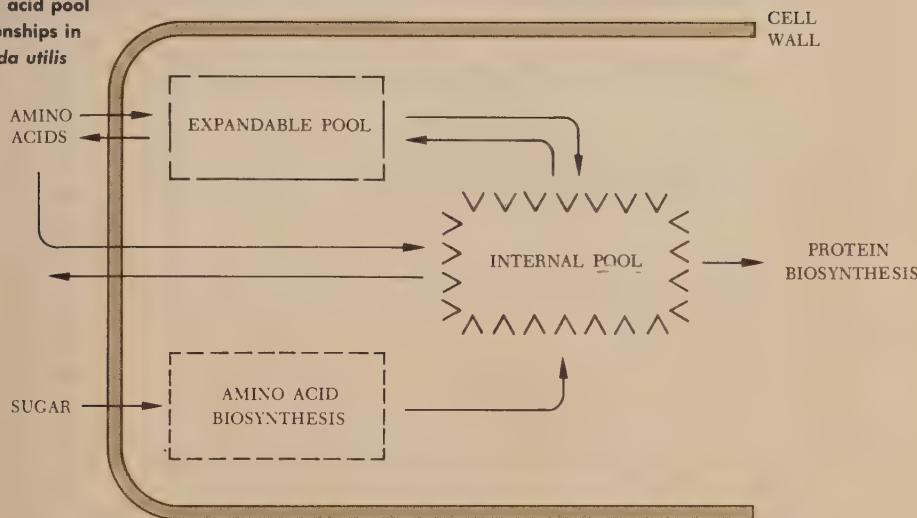
were measured at intervals, and the results were plotted in Fig. 12.5. These results have been interpreted in the following way. It is assumed that "pool glutamate" and "pool alanine" are converted to protein directly but at different characteristic rates. In contrast, the "pool arginine" must accumulate from another "pool" (known in this case to be glutamate) before marked incorporation into protein is noted. Glutamate is therefore denoted a "parental" amino acid, a precursor of another amino acid.

In such a system what might be the effect of adding amino acids to the medium? How would this exogenous source modify the relationships within the cell? Experimentation showed that if the yeast were furnished both fructose- $^{14}\text{C}$  and a parental amino acid, some competition occurred and less fructose carbon was incorporated. On the other hand, once an amino acid was present in a "pool," exogenous supplies did not modify its conversion to protein.

Other experiments led to the conviction that at least three processes contribute amino acids to the pools that supply the building units for proteins. Endogenous (in the cell) formation of parental amino acids, "active" concentration of exogenous amino acids, and diffusion of amino acids into the pools—all were implicated. The model proposed to correlate these results is presented in Fig. 12.6. The *expandable pool* is composed of the amino acids extractable with cold water. It readily exchanges amino acids with the medium; no amino acid interconversions take place; and the composition is highly variable with the environment and growth conditions.

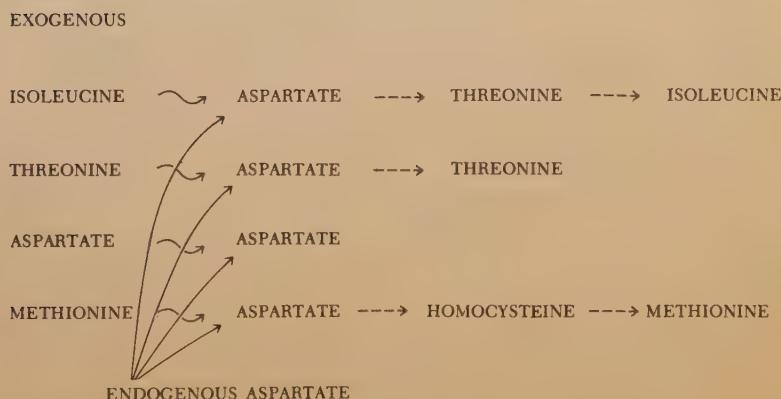
The *internal pool* is thought to consist of amino acids attached in some fashion to macromolecules and to some subcellular structure. The normal interconversions of amino acids, such as glutamate to arginine, are not modified by the presence of these amino acids in the medium. Exogenous amino acids do compete with those from amino acid biosynthesis but do not displace

**FIGURE 12.6**  
proposed model for  
amino acid pool  
relationships in  
*Candida utilis*



any that are already in the pool. It is also postulated that interconversions in this pool are interlinked as in Fig. 12.7. That is to say, the aspartate derived from biosynthesis within the cell may compete not only with the aspartate of the medium but also with amino acids derivable from aspartate. Also, this diagram signifies that isoleucine in the medium competes with the process of isoleucine production from aspartate but does not affect other reactions of aspartate. The validity of this model remains to be determined. Nevertheless, the model serves to emphasize the idea that metabolic reactions take place on organized subcellular structures. It also emphasizes that in addition to the elaborate controls of protein synthesis, to be seen later, there is a complex regulation of the amino acid supply.

**FIGURE 12.7**  
Interaction of amino  
acid sequences and  
supply in the internal  
pool



Metabolism may be thought of as a multiple-coupled system, and amino acid metabolism offers many examples. For pedagogical purposes it is necessary to examine the amino acids one by one, but it is well to remember the multiplicity of relationships.

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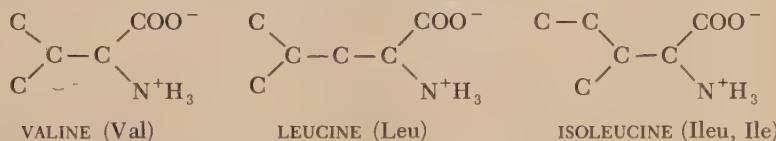
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# 13

## amino acid metabolism, part III

### valine, leucine, isoleucine

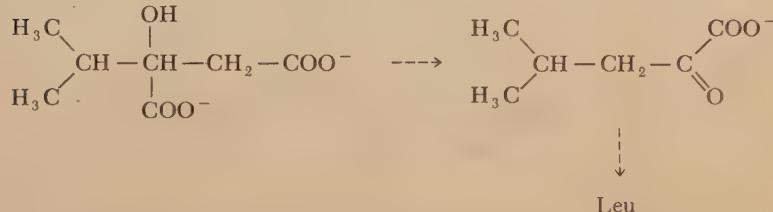
The synthesis of branched aliphatic structures in higher animals is not common. However, the proteins of higher animals, like those of other forms of life, contain three amino acids having branched chains.



The synthesis of valine and isoleucine in *Neurospora* (a fungus) and *E. coli* probably involves the reactions in Fig. 13.1.

The reaction following decarboxylation is similar to a pinacol rearrangement, shown in Fig. 13.2 for comparison.

A synthesis of leucine that has been proposed involves the reaction of the valine precursor  $\alpha$ -keto isovalerate and acetyl CoA to yield the following products:

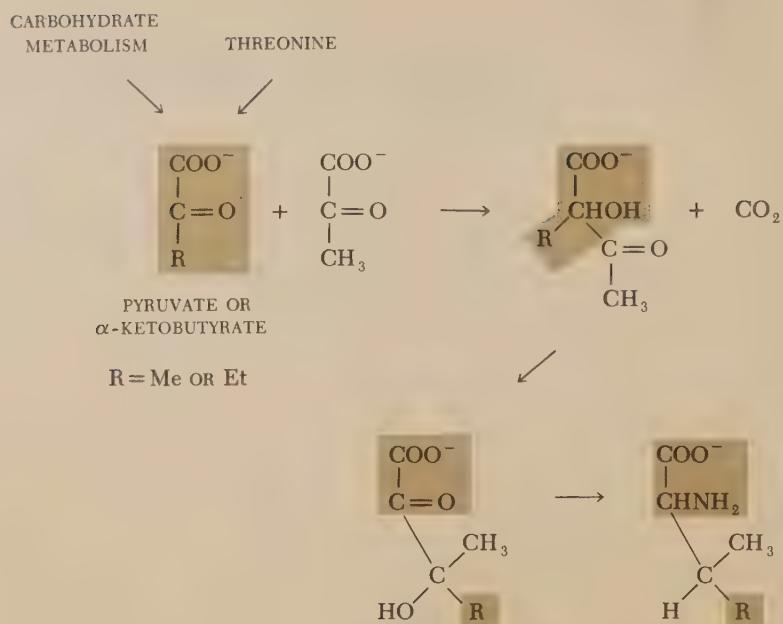


The utilization of these amino acids involves reactions as unexpected as those involved in the synthesis. In Figs. 13.3, 13.4, and 13.5 are outlined the reactions believed to describe the transformations of valine, leucine, and isoleucine in the tissues of higher animals.

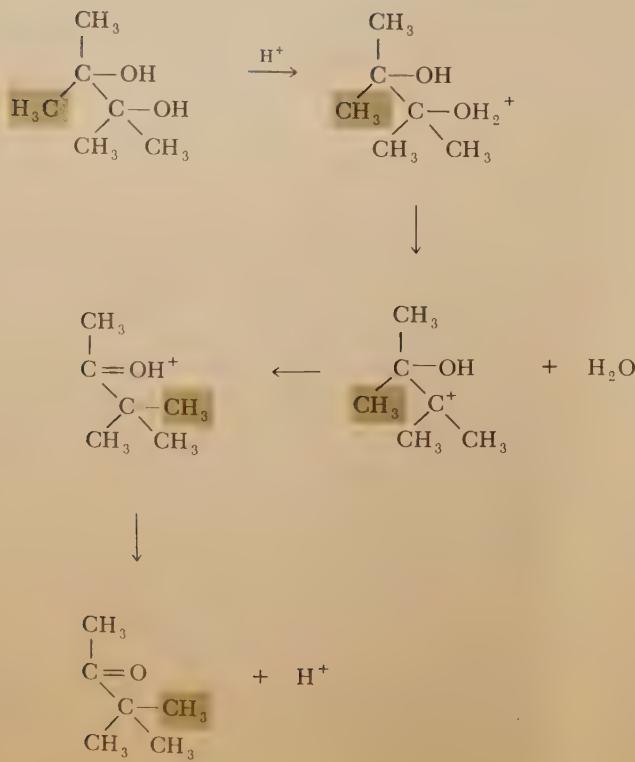
A rather impressive array of reactions seems to be required for the conversion of these branched chains to straight-chain four-carbon compounds. It should be noted that the reaction sequences also branch. Again, some, but not all, steps involve coenzyme A derivatives of acyl compounds as in fatty acid metabolism. The final disposition of carbon from each of these amino acids employs the "machinery" of the citrate cycle, although leucine may be considered a carbon source for fat synthesis.

In Sec. 8 the consideration of fatty oxidation was restricted to acids containing even numbers of carbon atoms. In the following diagrams it is obvious that odd-number carbon atom chains can also be oxidized. In general, the oxidation of an odd-number chain results in acetyl CoA and propionyl CoA. The latter is carboxylated to methyl malonyl CoA, an alternative source of succinyl CoA.

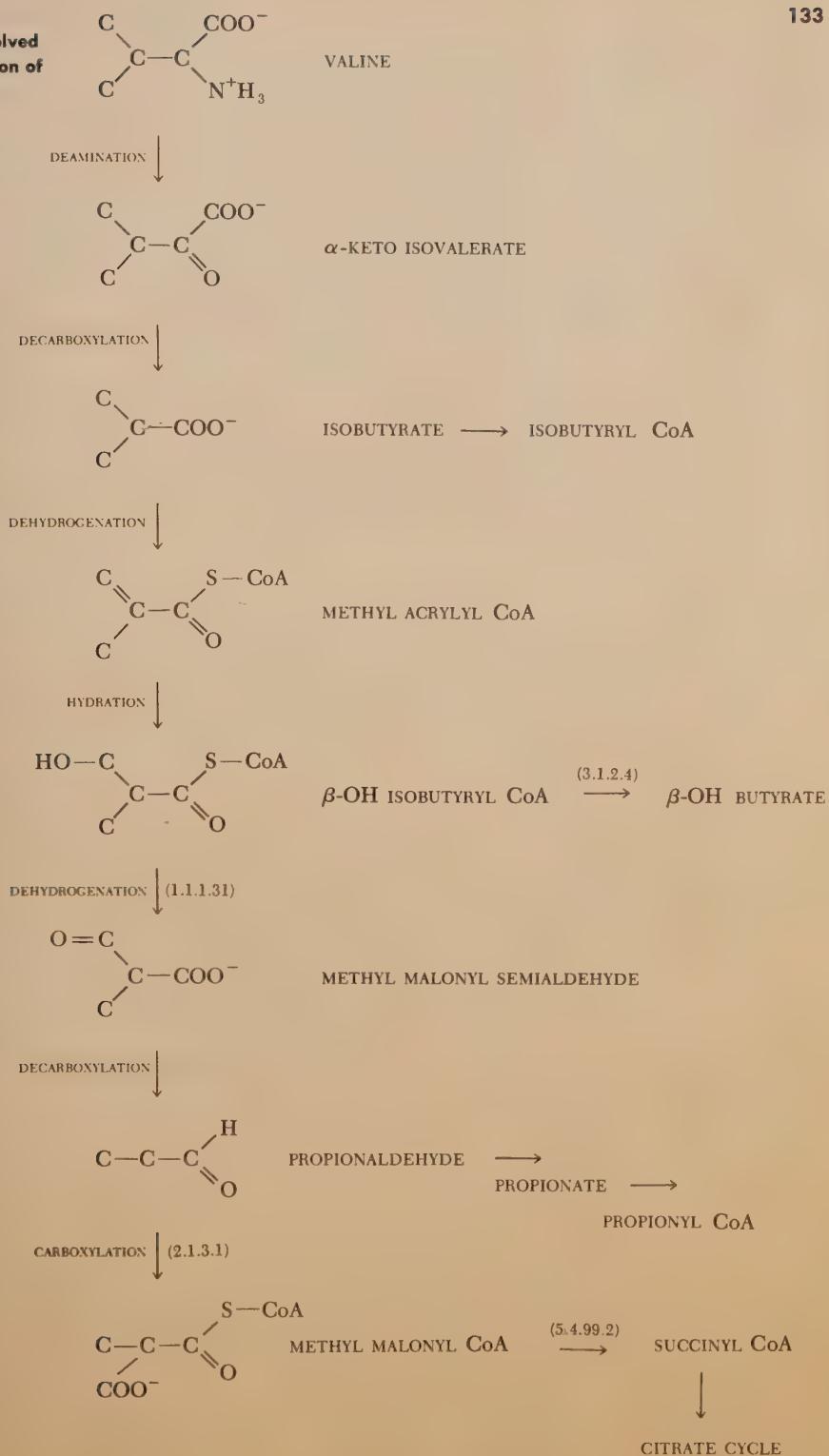
**FIGURE 13.1**  
biosynthesis of valine  
and of isoleucine



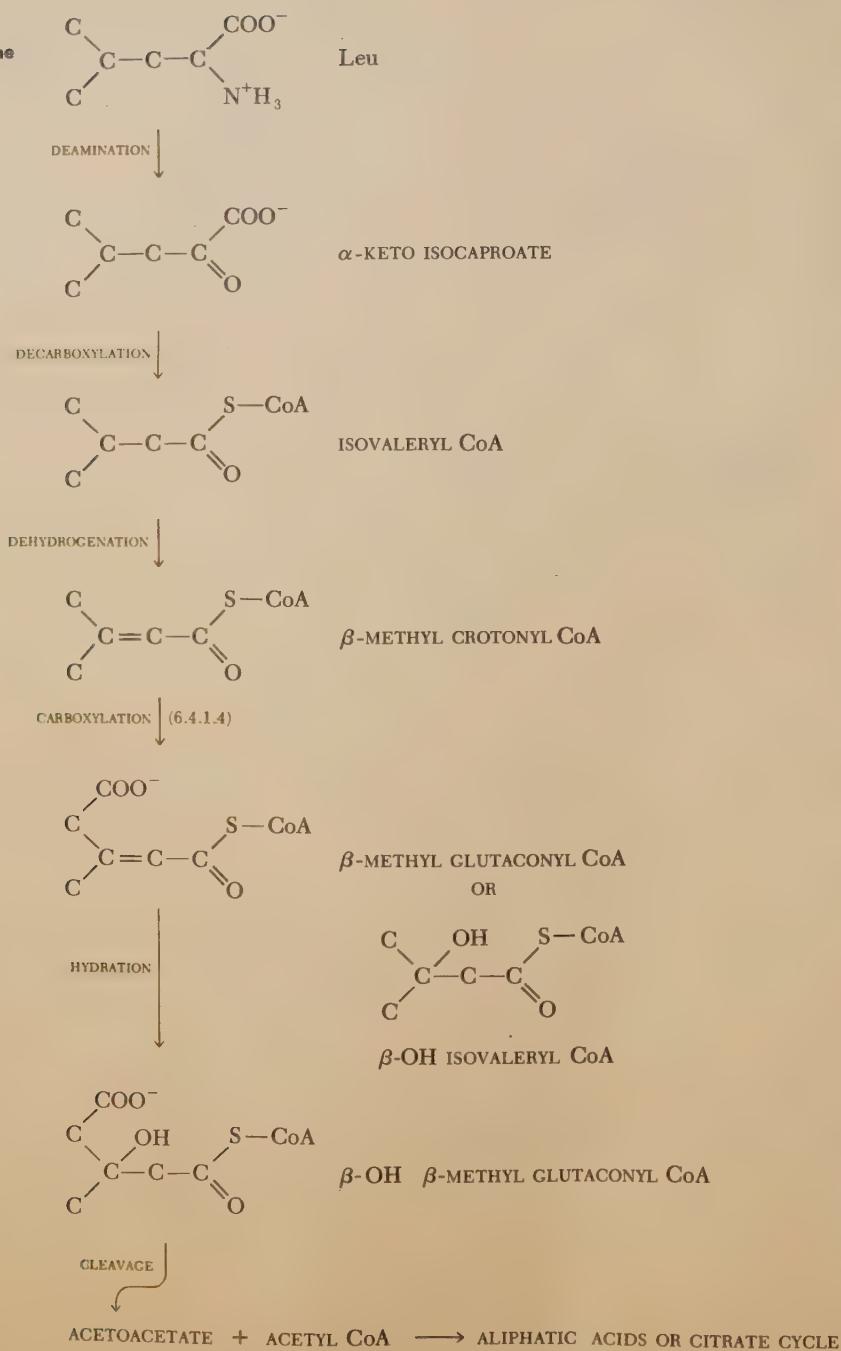
**FIGURE 13.2**  
pinacol  
rearrangement



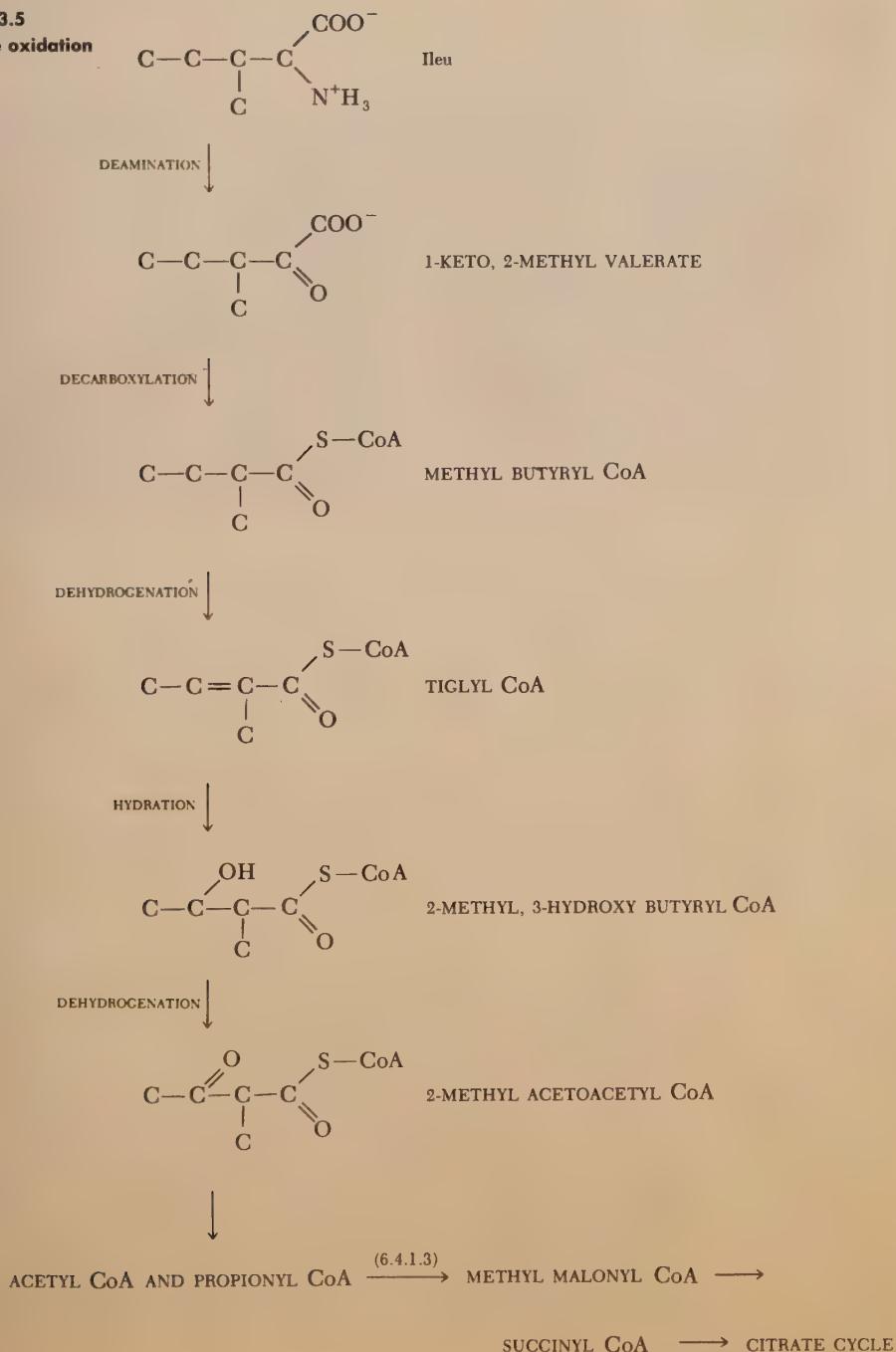
**FIGURE 13.3**  
reactions involved  
in the oxidation of  
valine



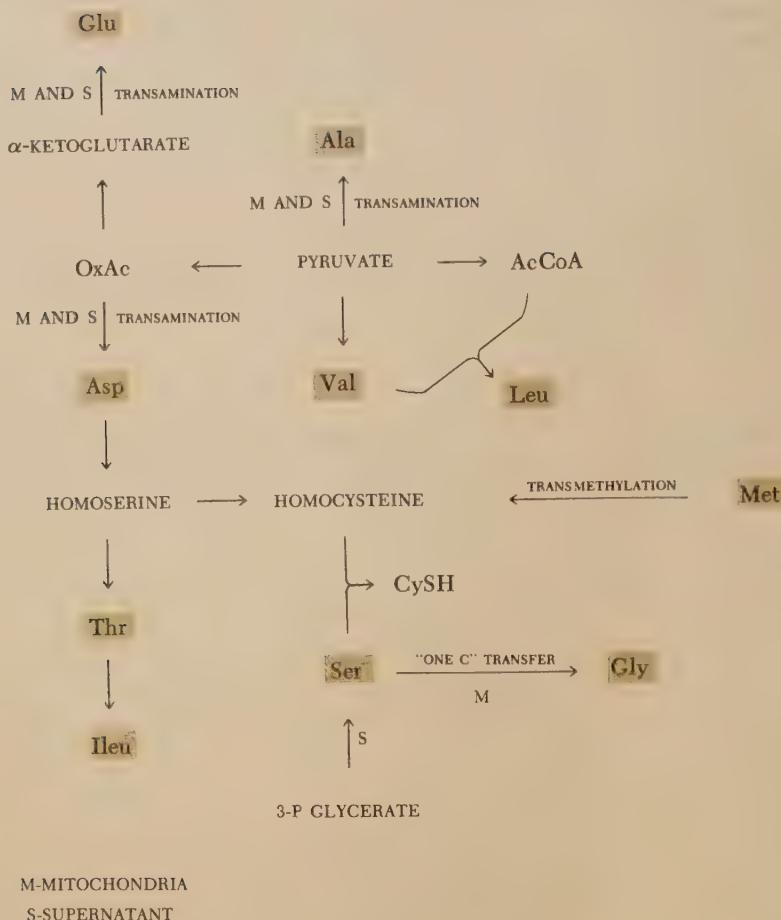
**FIGURE 13.4**  
reactions in leucine  
oxidation



**FIGURE 13.5**  
isoleucine oxidation sequence



**FIGURE 13.6**  
amino acid inter-  
relations



The syntheses of valine and isoleucine are closely coupled, but isoleucine synthesis is seen to require a product derived from threonine. Since the synthesis of leucine also involves an intermediate of valine metabolism, the synthesis of each is affected by the others. Studies on the control of these syntheses have revealed the kinds of feedback that might be anticipated. In bacteria, where these syntheses occur, L-threonine deaminase and the dihydroxy acid dehydrase, which are necessary to the formation of  $\alpha$ -ketobutyrate, are repressed only by the concerted action of valine, leucine, and isoleucine. For a discussion of this topic refer to the final section.

A brief and partial summary of amino acid interrelations is shown in Fig. 13.6. In those cases where a reaction has been demonstrated in a cell fraction it is so indicated.

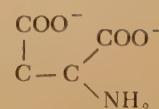
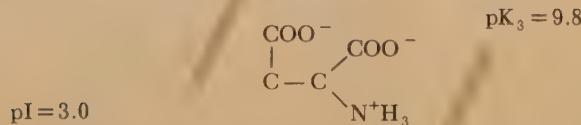
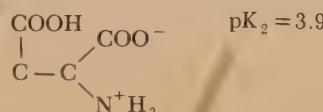
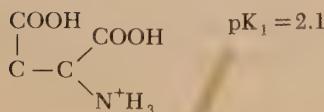
### aspartate and glutamate

The dicarboxylic amino acids, aspartic and glutamic acids, are often juxtaposed as in the scheme of Fig. 13.6. Both derive from the citrate cycle and easily exchange amino groups. Since they differ by only one  $-\text{CH}_2-$  group,

**aspartic and glutamic acids**


it might be thought that they would be chemically nearly indistinguishable. That is not so. Brief examination of three-dimensional models reveals that five carbons permit a greater variety of interactions than four. Empirically it was discovered long ago that glutamate can be separated from mixtures as an insoluble hydrochloride and that aspartate can be isolated as the insoluble copper salt.

The ionic behavior of both is more complex than that of the amino acids considered so far. There are three dissociable groups; hence three inflections in the titration curves and four ionic species are possible.



The amino acids deriving directly from the citrate cycle are certain to be in rapid flux, and the lifetime of an individual molecule in the pool is likely to be brief. In *Chlorella* (an alga) the synthesis of these amino acids appears to occur within the chloroplast. In other photosynthetic organisms this may or may not be so. In Table 13.1 it is evident not only that the first three amino acids are synthesized rapidly but that there is a corresponding uptake of inorganic  $\text{NH}_4^+$  from the medium. (Multiply the figures in the last column by the number of carbons in the amino acid involved.) The rates of labeling for the first four amino acids in this table reach a maximum as soon as those for the intermediates of carbohydrate photosynthesis. This suggests that these four are the amino acids primarily involved in nitrogen incorporation and that other amino acids synthesized derive their nitrogen from them by transamination.

In the animal there is facile transamination from aspartate to  $\alpha$ -ketoglutarate. In the event of a large influx of aspartate, oversaturation of the glutamate pool is prevented by the deaminating action of GLUTAMATE DEHYDROGENASE (1.4.1.3), an enzyme confined to mitochondria. An hypothesis that has been advanced invokes this mechanism for the disposition of most of the nitrogen arising from oxidative deamination. If this hypothesis is valid, the mitochondrion is a pacemaker for nitrogen excretion.

The metabolism of glutamate varies from one cell type to another. The number of transaminases is large, and at least ten amino acids are donors of amino groups to  $\alpha$ -ketoglutarate to form glutamate. In the brain tissue of animals there is an enzyme (4.1.1.15) that catalyzes the decarboxylation of glutamate to  $\gamma$ -amino butyric acid. The singular occurrence of this amino acid, together with its property of inhibiting nerve function, has attracted the attention of those studying brain metabolism. Glutamate and its  $\gamma$ -amide, glutamine, are also found in quantity in the brain, and there is a substantial record of experimentation on this topic as well.

Only one function of glutamate in the brain seems satisfactorily established. Its conversion to glutamine serves to remove ammonia from nerve

**TABLE 13.1**  
**Rates of amino acid synthesis in Chlorella\***

amino acid	calculated rate of synthesis micromole C	uptake micromole $\text{NH}_4^+$
alanine	2.7	0.9
glutamate	1.0	0.2
aspartate	0.9	0.2
serine	0.5	0.2
glycine	0.04	0.02

\* Table based on data from Calvin and Bassham, *The Photosynthesis of Carbon Compounds*. By permission of W. A. Benjamin, Inc. From M. Calvin and J. A. Bassham, *The Photosynthesis of Carbon Compounds*, 1962, Table 1, p. 31.

tissue, where it is generated by metabolism. The fine details of mechanism are unknown. The toxicity of  $\text{NH}_4^+$  is easily demonstrable by direct administration to animals. Rats develop violent convulsions when the concentration of  $\text{NH}_4^+$  in the brain reaches  $6 \times 10^{-3} M$ . For rabbits the lethal concentration in the blood is 5 mg/100 ml ( $3.3 \times 10^{-3} M$ ). It is curious that as long ago as 1922 Tashiro<sup>\*</sup> observed stimulated nerve in vitro to release  $\text{NH}_4^+$ . It may be that the normal sequence of events during passage of a nerve impulse requires an environment buffered toward  $\text{NH}_4^+$ .

**amides** The formation of the amides asparagine (Asn) and glutamine (Gln) requires energy. In the brain, GLUTAMINE SYNTHETASE (6.3.1.2) catalyzes an ATP-coupled reaction localized in the microsomes. No similar reaction for asparagine has been described for animal cells. In plants the relations vary as exemplified in Table 13.2.

It is likely that such numbers are the resultant of several competing processes, but the conclusion is clear: the quantitative relations vary from plant to plant. Asparagine may be regarded as being primarily a plant product, and yet it does occur, as does glutamine, in animal protein. The amide groups of protein can be detected by treatment with concentrated HCl at 37°, under which conditions hydrolysis takes place. The amide nitrogen of glutamine is released by treatment with nitrous acid, but asparagine does not react in this way. The deamidation of glutamine by GLUTAMINASE (3.5.1.2) (mitochondria) is a reaction with  $\Delta G^\circ = -3$  kcal. Both glutaminases and asparaginases are widely distributed.

\*In the experiments reported frog sciatic nerve was used. Unstimulated nerve was found to produce both  $\text{CO}_2$  and  $\text{NH}_3$ , and both volatile metabolic products were released in greater quantity upon stimulation. Tashiro concluded speculatively that the  $\text{NH}_3$  was produced directly from a protein reaction.

TABLE 13.2  
amide formation  
in plants\*

compound	Relative radioactivity found in:	
	wheat leaves	bean leaves
	supplied with	supplied with
aspartate	—	32
asparagine	50	26
glutamate	75	4
glutamine	75	10
alanine	1	34

By permission of F. C. Steward and the Elsevier Publishing Co., Amsterdam. From F. C. Steward and R. G. S. Bidwell, in *Amino Acid Pools*, J. T. Holden (ed.), 1962, Table 1.

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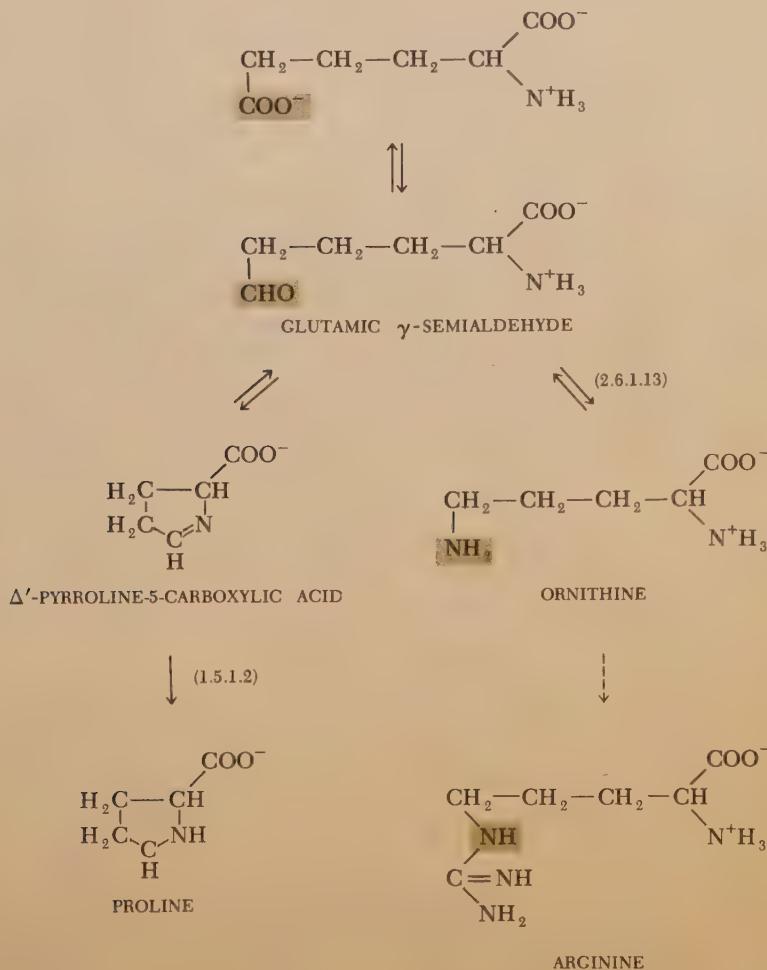
# 14

## amino acid metabolism, part IV

### arginine

As noted in the discussion on amino acid pools, arginine (Arg) is a metabolic derivative of glutamate. It is often referred to as a basic amino acid, basic in the sense that the pI is 10.8. This high value reflects the presence of the guanidino group having a pK value of 12.5. The pK values of the carboxyl and amino groups are 2 and 9, respectively. The five-carbon chain joined to the guanidino group is derived from glutamate and is also related to proline (Pro) as shown in Fig. 14.1. In some organisms the intermediates between glutamate and ornithine are *N*-acetyl derivatives.

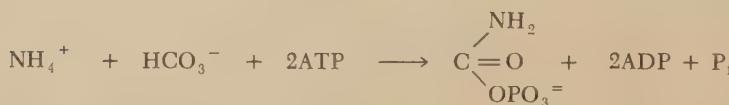
**FIGURE 14.1**  
derivation of arginine  
and proline from  
glutamate



**urea synthesis**

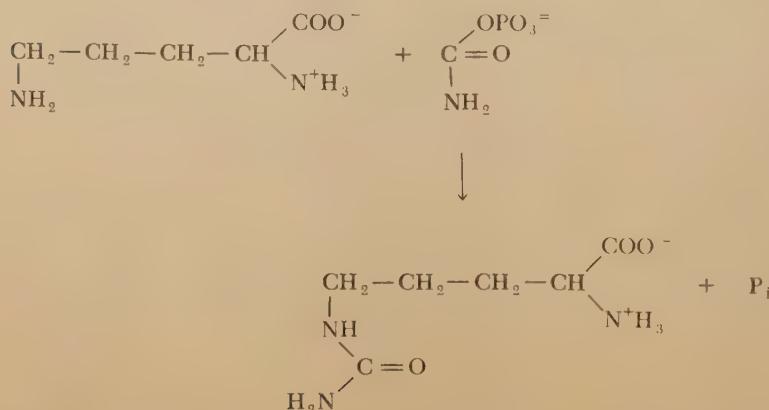
It was stated previously that in animals there is a continual nitrogen loss. The main excretory product is urea, and arginine is the immediate precursor. The formation of urea is a continuing activity in the liver. In many animals the urea is moved out of the liver into the circulatory system and continually leaves the circulation through the kidney tubules. The blood level of urea in mammals is low, although urea is not toxic even at fairly high levels. (Difficulties accompanying uremia are usually due to kidney failure rather than the deleterious effects of urea.) In some aquatic animals urea is a component of a physiological mechanism that aids in adjusting the internal osmotic relationship and minimizing environmental pressures.

Current opinion holds that the nitrogen of amino acids being utilized for energy, or for some kinds of syntheses, is transferred by transamination to  $\alpha$ -ketoglutarate, forming glutamate, which in turn is deaminated by liver glutamate dehydrogenase. Since the ammonia level of the liver, as well as of other tissues, is low, it may be presumed there is a facile conversion of the ammonia into some other compound. The livers of ureotelic (urea-forming) animals contain the enzyme CARBAMYL PHOSPHATE SYNTHETASE (2.7.2.5) that catalyzes the reaction



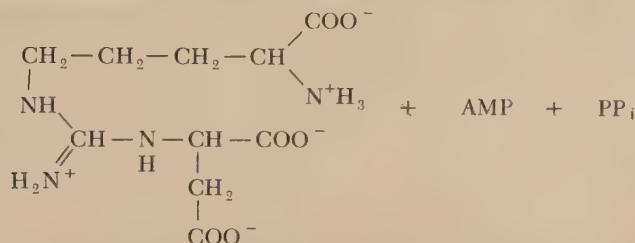
The mechanism of this reaction, irreversible as shown and requiring acetyl glutamate as a cofactor of unknown function, has not yet been determined. It is inhibited by sodium ions and accelerated by potassium ions. The  $K_m$  values for ATP, acetyl glutamate, and  $\text{NH}_4^+$  are each about  $10^{-4} \text{ M}$ .

In the scheme in Fig. 14.1 ornithine (not a protein component) is seen to derive from glutamate. This compound is an acceptor for carbamyl phosphate, and the product formed has the trivial name of citrulline.



The enzyme catalyzing this reaction, CARBAMYL TRANSFERASE, (2.1.3.3) occurs in a wide variety of tissues. Citrulline, like ornithine, is not incorporated into protein, and for both the steady-state concentration is probably quite low.

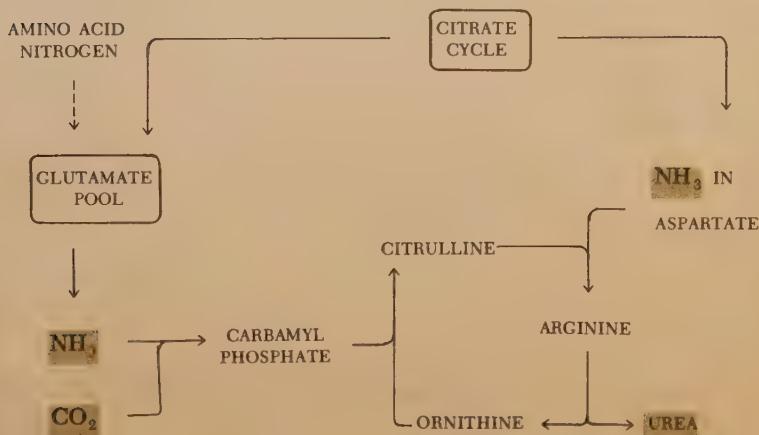
Citrulline interacts readily (6.3.4.5) with aspartate and ATP to form arginino succinic acid:



This compound is formed in a variety of tissues and is in equilibrium with arginine and fumaric acid. However, where ARGINASE (3.5.3.1) is present, as in liver, and arginine is continually being hydrolyzed to ornithine and urea, arginino succinic acid is continually dissociated. These reactions are summed up in Fig. 14.2. The process is cyclic with respect to carbon except for the bicarbonate that becomes part of the urea. Inspection of the equations shows that two  $\text{ATP} \rightarrow \text{ADP}$  and one  $\text{ATP} \rightarrow \text{AMP}$  transformations are coupled, since urea synthesis requires energy. All these reactions can be demonstrated within the liver mitochondrion except the arginase reaction. Arginase is found within nuclei and microsomes, but it occurs mainly in the supernatant cell fraction. It is not certain yet that the citrulline-to-arginine reaction occurs *only* within mitochondria. It is also an interesting observation that all metazoan<sup>\*</sup> cells *in culture* require either citrulline or arginine for growth. Carbamyl phosphate is an intermediate not only in urea synthesis but also in the biosynthesis of pyrimidines (Sec. 17) via carbamyl aspartate (ureido succinate). This pathway represents a competing reaction opportunity for amino acid nitrogen, especially during growth or regeneration. The incorporation of amino acid nitrogen into the ring structure of pyrimidines contributes to the positive nitrogen balance characteristic of young animals.

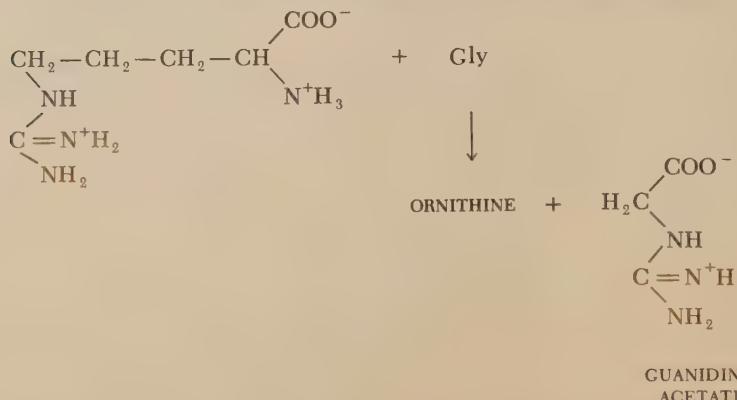
\*The metazoa includes all animals except protozoa. Hence such animals are characterized by differentiated cells.

**FIGURE 14.2**  
urea formation in  
liver

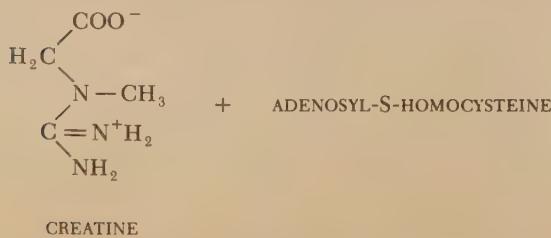


The comparative biochemistry of nitrogen excretion is a well-studied topic. It reflects a richness of variation that contrasts with the unity characterizing some other aspects of metabolism. Nitrogen is excreted in several compounds; some organisms excrete NH<sub>3</sub>, some urea, some uric acid, and some allantoin. The details must be sought elsewhere, preferably in works treating both the physiological and biochemical aspects of the subject.

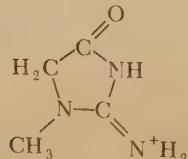
**creatinine formation** In addition to urea another nitrogenous compound is continually excreted in the urine of some animals, including man, and it also is derived from arginine. This compound, creatinine, is the end product of the ensuing processes. First, in the kidney there is a TRANSAMIDINASE (2.1.4.1) which catalyzes



Next, in the liver, to which it is carried by the circulatory system, guanidino acetate becomes an acceptor for the labile methyl group in adenosyl methionine. Hence there is formed

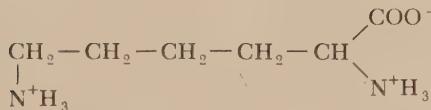


In muscle, creatine interacts with the ATP/ADP system and creatine phosphate results. This is the source of the cyclic anhydride, creatinine,

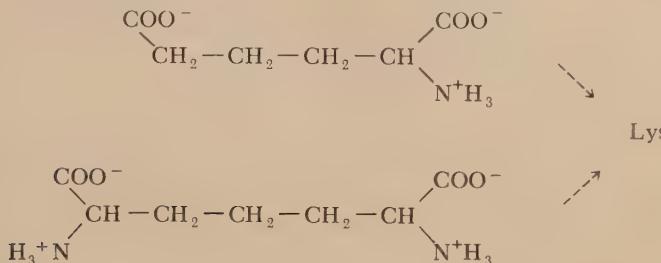


that is produced continually. The amount excreted per day can be correlated with muscle mass.

**lysine** Lysine (Lys) is also referred to as a basic amino acid, since the  $\epsilon - \text{NH}_3^+$  group does not lose its charge until a  $\text{pH} > 10$  has been reached. This group is often referred to verbally by protein chemists as an "epsilon amino group," but the student should keep in mind that the *charged* form is usually the one involved.

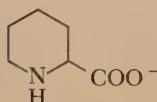


The synthesis of lysine differs with the organism. In fungi the precursor is  $\alpha$ -amino adipic acid; in bacteria it is  $\alpha, \epsilon$ -diamino pimelic acid.

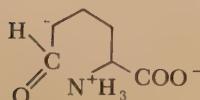


Neither precursor is found in proteins, but the latter is a characteristic component of many bacterial cell walls (Sec. 30).

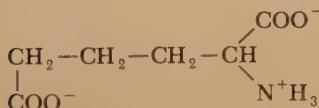
It was stated in a preceding section that lysine must be furnished animals as such; that is, the keto analog is insufficient. Moreover,  $^{15}\text{N}$  is not incorporated into lysine by transamination from other labeled amino acids, nor does deamination occur by a previously described process. It is known that the product, presumably  $\alpha$ -keto,  $\epsilon$ -amino caproate, is converted into the ring-containing compound, pipecolic acid,



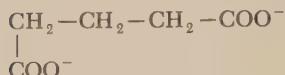
further transformed to  $\alpha$ -amino adipic semialdehyde,



thence to  $\alpha$ -amino adipate,



in turn to keto adipate, and finally to glutarate,



The carbon of glutarate eventually enters the pool of acetyl CoA.

Many practical nutritional problems are posed by the need for lysine. It is essential for the good health of humans, and yet the percentage is very low in all cereals. This is a matter for concern, since cereals form the bulk food supply of the world population. At one time it was proposed to add synthetic lysine to flour to be shipped abroad as a food supplement. Unfortunately, this remedy was shown to be inadequate. It is necessary to maintain a certain lysine-tryptophan ratio, and unless the entire diet is prescribed, the use of an amino acid supplement is unsatisfactory.

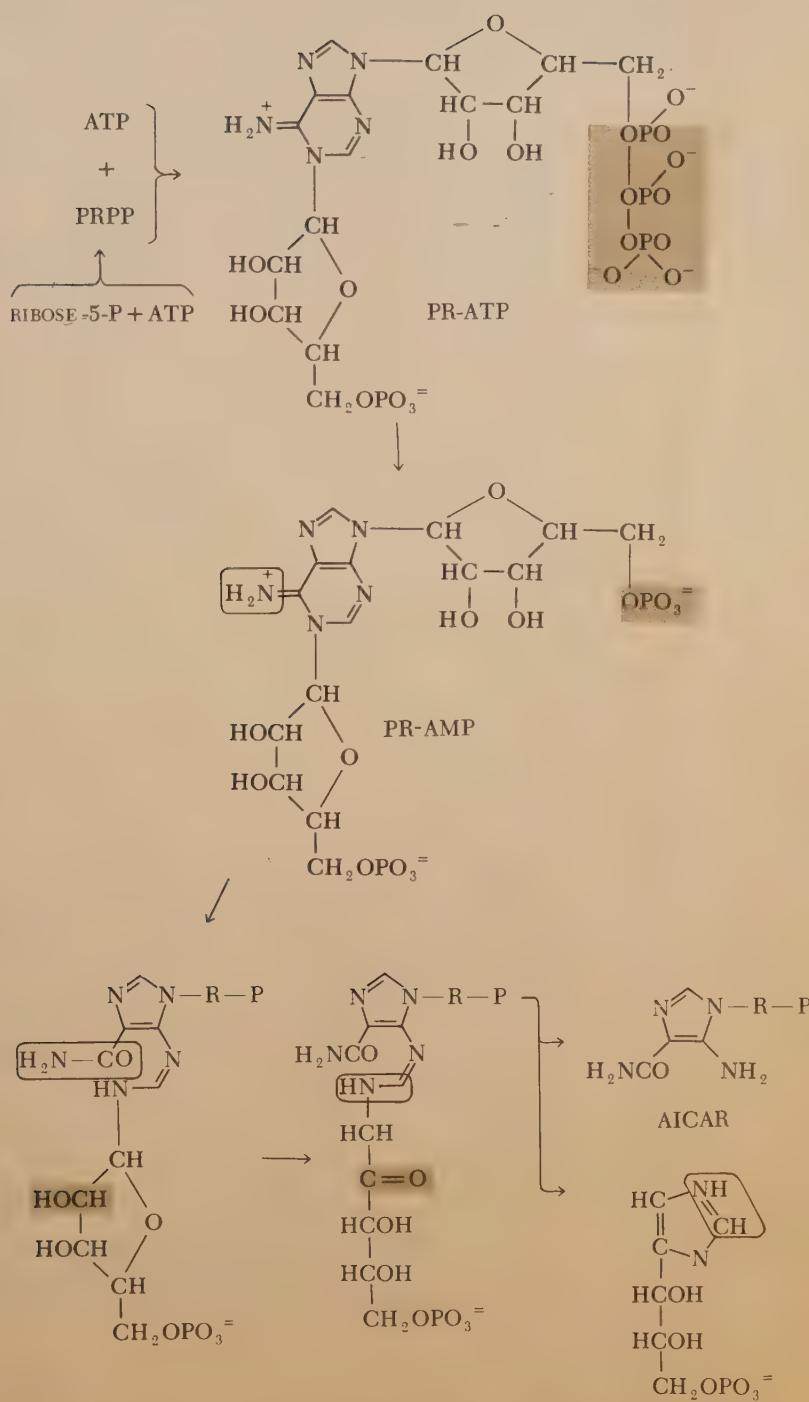
### histidine

Histidine (His), like arginine and lysine, contains six carbon atoms and has an isoelectric point (7.6) greater than 7. Like cysteine, it has received a good deal of attention because of its suspected participation in enzyme catalysis.

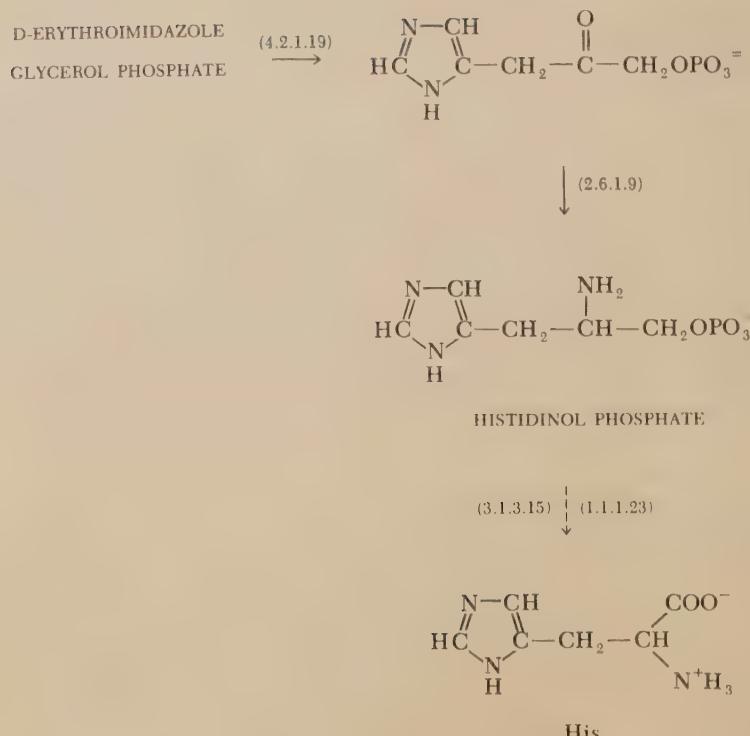
The reaction sequence that yields histidine is known with reasonable certainty, and part of it is sketched in Fig. 14.3. The synthesis is unusual in that the amino acid carbon is furnished by a purine. A derivative of ATP, phosphoribosyl ATP, results from the reaction with phosphoribosyl pyrophosphate (PRPP). After the loss of a pyrophosphate group, the pyrimidine ring is opened and an isomerase catalyzes the production of a ribulosyl derivative. This intermediate accepts a nitrogen from glutamine and two products are formed: AICAR (Sec. 17), an intermediate in purine synthesis, and imidazole glycerol phosphate. In Fig. 14.4 is shown the remainder of the reaction sequence. The imidazole glycerol phosphate is dehydrated to the acetol phosphate, which in turn participates in a transamination reaction to form histidinol phosphate. After the loss of the phosphate group and an oxidation requiring two equivalents of NAD, the synthesis of histidine is completed. This is a most unexpected concatenation of reactions involving purines, pentoses, phosphorylation, transamination, and oxidation. Studies of "histidine-less" mutants have shown that some of the intermediates postulated actually do accumulate. As a result, some of these reactions are well established. A reasonably complete description of histidine synthesis has been given in view of its relation to the operon hypothesis. Studies of this synthesis indicate polarity (Sec. 31). In brief, this system has provided evidence that the synthesis of the enzymes involved takes place serially in the same order as the reaction sequence and that the structural genes are ordered in a linear fashion corresponding to the reaction sequence.

The knowledge of histidine metabolism in animals is strewn with uncertainties. Histidine is essential in the diet of growing animals, but it is not essential for adults. Presumably the growing animal has such a continual requirement for a high rate of purine synthesis (for nucleic acids) that insufficient intermediates are available for histidine synthesis. Free histidine is

**FIGURE 14.3**  
histidine synthesis in  
*Salmonella*



**FIGURE 14.4**  
formation of histidine  
from imidazole  
glycerol phosphate



rapidly oxidized in tissues by a series of reactions, the stoichiometry being represented as



Several histidine derivatives may be demonstrated in various tissues. Of these, perhaps the best known is histamine, formed by the action of HISTIDINE DECARBOXYLASE (4.1.1.22). Histamine is both a vasodepressor and a stimulator of gastric juice secretion. It is involved in allergic responses and in local inflammation, is liberated in the syndrome called *shock*, and is at least one of the substances implicated in the phenomenon of pain. The use of antihistamines, of course, is well known, although their mechanism of action may have nothing to do with histamine.

**comment**

In the amino acid relationships outlined in Secs. 11 to 13 many references are made to carbohydrate and fat metabolism, or to methyl transfer. In the case of the basic amino acids there are relationships to ring systems. Two intermediates, carbamyl phosphate and phosphoribosyl pyrophosphate, are also involved in pyrimidine and purine syntheses. The possible catalytic function of the imidazole group was touched upon in Sec. 2.

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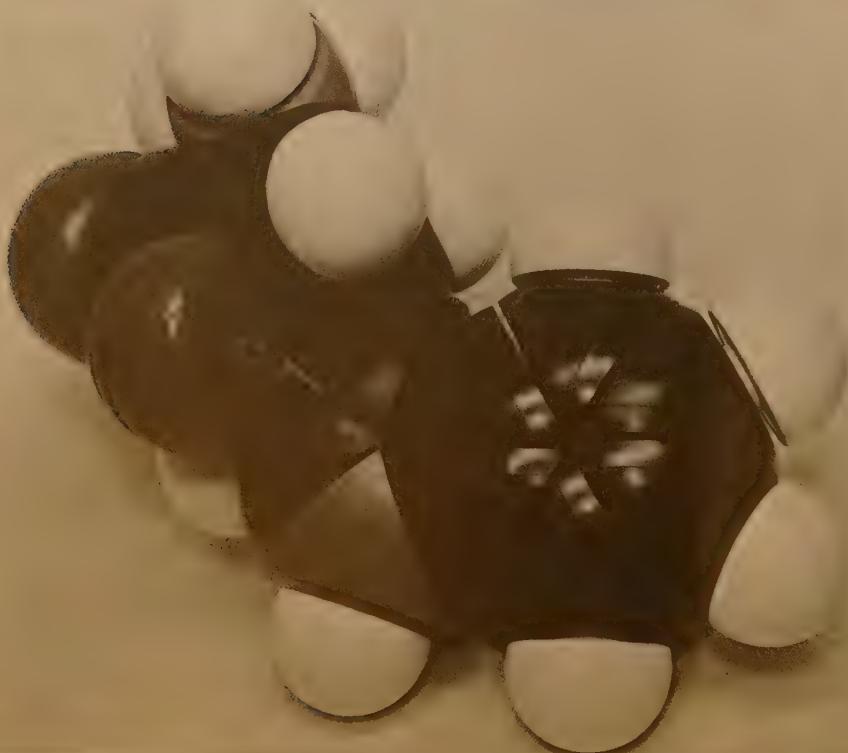
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# 15

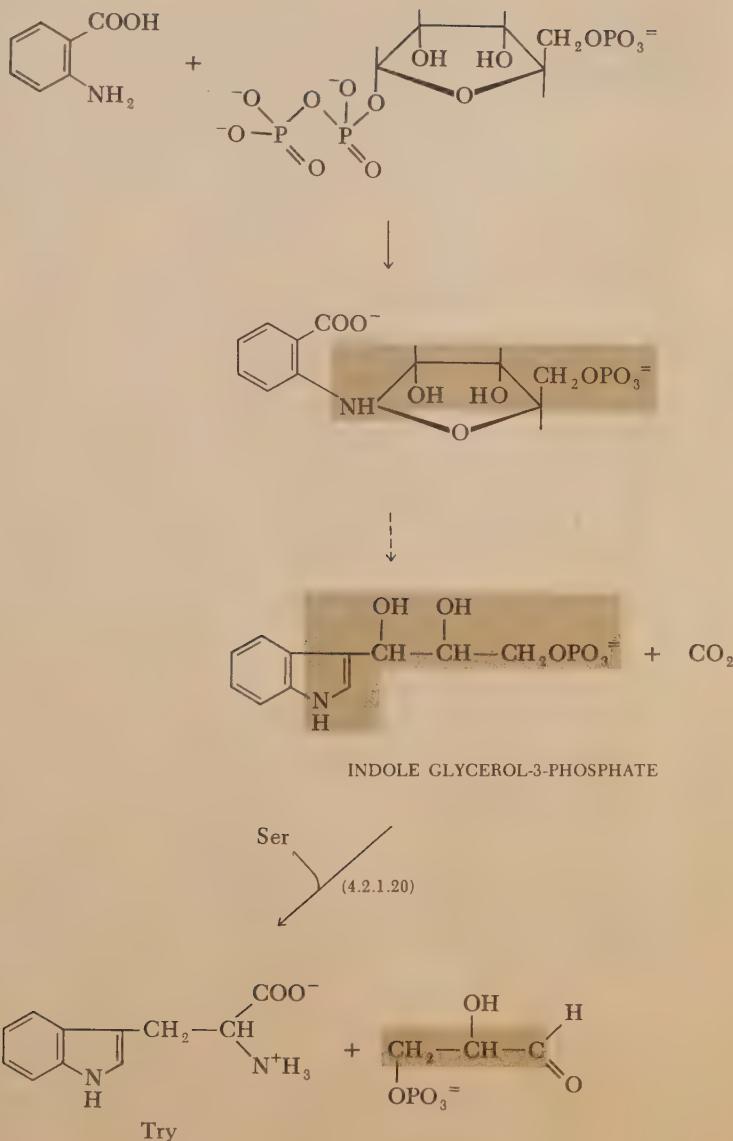
## amino acid metabolism, part V

Tryptophan (Try) (Trp) was discovered in 1901 in the laboratory of Frederick Gowland Hopkins, one of the great figures in biochemistry and responsible for the growth of a school of biochemical thought at Cambridge University. Tryptophan was early recognized as an essential component in animal diets. Its synthesis in *Neurospora* and in bacteria has been extensively investigated. The most direct precursor of the benzenoid ring in tryptophan is anthranilic acid, which is synthesized, together with several similar compounds, from carbohydrate (Figs. 15.4 and 15.6). As outlined in Fig. 15.1, anthranilic acid is condensed with 5-phosphoribosyl 1-pyrophosphate (PRPP) to form an intermediate that undergoes decarboxylation and ring closure. The indole glycerol 3-phosphate resulting participates in an unusual reaction in which the side chain is exchanged for a molecule of serine (not deaminated). The enzyme that catalyzes this reaction, TRYPTOPHAN SYNTHETASE (4.2.1.20), appears

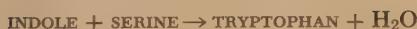
tryptophan



**FIGURE 15.1**  
biosynthetic reactions  
in tryptophan  
synthesis



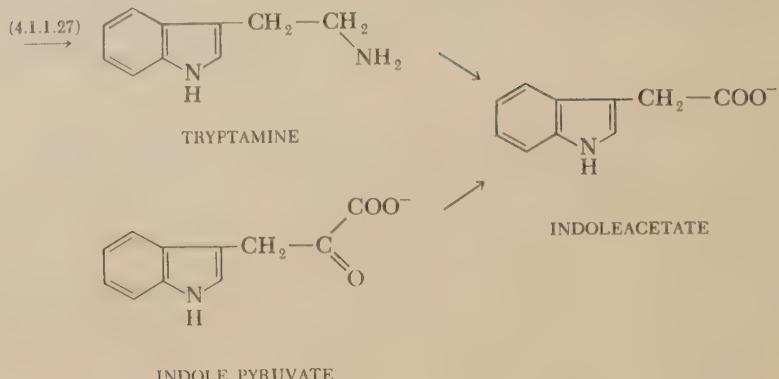
to catalyze two other reactions:



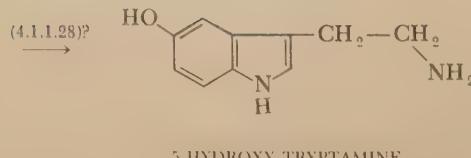
Pyridoxal phosphate is a coenzyme for the reactions resulting in tryptophan; it stimulates the latter (aldolytic) reaction presumably by affecting the conformation of the protein. The *E. coli* enzyme has two protein components; the *Neurospora crassa* enzyme one. The enzyme has been used to study the effects of mutation on its amino acid sequence and will be discussed in Sec. 23.

**FIGURE 15.2**  
tryptophan derivatives involving the side chain

## PLANTS AND SOME BACTERIA

SOME BACTERIA, INCLUDING *E. coli*

## ANIMALS

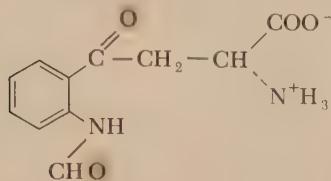


5-HYDROXY TRYPTAMINE

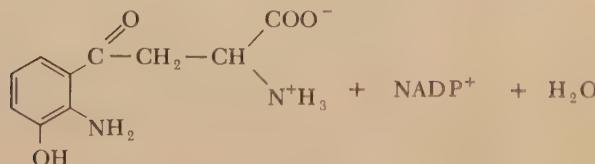
The intermediary metabolism of tryptophan is richly varied, and several of the intermediates have remarkable physiological properties. The modifications involving only the side chain are noted in Fig. 15.2. Indoleacetate has been the subject of much investigation because it acts as a plant growth hormone. The formation of indole in bacteria by TRYPTOPHANASE action is of interest, since this activity is reciprocal with that of tryptophan synthetase and presents an example of a metabolic control mechanism. The trivial name of 5-hydroxy tryptamine is serotonin. This substance affects intestinal motility, nerve function, and kidney function; it produces vasoconstriction in shock; and it is believed to be an active agent in immune reactions. Although a decarboxylase for 5-hydroxy tryptophan exists in various animal tissues, some doubt about the mechanism of serotonin formation remains.

**Try ring cleavage**

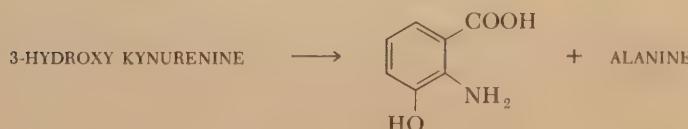
The main oxidative pathway for tryptophan in animals is probably the following. Ring cleavage is promoted by the enzyme TRYPTOPHAN OXYGENASE (PYRROLASE) (1.13.1.12). (That is, molecular oxygen is incorporated into the product, *N*-formyl kynurenone.)



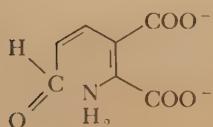
This enzyme is widely distributed in animals and is found in insects, bacteria, and fungi. In the liver it is found in the supernatant fraction and not in the subcellular particles. The formyl group is hydrolyzed in the presence of FORMAMIDASE (3.5.1.9) (also in the supernatant fraction of liver preparations). The next reaction, catalyzed by KYNURENINE-3-HYDROXYLASE (1.14.1.2) (mitochondrial) is extraordinary in that both NADPH and O<sub>2</sub> are required.



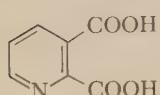
A scission of alanine from the aromatic ring follows, potentiated by KYNURENINASE (3.7.1.3) (found in liver, fungi, bacteria), for which pyridoxal phosphate is a cosubstrate.



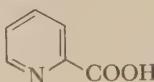
The 3-hydroxy anthranilic acid formed undergoes a ring cleavage catalyzed by an OXYGENASE (1.13.1.6) (isolated from liver) with the formation of 2-acrolyl-3-amino fumarate (2-amino-3-carboxymuconate semialdehyde).



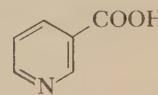
This intermediate is believed to be the precursor of the ring compounds



QUINOLINIC



PICOLINIC



NICOTINIC ACID

At present it is contended that the cyclization of 2-acroleyl-3-amino fumarate to quinolinic acid is nonenzymic. If this is correct, it is exceedingly important, since the question of control is involved. Control may derive from the relative velocities of the two reaction paths to picolinic and nicotinic acids. The action of **PICOLINIC CARBOXYLASE** results in a series of compounds including  $\alpha$ -amino muconic acid and glutaryl CoA (Fig. 15.3) terminating in acetyl CoA.

### NAD synthesis

Competing with this pathway is the condensation of quinolinic acid with phosphoribosyl pyrophosphate (ribose-5-P transfer) in the presence of a **TRANS-PHOSPHORIBOSYLASE** found only in liver and kidney. The desamido derivatives of NMN and NAD are formed, and the latter is converted to NAD in the presence of **NAD SYNTHETASE**.



Nutritional studies long ago showed that animals do not require the nicotinamide portion of the vitamin B complex if an appropriate diet is fed. The evidence for the direct relationship between tryptophan and nicotinamide (or niacin) is now at hand.

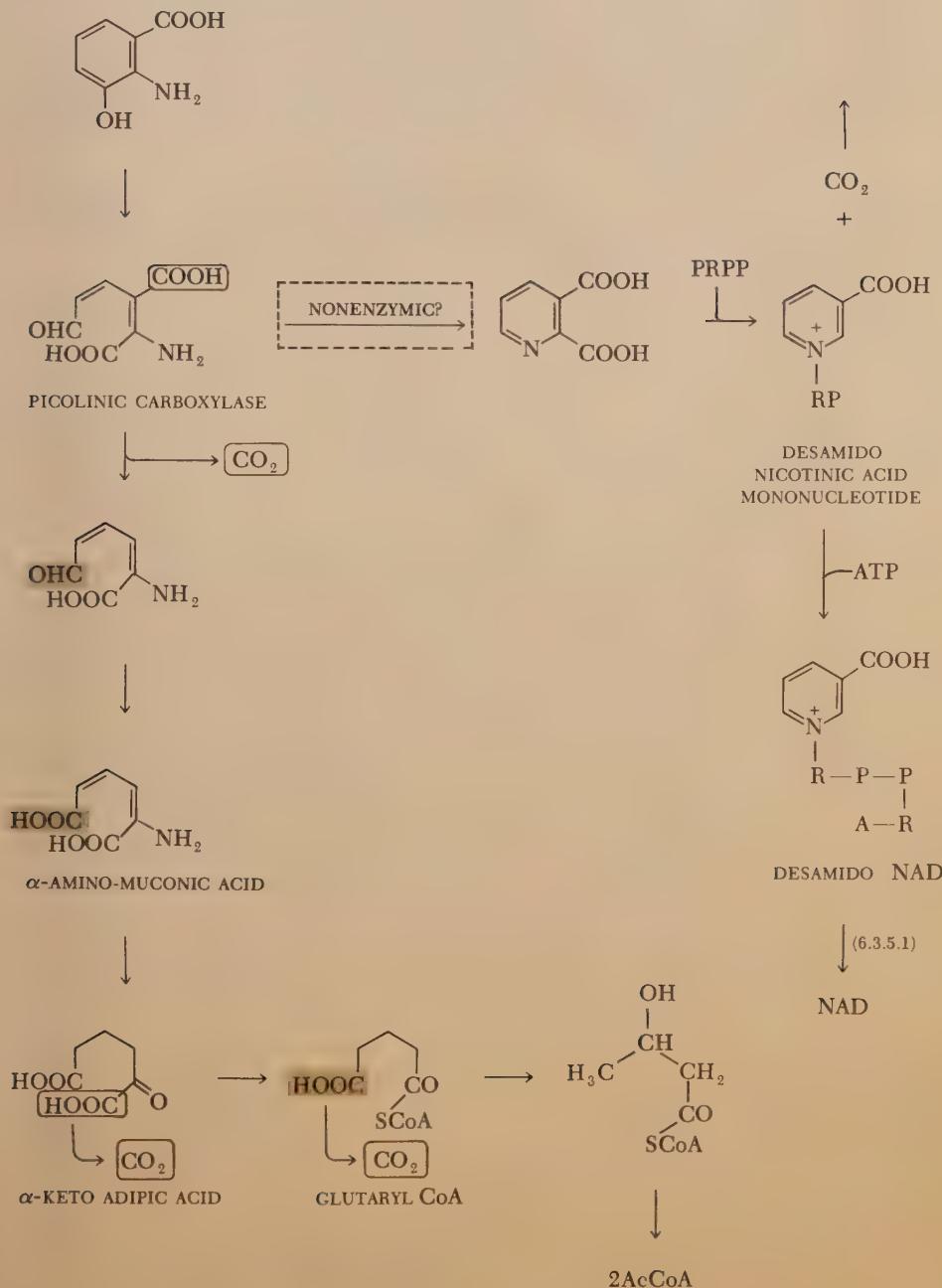
In most tissues the bulk of tryptophan carbon does not form ring compounds. The  $\alpha$ -keto adipate formed merges with that arising from lysine oxidation, and the carbon enters the acetyl CoA pool. Other reactions more strictly species-specific will be considered later.

### aromatic acid syntheses

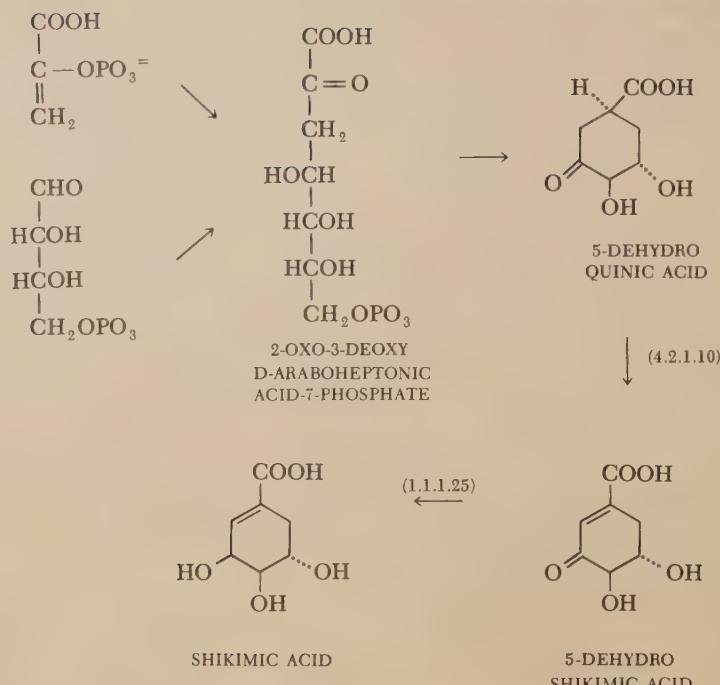
The syntheses of the aromatic amino acids phenylalanine (Phe) and tyrosine (Tyr) involve some of the precursors of tryptophan. A series of *E. coli* and *A. aerogenes* mutants showing growth requirements for either tyrosine, phenylalanine, tryptophan, or para amino benzoic acid has been isolated. It was found that the growth requirements for each of these different mutants could be met by a single compound, shikimic acid. This intermediate is a “branch point” in the disposition of carbon derived from carbohydrate in mutant microorganisms<sup>o</sup> (Fig. 15.5). Shikimic acid accumulates in the culture

<sup>o</sup>There are several techniques for isolating mutants; the penicillin-enrichment method is historically important and much employed. It is based on the observation that penicillin kills only growing cells. In executing the method, a bacterial culture is irradiated to induce mutation, transferred to enriched media, and again transferred to a minimal medium lacking a nitrogen source. After a few hours incubation, growth of the wild-type (nonmutant) bacteria is encouraged by adding a source of nitrogen, and penicillin is added to kill the nonmutants. This procedure results in a culture containing no viable cells except mutants, which may now be grown on an enriched medium.

**FIGURE 15.3**  
**reactions stemming**  
**from 3-hydroxy-**  
**anthranilate in**  
**mammalian liver**



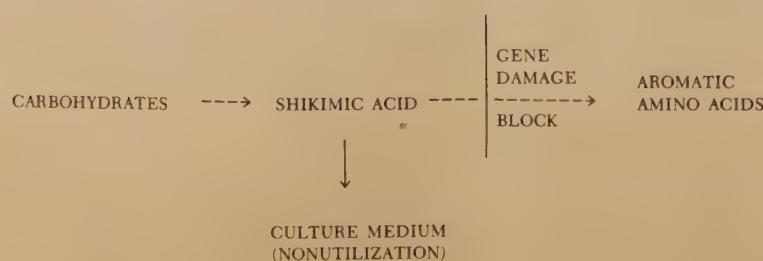
**FIGURE 15.4**  
reactions in the  
synthesis of shikimic  
acid from glucose



filtrate of cells that require the aromatic amino acids for growth. Figure 15.5 represents a generalization which has been found useful experimentally. Examination of mutant-culture filtrates, chromatographically or otherwise, has led to the recognition of intermediates normally present only in small quantity inside the cell. The careful exploration of this technique has helped to determine the relations shown in Fig. 15.6.

In plants, shikimic and prephenic acids are the precursors of aromatic alcohols (Fig. 15.7). These intermediates accumulate briefly during early growth in a few instances, and they have been shown to undergo dehydrogenation to lignin. The term lignin, like starch, is a generic one and includes several variations on a theme. Despite substantial gains in knowledge, the structure of the polymeric variations of lignin cannot be written with confidence. A recent suggestion by Freudenberg is pictured at the bottom of Fig. 15.7. About 25% of dry wood is lignin, found in the cellulose fibril matrix of cell walls and in the intercellular spaces. It is bonded to polysaccharides

**FIGURE 15.5**  
diversion of inter-  
mediates by genetic  
change



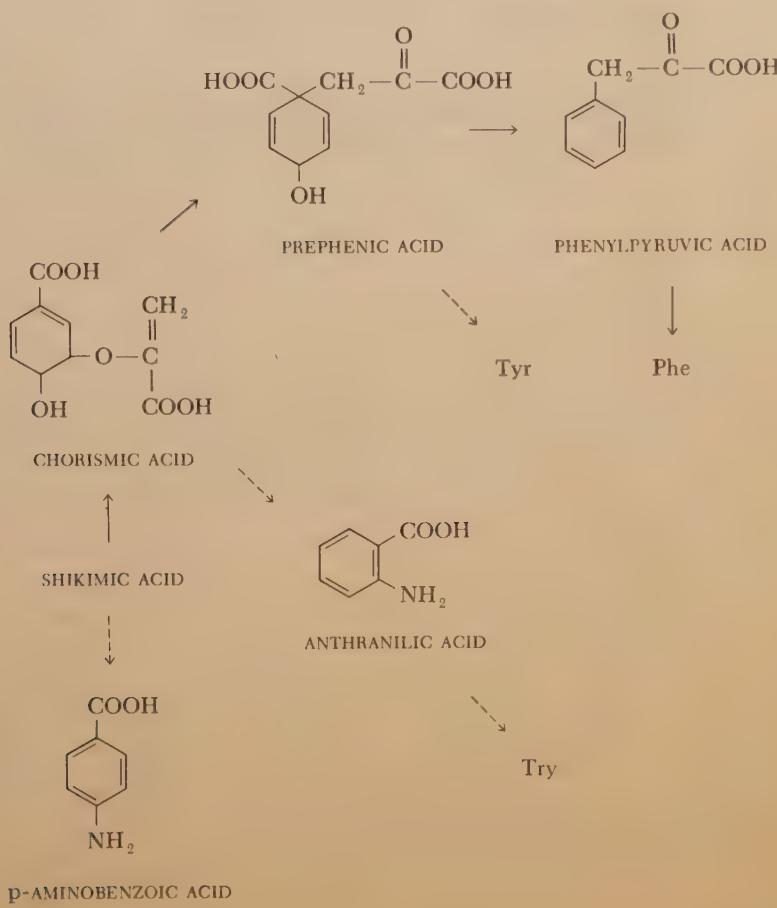
in various ways but can be dissociated at low pH. The metabolic significance of the coupling of lignin formation and polysaccharide formation is obscure.

That gene damage may result in the excretion of metabolic intermediates may be observed in animals as well as in microorganisms. An important part of nineteenth century medicine was urine examination as a part of laboratory diagnosis. Methods were eventually developed for the analysis of blood, so that the cardinal position of urinalysis changed. However, urine examination occasionally revealed unexpected compounds, and these were, in some cases, related to hereditary disorders. Today the routine examination of urine samples for unusual components is done chromatographically.

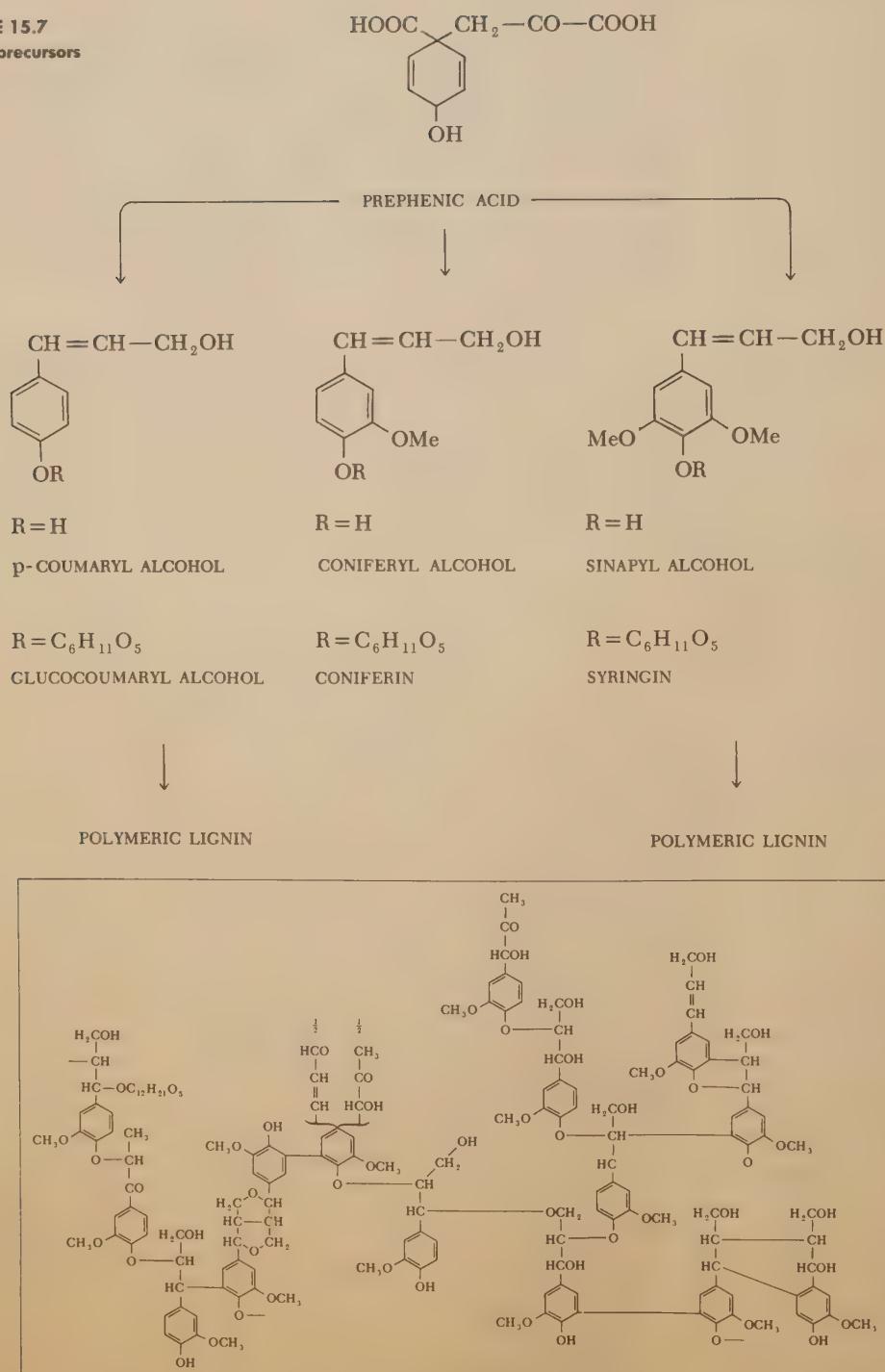
### **phenylketonuria**

The excretion of phenylpyruvic acid (phenylketonuria) is recognized as an ominous sign. Children excreting this substance are found to be not merely unwell but, distressingly, unable to develop mentally. The condition, phenylpyruvic oligophrenia, is characterized by lack of the enzyme that normally converts phenylalanine irreversibly to tyrosine. Normally, adequate phenylalanine can replace tyrosine in the diet; the reverse is not true. A liver enzyme

**FIGURE 15.6**  
biosyntheses of  
aromatic amino acids  
and p-aminobenzoic  
acid



**FIGURE 15.7**  
lignin precursors

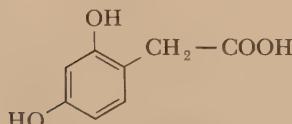


system (1.14.3.1) catalyzes the incorporation of molecular oxygen into the tyrosine hydroxyl with the concurrent oxidation of NADPH and the coenzymic participation of a tetrahydro pteridine (Sec. 29). Diets deficient in phenylalanine allow individuals with this defect, seemingly genetic, to develop more normally. If phenylpyruvate is administered to normal individuals, it is decarboxylated and phenyl acetate is excreted.

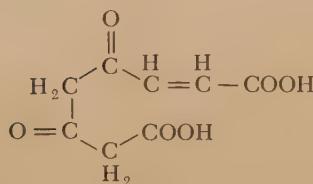
Phenylpyruvic oligophrenia has continued to attract the attention of research scientists because it furnishes a model for testing an idea, the idea that a metabolic deficiency, due to gene damage, is responsible for a mental deficiency. If it is, then it should be possible to simulate the condition in animals; and in support of this idea a recent report states that experimental phenylketonuria has been produced in monkeys (REF. 6). The initial results indicated that central nervous disturbances were connected with metabolic events. Individuals suffering phenylketonuria characteristically develop elevated levels of phenylalanine in the blood; overfeeding with phenylalanine produces experimental phenylketonuria. This points to phenylalanine (or a derivative produced because of an insufficient hydroxylase activity) as the substance responsible for the pathology observed.

### **alcaptonuria**

Urinalysis also offered the clue to the reactions in which tyrosine is oxidized further. An occasional patient was found to excrete a urine that became black on standing. This condition, named alcaptonuria, is characterized by the excretion of homogentisic acid.



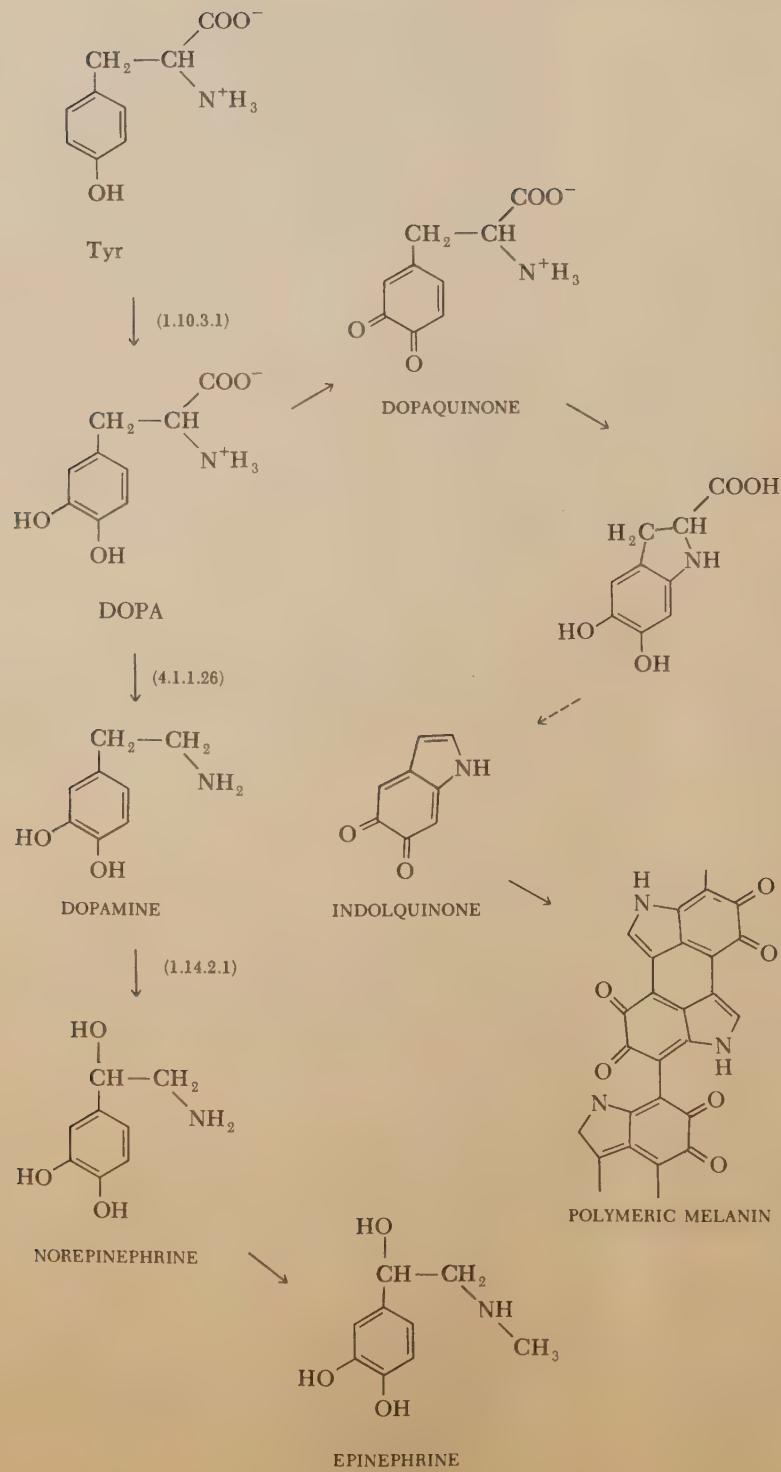
which, upon oxidation by air, results in a black pigment. Again the defect was found to be hereditary and due to the lack of the enzyme HOMOGENTISATE OXYGENASE (1.13.1.5) that normally catalyzes the production of 4-maleyl acetoacetate.



This, in the presence of an isomerase (5.2.1.2) and a hydrolase (3.7.1.2), is converted to fumarate and acetoacetate.

Tyrosine is converted by a set of enzymes in the adrenal medulla, an animal endocrine gland, into epinephrine (or adrenalin, a proprietary name). The

**FIGURE 15.8**  
metabolic products  
related to tyrosine



reactions involved are given in Fig. 15.8. Epinephrine has a variety of pharmacological properties and, together with norepinephrine, is the stimulator of the adrenergic nerve system in animals. The participation of epinephrine in the hormonal control of carbohydrate metabolism was noted in Sec. 5.

A quite different set of reactions follows from dihydroxy phenylalanine (DOPA) in other tissues. Through a series of oxidations catalyzed by tyrosinase (1.10.3.1) and cyclization, terminated by polymerization, characteristic black or brown pigments, melanins, are formed (Fig. 15.8). These pigments occur in the skin of most animals and are also formed when potatoes or certain fungi are cut and exposed to the air.

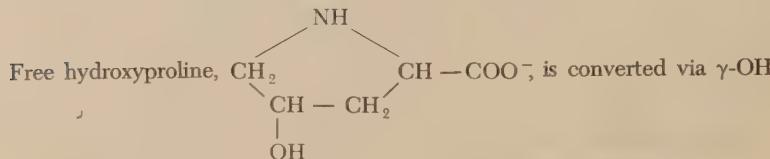
**proline and hydroxy-proline** The two amino acids remaining to be discussed are not  $\alpha$ -amino acids in the strictest sense. The synthesis of proline (Pro) already has been noted as following the cyclization of a glutamate derivative. Hydroxyproline (Hyp) (OH-Pro) is derived from proline by an irreversible reaction that has resisted many efforts to describe its mechanism. Hydroxyproline constitutes roughly 13% of the protein collagen that, in turn, accounts for about 20 to 25% of the total body protein. Thus, this amino acid represents about 3% of the total weight of body protein and is an important dietary constituent for carnivores, although it is not incorporated into protein as such.

In plants it is far less abundant. However, it has been shown that hydroxyproline is in the primary cell wall of plants in peptide linkage. Proline is rapidly assimilated by actively proliferating plant tissue and is converted into a protein which in turn becomes the substrate for the conversion of proline to hydroxyproline. A similar mechanism appears to be operative in collagen synthesis. The oxygen in the hydroxyl group has been shown to derive from  $O_2$ . The reaction of protein as a substrate in this reaction is unusual. As noted previously, the amino acid residues in proteins are generally not involved in such reactions.

In the animal, ascorbic acid (vitamin C) is necessary for a maximum rate of conversion of Pro to Hyp.<sup>a</sup> Guinea pigs deficient in ascorbic acid are unable to repair tissue damage properly, and impaired hydroxyproline formation is the earliest manifestation of this vitamin deficiency. When scorbutic animals are fed ascorbic acid, there is an immediate and rapid synthesis of hydroxyproline. Collagen biosynthesis, as measured by hydroxyproline synthesis in subcutaneously implanted polyvinyl sponges, has been studied in normal and in scorbutic guinea pigs. In scorbutic animals an implanted sponge containing ascorbic acid allowed synthesis, whereas a sponge without ascorbic acid did not. It seems likely that ascorbic acid is *directly* involved in this reaction. Other studies of collagen biosynthesis in cell-free systems prepared from chick embryo homogenates suggest another mechanism of hydroxyproline formation. In aerobic conditions enhancement of hydroxyproline bonding in peptide linkage was noted when sRNA-proline-<sup>14</sup>C was added (Sec. 19). There

<sup>a</sup> Ascorbic acid is also involved in the conversion of dopamine to norepinephrine (Fig. 15.8). Again the oxidizing agent is molecular oxygen. The mechanism is unknown, and it is not known whether this is a rather general reaction or one that is highly restricted.

was evidence that the conversion of proline to hydroxyproline occurred while the proline was bonded to the nucleic acid.



**comment** In these amino acid metabolism sections I have attempted to exclude those reactions which, however interesting, are restricted in occurrence. In this section on the aromatic amino acids there are some exceptions. Obviously, every student should know that epinephrine derives from tyrosine, and everyone might be expected to learn that lignin, a substance of very common occurrence, is formed from precursors originating in amino acid metabolism. Many other examples could be given; one such as thyroxin, also a tyrosine derivative but formed only in thyroid tissue.

The existence of long reaction sequences increases the chance that mutations will occur and that metabolites ordinarily unseen will be excreted in moderate amounts. The concept of "inborn errors," or genetic defects in metabolism, is credited to Garrod, and it became an important idea in diagnosis. It was not until the research of B. D. Davis, however, that advantage was taken of the opportunity to use bacterial mutants produced purposely to examine the relation between "error" and mutation.

In the metabolism of the aromatic amino acids there are several reactions in which molecular oxygen participates directly. For the most part the mechanisms involved are unknown. Obviously, such reactions are not coupled directly to mitochondrial events or to "energy metabolism," and one wonders what type of control mechanism exists.

The extraordinary metabolism of hydroxyproline will be appreciated more fully after an introduction to the structure of the biopolymers. The fact that hydroxylation seems to occur after proline is incorporated into a macromolecule will relate to the modification of purines and pyrimidines after incorporation into soluble ribonucleic acid. Moreover, it will be seen that the hydroxyproline-containing protein, collagen, has a structure quite unlike that of other proteins but has interchain relations resembling those of nucleic acids in some aspects.

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# 16

## from small molecules to heteropolymers

In the preceding sections attention has been on the chemistry of "small" molecules with molecular weights less than a thousand. Enzymes have been considered, but not their detailed structure. It has been preferable to accept them as functional entities and to delay structural considerations. Starch and glycogen have been considered briefly and have been shown to be homopolymers with variation deriving from a few types of bonding. They do not have discrete molecular weights, but form aggregates and polymers having a range of sizes.

Glucose has been seen as the prime carbon source for heterotrophs;  $\text{CO}_2$  serves for organisms capable of photosynthesis. The mechanisms of biological oxidation and the coupling of syntheses with the ATP/ADP system seem to be similar in a wide variety of organisms. There is no accumulation of nitrogen similar to the accumulation of carbon and hydrogen in fat. If there were, we might observe instant growth as well as the instant action that is characteristic of many organisms. But, as will be seen, growth involves some processes that extend over time periods much longer than those characteristic of intermediary metabolism. It is a process which may produce gross changes in the organism and, in short, has many more consequences than action alone.

Most of the chemistry so far discussed is "small-molecule" chemistry fundamentally similar to that treated in the usual courses of organic chemistry. But compounds singular to living cells are not small molecules. They are macromolecules, for the most part heteropolymers. It is to be expected that the most complicated macromolecules would contain several types of constituents, and indeed the proteins are constituted from the many amino acids just considered.

The biological macromolecules to be considered exhibit two kinds of chemical behavior: that deriving from the properties of the smaller component units and that of a physical-chemical nature reflecting the molecular size and architecture.

What we know about the mechanism by which amino acids are polymerized or assembled into proteins is largely the effort of the last twenty years, although the subject has been studied for a much longer period. What we know of the chemistry and the structure of proteins has been accumulating for more than fifty years, but again there has been a sharp increase in comprehension during the recent past.

### mono- dispersity

Proteins, unlike most polymeric substances, are monodisperse; that is to say, any molecule of a protein like insulin, for example, has the same molecular weight as any other. This property is not exhibited by polysaccharides, or lignin, or any other biopolymer except DNA and possibly some types of RNA.

Monodispersity is very rare among synthetic polymers. Proteins may exist in several molecular-weight states, but each possesses characteristic polypeptide chains, often several in number, and occasionally of more than one kind. For example, it has been found that the enzyme aldolase probably contains three polypeptide chains normally associated in one complex and probably identical. Hemoglobin contains two pairs of identical-chain varieties. Alpha-chymotrypsin contains three polypeptide chains, each differing from the others. The application of genetic methods can often settle the question of the number of kinds of chains, since the synthesis of each type of chain is believed to be under the control of a separate gene.

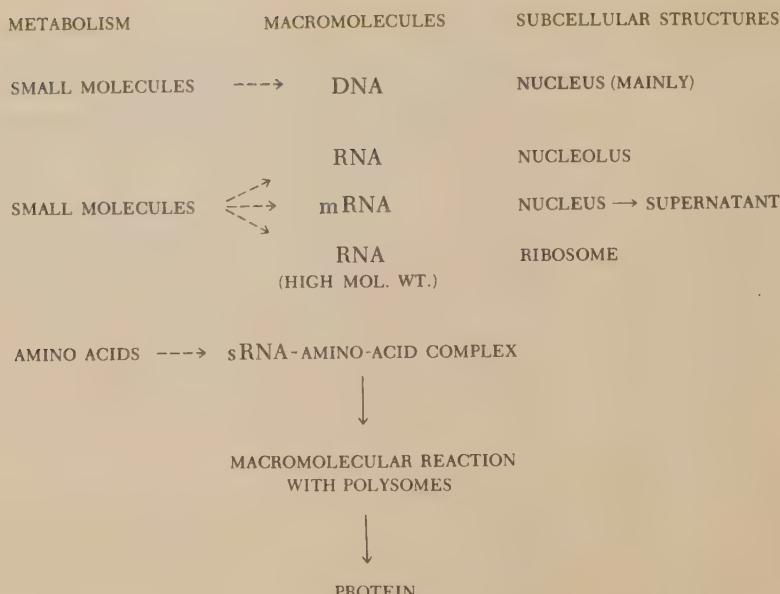
The soluble proteins to be considered may be viewed as metastable associations of polypeptide chains in a conformation corresponding to the most stable thermodynamic state. Evidence now at hand allows the conclusion that the characteristic conformation of the polypeptide chain is largely dictated by the amino acid sequence. It is precisely this sequence which is controlled by the genetic mechanism. The transfer of the "code" for the amino acid sequence from the gene to the site or process of protein synthesis is a study that has preempted much attention recently. The code contained in a replicating molecule is transmitted to coupled reactions that convert the proper carbon sources into characteristic biopolymers, many of them with enzymic activity.

The conviction that deoxyribonucleic acid, DNA, is *the* genetic material is virtually universal. At the moment there is no compelling reason to believe otherwise, although RNA replaces DNA in some viruses. In higher organisms DNA is contained mainly in chromosomal structures; in others it is not. The coding is believed to be inherent in the sequences of purine and pyrimidine bases. The simplifying assumption that the gene, originally a biological concept, is no more than a region of a macromolecule still disturbs some biologists, but it has proved to be a very satisfactory working hypothesis.

### **microsomes, ribosomes**

There is also excellent evidence that much of the synthesis of soluble protein takes place in or on the microsome (ribosome, polysome). Some of the terminology tends to be confusing because it has arisen from different disciplines. For example, the term microsome is an operational one. If a suspension of broken metazoan cells is centrifuged at speeds sufficient to sediment cell walls, nuclei, and mitochondria, there is left a supernatant fluid containing material termed *endoplasmic reticulum* by cytologists. This subcellular fraction, sedimentable at 50,000 to 100,000 times gravity, forms a pellet that can be resuspended and further fractionated in a density gradient. It appears to consist of membrane material, largely lipoprotein, and a denser ribosome fraction containing ribonucleic acid, protein, and small amounts of other substances. In the bacterial cell there is no endoplasmic reticulum and hence no microsomes in the above sense, but there are ribosomes. In preparing ribosomes from *E. coli*, sodium deoxycholate is added to the post-mitochondrial supernatant fraction in order to dissociate them from other structures. The preparation is centrifuged at 105,000 times gravity for 2 hr in order to sediment the particles. Further differential centrifugation in density gradients shows

**FIGURE 16.1**  
some relations  
among small  
molecules and  
macromolecules



that particles of various sizes are present. The smallest are now called monosomes and the larger aggregates of ribosomes, polysomes. It is the latter that effect protein synthesis, a process to be discussed further in Secs. 19 and 20.

There is no doubt that these cytoplasmic particles are the major site of protein synthesis; there is also no doubt that synthesis occurs in several regions of the cell. Synthesis has been demonstrated in mitochondria, in the nucleus, and in cell membranes where ribosomes are present but far more difficult to demonstrate.

There is substantial agreement that the substances involved in the activation of the amino acids prior to polymerization, in the transfer of the sequence code, and in the ribosomal apparatus are ribonucleic acids. The relationships hypothesized are shown in Fig. 16.1.

### macro- molecular interaction

It is intended here to emphasize that protein synthesis is a process involving not only small-molecule-macromolecule interactions but macromolecule-macromolecule interaction as well. As presently conceived, the synthesis of protein is hardly a chemical reaction in the classical sense. The species that react do so only in a rigidly directed manner, so that protein synthesis might better be thought of as a process. Before turning to the details of macromolecular reactions, it is desirable to review the metabolism of the purines and pyrimidines, which form the nonprotein macromolecules DNA and RNA. Then the properties of the macromolecules and finally the synthetic process itself will be outlined.

Although it is not common to emphasize the differences between the metabolism of small molecules and of large complexes, such differences exist. Substances such as glycolysis intermediates, phospholipids, and amino acids

are in a state of rapid flux. By contrast, proteins are reasonably stable; that is to say, the average life of a given protein molecule is relatively much longer. Between the two extremes are some nucleic acids, some complex polysaccharides, and some small molecules such as coenzymes. Different types of control are involved at different regions in this spectrum. It is sometimes stated that protein synthesis is the "most important process" in the cell. In a living cell, with its intricate web of interaction, it is impossible to select "the most important process." The view taken here is that all main types of processes in rapid flux should be considered first, since they must be exploited to form the more complex functional aggregates which, in turn, interact in their own characteristic fashions.

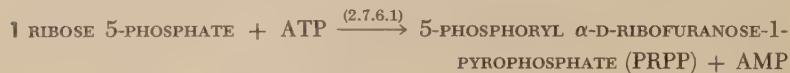
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# 17

## the biosynthesis of purines and pyrimidines

It was stated in Sec. 6 that ribose 5-phosphate is an ingredient in nucleotide synthesis. Not only is it recognizable as such in ribonucleotides but it is also directly involved in the long series of reactions necessary for purine ring formation. Evidence for the following reaction series has been assembled from experiments with a variety of cells and tissues.



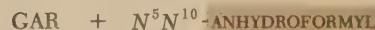
### 2 Formation of an *N*-glycoside



### 3 Beginning of a five-member ring synthesis and amide formation

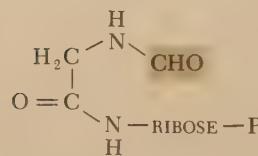


### 4 One-carbon transfer to form substituted formamide



TETRAHYDROFOLIC

↓  
(2.1.2.2)

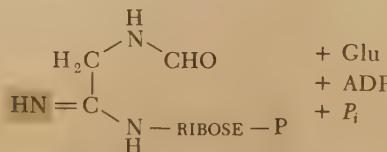


FORMYL GLYCINAMIDE RIBOTIDE (FGAR)

### 5 Amidination

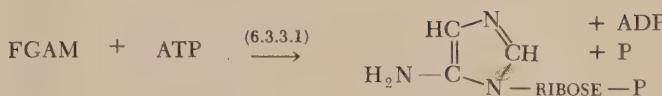


↓  
(6.3.5.3)



FORMYL GLYCINAMIDINE RIBOTIDE (FGAM)

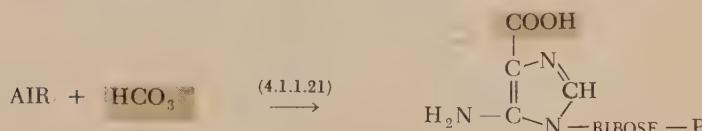
### 6 Cyclization



7

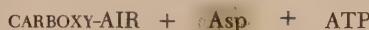
5-AMINO IMIDAZOLE RIBOTIDE (AIR)

Initiation of six-member ring synthesis by carboxylation

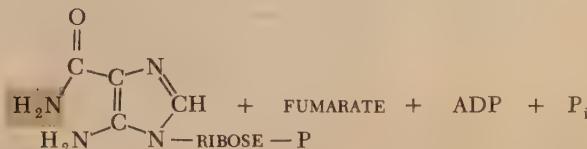


5-AMINO-4-IMIDAZOLE  
CARBOXYLIC ACID RIBOTIDE  
(CARBOXY-AIR)

### 8 Formation of amide



$\downarrow (6.3.2.6)$



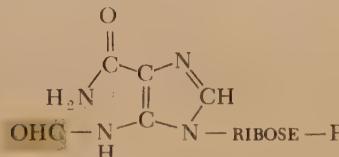
5-AMINO-4-IMIDAZOLE  
CARBOXAMIDE RIBOTIDE  
(AICAR)

AICAR was referred to in Sec. 14 as an intermediate in histidine synthesis. It also accumulates in the medium of sulfathiazole-treated *E. coli*.

### 9 Formation of substituted formamide by one carbon transfer



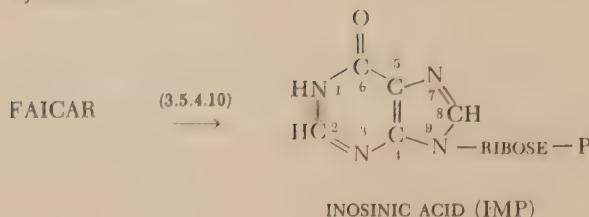
$\downarrow (2.1.2.3)$



5-FORMAMIDO-4-IMIDAZOLE  
CARBOXAMIDE RIBOTIDE (FAICAR)

Note that the tetrahydro folate derivative involved as a formyl donor in this step is not identical with that in step 4.

## 10 Cyclization



Inosinic acid is a nucleotide (purine-sugar-P) that has been known for a very long time. It was isolated by Liebig from meat extract in 1847. The purine structure synthesized here is known as hypoxanthine, and the nucleoside (purine-sugar) is known as inosine.

ribose-5-phosphate



5-phosphoryl  
ribofuranose  
1-pyrophosphate  
(PRPP)



*5'-phospho  
ribosylamine (PRA)*



*Formyl glycaminide  
ribotide (FGAR)*



formyl glycaminidine  
ribotide (FGAM)



5-amino imidazole  
ribotide (AIR)



-amino 4-imidazole  
carboxylic acid  
ribotide  
(carboxy-AIR)



-amino 4-imidazole  
carboxamide ribotide  
(AICAR)



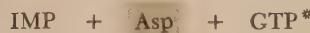
5-formamido  
4-imidazole  
carboxamide ribotide  
(FAICAR)



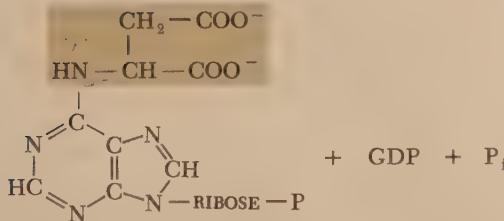
inosinic acid (IMP)



Although inosinic acid is the end product of this series of synthetic reactions, it is not found incorporated in nucleic acids except in sRNA. The syntheses of the purines that *are* to be found in these macromolecules are shown in what follows.

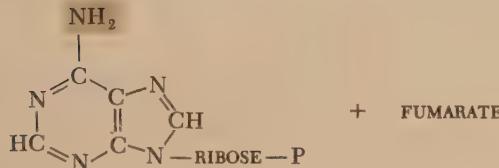


$\downarrow$   
(6.3.4.4)



SUCCINYL ADENYLYC ACID

$\downarrow$   
(4.3.2.2)



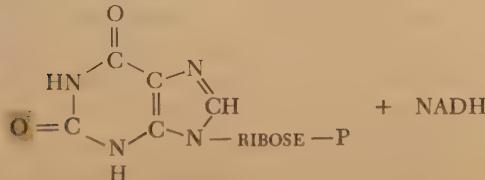
ADENYLYC ACID (AMP)

<sup>o</sup>Guanosine triphosphate is analogous to ATP. It is a derivative of guanine, whose synthesis is described next. A neat control device is involved in that guanine is dependent on adenine synthesis, and vice versa.

The nucleotide adenylyc acid is also adenosine 5'-phosphate. The nucleoside *adenosine* is the riboside of the purine *adenine*. Already familiar are adenosine diphosphate (ADP) and triphosphate (ATP).

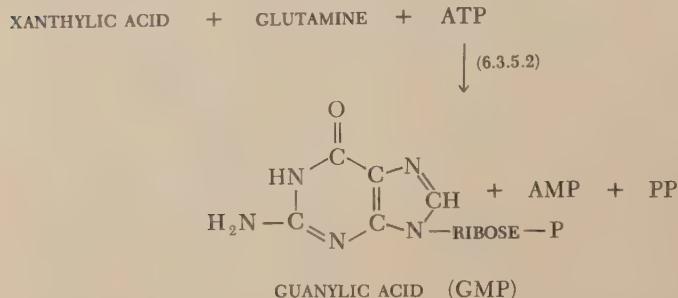


$\downarrow$   
(1.2.1.14)



XANTHYLYC ACID

Xanthyllic acid is also xanthosine 5'-phosphate, or xanthosine monophosphate. The purine structure is xanthine.



Guanylic acid, or guanosine monophosphate, and adenosine monophosphate are the usual purine derivatives found in ribonucleic acids. The purine structure is guanine. There are, in addition to GMP, also GDP and GTP.

It is a simple exercise to add up the precursor requirements for the synthesis of a molecule of AMP. The list includes ribose 5-phosphate, four molecules of ATP, two molecules of glutamine, glycine, two different forms of formyl tetrahydrofolic acid, HCO<sub>3</sub><sup>-</sup>, two molecules of aspartate, GTP, and at least twelve different enzymes. It is also necessary that this synthesis proceed with facility in a wide variety of conditions and in a wide variety of tissues. It may be assumed that the complexities evident in this description are dictated by the need for control. It has been shown that adenine can arise from a potpourri of ammonia and carbonate reacting in an ultraviolet discharge; hence we can assume that its structure is thermodynamically probable.

Many organisms ingest purines in the diet. It is a matter of some interest whether the purines are used as such or whether they must be oxidized or modified. It has been found that RIBONUCLEOTIDE PYROPHOSPHORYLASE (2.4.2.7) catalyzes adenine + PRPP → AMP + PP, that RIBONUCLEOTIDE PHOSPHORYLASE catalyzes adenine + ribose-1-P → adenosine and P<sub>i</sub>, and that ADENOSINE KINASE (2.7.1.20) catalyzes adenosine + ATP → AMP + ADP. There are present other enzymes that promote similar reactions with other purines as well. No useful generalization can be provided, since these reactions proceed in various tissues at differing rates.

### deoxyribo-nucleotides

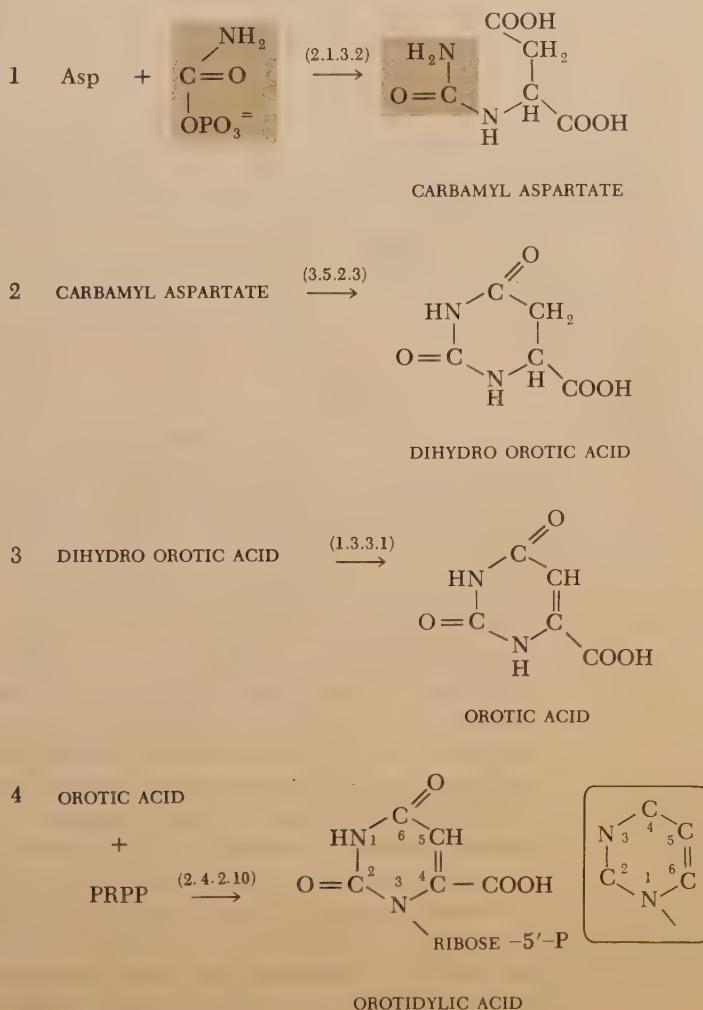
As noted, the ribonucleotides of adenine and guanine are chiefly those found in ribonucleic acids, a class of biopolymers containing many types of molecules but distinguished, as a class, by the presence of ribose. There is also another set of purine nucleotides differing in that the sugar component is 2-deoxy ribose. Such nucleotides are designated dAMP and dGMP. These two purine nucleotides are components of deoxyribonucleic acid. Present opinion is that these nucleotides are not synthesized as such but are derived from the corresponding ribonucleotides. Enzymes that catalyze the reduction of ribonucleotide diphosphates to deoxyribonucleotide diphosphates have been demonstrated in a variety of cells. An aldolase-like enzyme that catalyzes

the addition of acetaldehyde to glyceraldehyde-3-phosphate with the formation of 2-deoxy-D-ribose 5-phosphate has been described. Also, a mutase that effects the conversion of deoxyribose-5-P to deoxyribose-1-P has been described. Recent evidence does not support the participation of either of these latter two enzymes in the formation of deoxyribonucleotides.

In all naturally occurring ribo- and deoxyribonucleosides the sugars are bonded to N-9 of the purines in a  $\beta$ -glucosidic linkage. Recently, however (REF. 2), 3-isoadenosine, adenosine in which the ribosyl group is at N-3, has been synthesized, and the phosphate derivatives of this compound have been found to replace the usual adenosine coenzymes in some enzyme systems. The other major components of nucleic acids are the pyrimidine nucleotides. The syntheses of these compounds do not entail as many steps (Fig. 17.1) as

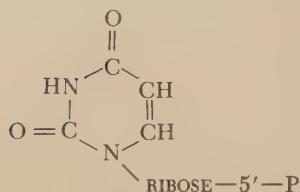
### pyrimidine synthesis

**FIGURE 17.1**  
biosynthesis of orotidyllic acid. In this formula orotidyllic acid is numbered according to the convention previously used and to be found in the older literature. On the right of the formula is the newer numbering convention, which is used in this book.



the purine syntheses entail. As recorded in Sec. 14, aspartate is an acceptor of carbamyl phosphate and participates in a reaction competitive with urea synthesis. Orotidylic acid, or orotidine 5'-phosphate, is the first pyrimidine nucleotide formed in the synthesis. As in the purine synthesis, the first product is not found in a biopolymer. The two major pyrimidines found in RNA molecules are uracil and cytosine, and their syntheses, originating from orotidylic acid, are interrelated.

Decarboxylation (4.1.1.23) of orotidylic acid yields uridylic acid (uridine monophosphate, uridine-5'-P, UMP).

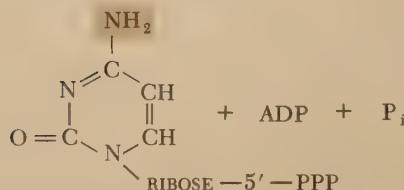


UMP

Further phosphorylation by the ATP system yields UDP and UTP. The triphosphate, UTP, serves as a precursor for cytosine derivatives.



$\downarrow$   
(6.3.4.2)



CYTIDINE TRIPHOSPHATE (CTP)

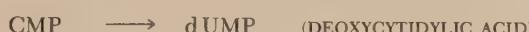
In this case the triphosphate appears first, and it is from this compound that cytidine monophosphate (cytidine-5'-P), or cytidylic acid, arises.

It will be shown in Secs. 18, 19, and 30 that these pyrimidine triphosphates, and indeed the purine triphosphates as well, are the direct precursors of nucleic acids and are also participants in the syntheses of certain sugars and some lipids. Without doubt these compounds are chiefly incorporated into polymers at the time the cell is in growth. When growth ceases or slows, they are participants in other reactions.

The pyrimidine deoxyribonucleotides include not only derivatives of uracil and cytosine but also thymine. Thymine, or 5-methyl uracil, is found in DNA but not in RNA. Both DNA and RNA preparations from various sources have been found to contain small amounts of methylated (5-methylcytosine, for example) or hydroxymethylated (5-hydroxymethylcytosine) pyrimidines

**FIGURE 17.2**  
**deoxyribonucleotide**  
**syntheses**

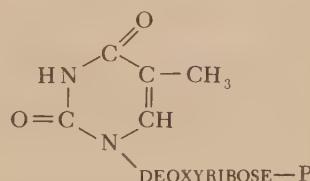
## REDUCTION via NADP-LINKED ENZYMES:



## DEAMINATION (3.5.4.12):



## METHYLATION via FOLIC-ACID DERIVATIVE:



THYMIDYLIC ACID OR THYMIDINE  
MONOPHOSPHATE

and purines. In many cases these groups appear to be added to the bases after the bases are in polymeric form.

The biosyntheses of some monomeric deoxyribonucleotides are noted in Fig. 17.2.

In Fig. 17.3 are shown the formulas for purine and pyrimidine compounds found less commonly.

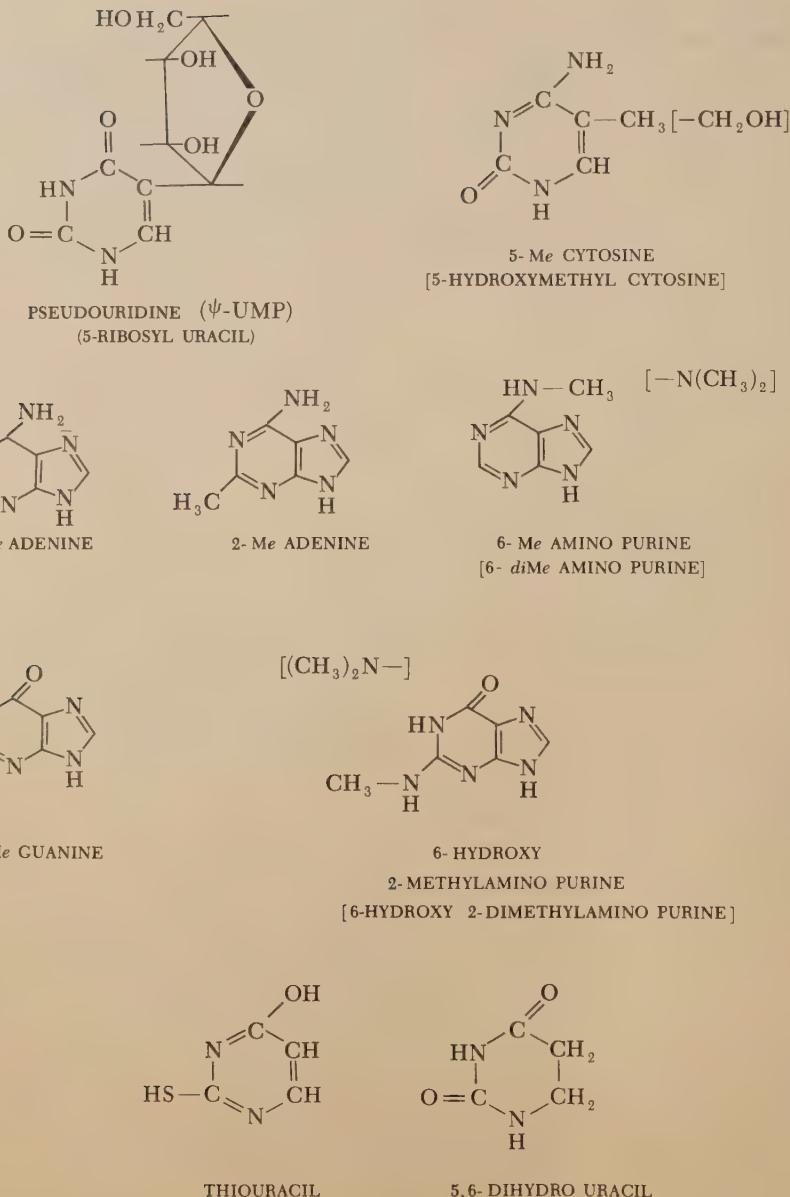
The utilization of free pyrimidines, or their nucleosides, is highly variable. Cytosine is remarkably resistant to assimilation into nucleotide. Uracil and thymine can be assimilated under some conditions. Experimentally, thymidine has been useful because it *can* become part of the DNA structure, and hence labeled thymidine has been used to tag DNA *in vivo* (Sec. 25).

The above outline is a minimal discussion of the syntheses and interrelations of the purine and pyrimidine ribo- and deoxyribonucleotides. I have omitted all discussion of mechanisms, of sites of syntheses, and of the enzymes involved. These omissions should not go unnoticed but should be understood to represent an attempt to remain succinct.

The purine and pyrimidine biosyntheses represent a coupling of carbohydrate metabolism with some special reactions of glycine, aspartate, and glutamine. It should be kept in mind that the latter two amino acids are readily formed from citrate cycle intermediates but that the formation of glycine depends on a folic acid enzyme catalysis. Note that folic acid is involved in other parts of the syntheses as well. This compound, or class of compounds, can obviously limit growth in a very direct way if it is not available.

The laboratory syntheses of the purine and pyrimidine nucleosides were

**FIGURE 17.3**  
pseudouridine and  
the "minor bases"



devised by Kossel, Levene, and Fischer during the formative years of biochemistry. During the last twenty years methods have been elaborated for the synthesis of the nucleotides as well. The further development of methods for synthesizing oligo- and polynucleotides has received the attention, in the recent past, of Todd, Khorana, and several other bioorganic chemists. The availability of such compounds, prepared by classical organic techniques, has been very helpful in biochemical experimentation.

### anti-metabolites

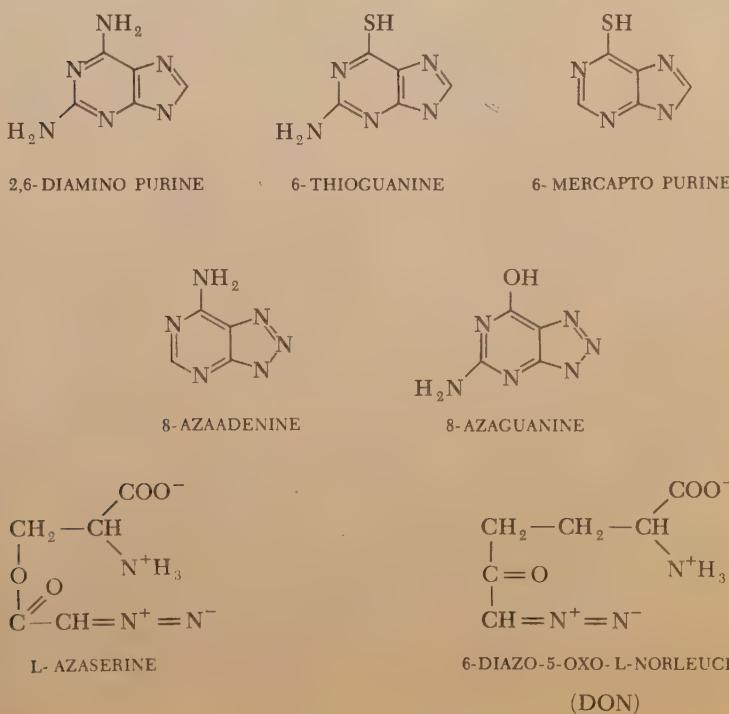
The use of inhibitors in studying enzyme kinetics is well established and profusely documented. The extension of such studies to the intact animal is a type of experimentation which readily suggests itself. As the details of intermediary metabolism proliferated in our technical knowledge, so too there accumulated a vast number of analogs to be tried.

It can be readily appreciated that the administration of a coenzyme analog may have profound effects if it bears the proper specificity references but differs in function, either qualitatively or quantitatively. Evidence that will be presented in sections that follow indicates purine or pyrimidine analogs may also have profound effects, particularly if they are incorporated into nucleic acids. Among the many substances that have been screened for carcinostatic activity are those whose formulas are given in Fig. 17.4. Both 6-thioguanine and 8-azaguanine have been found to be incorporated into RNA and to be effective in slowing or stopping the growth of some types of cancer.

The effectiveness of azaserine and DON in slowing the growth of sarcoma seems to rest in an inhibition of FGAM synthesis from FGAR. This reaction is an amido transfer from glutamine, and these drugs inhibit other such reactions as well.

In a subject such as this it is difficult to know whether we are discussing biochemistry or pharmacology, and perhaps it does not matter. The two disciplines are organized with somewhat different goals, and where they overlap there is reinforcement (REF. 4).

**FIGURE 17.4**  
purine analogs and  
inhibitors of purine  
synthesis



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# 18

## DNA

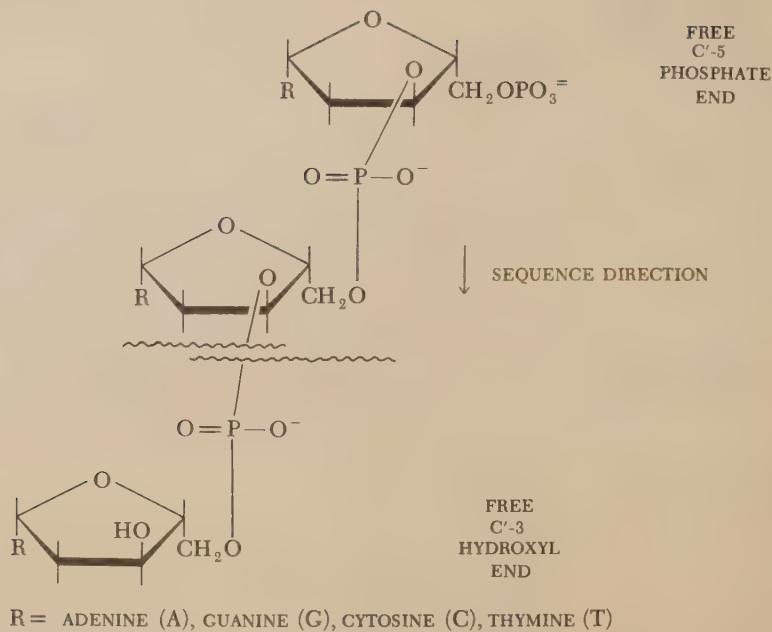
Deoxyribonucleic acid is a dimer of two linear structures, each about  $10^4$  to  $10^8$  nucleotide units in length. These "chains" can be considered to be composed of deoxyribose "links" held together by phosphodiester bonds as in Fig. 18.1. Such a linear array could have a very large number of arrangements in space, but it would hardly be expected to be a random structure. The Watson-Crick hypothesis (REF. 1) stated that it is a spiral and that the usual DNA structure consists of a pair of antiparallel  $\rightleftharpoons$  "chains". The structure was perceived to be stabilized by stereochemically specific interactions—hydrogen bonding—between the purine and pyrimidine components as diagrammed in Fig. 18.2.

The model requires that moles of A + G equal moles of T + C, and this is consonant with the analytical data. Alternatively, it could be stated that there is an equivalence of bases with an amino group at ring-carbon 6 to those with a carbonyl at ring-carbon 6. Thus, on a molar basis, A = T and G = C.

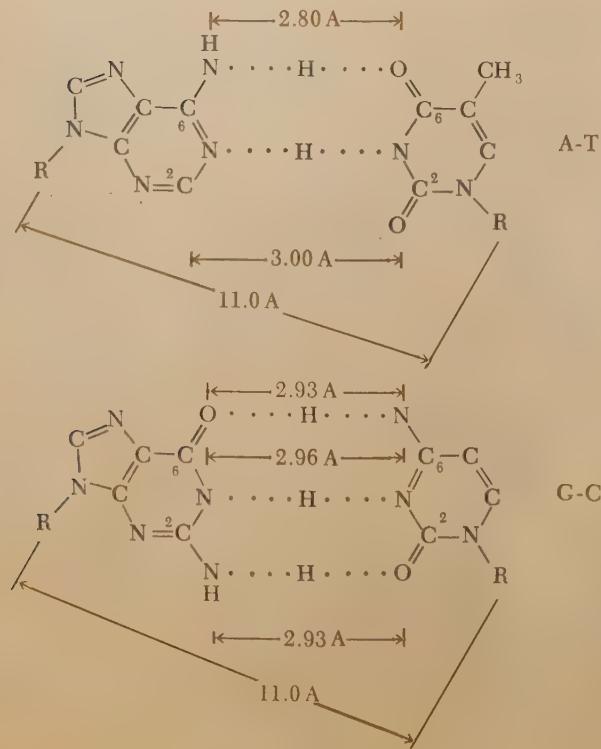
model trideoxy-nucleotide



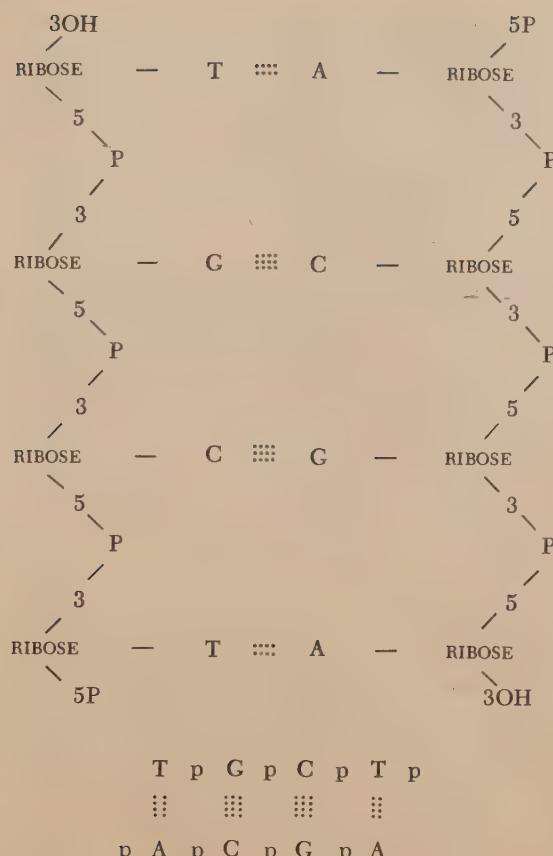
**FIGURE 18.1**  
some aspects of DNA  
structure



**FIGURE 18.2**  
hydrogen bonding  
between purine and  
pyrimidine bases in  
DNA



**FIGURE 18.3**  
complementary but  
nonidentical  
hydrogen-bonded  
chains

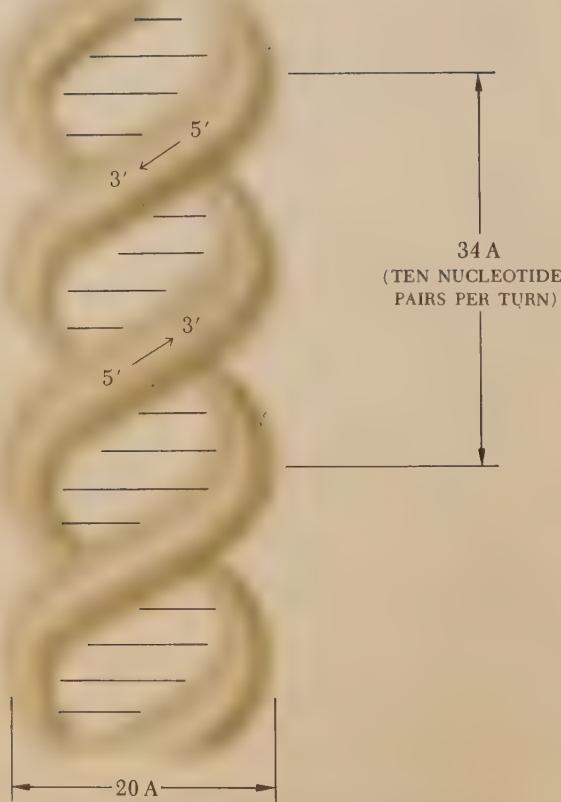


It follows that the sequence of bases in one chain of the pair rather narrowly defines that of a complementary chain, and it is explicit that the two chains must be nonidentical. This point is illustrated in Fig. 18.3.

In Sec. 21 there is a brief discussion of the X-ray method used to probe the structures of macromolecules. It was the periodicities observable in the X-ray diffraction data that formed the basis for suggesting that DNA is helical. This, in itself, was by no means new, since polypeptide helices had been suggested on the basis of X-ray data long before. The novel feature was the complementarity of two chains. Again, the stabilization of a helical array by hydrogen bonding was hardly original, since such bonding had been invoked for the helices of polypeptides. It was recognition of the complementarity of the purines and pyrimidines that was so revealing and was solidly based on analytical data already in the literature.

As seen in Fig. 18.4, the right-handed helical strands wind about an axis to which the purine-pyrimidine arrays are normal. (The left-handed helix is stereochemically unfavorable.) The base pairs are close to the axis, and the

FIGURE 18.4  
helical nature of  
DNA



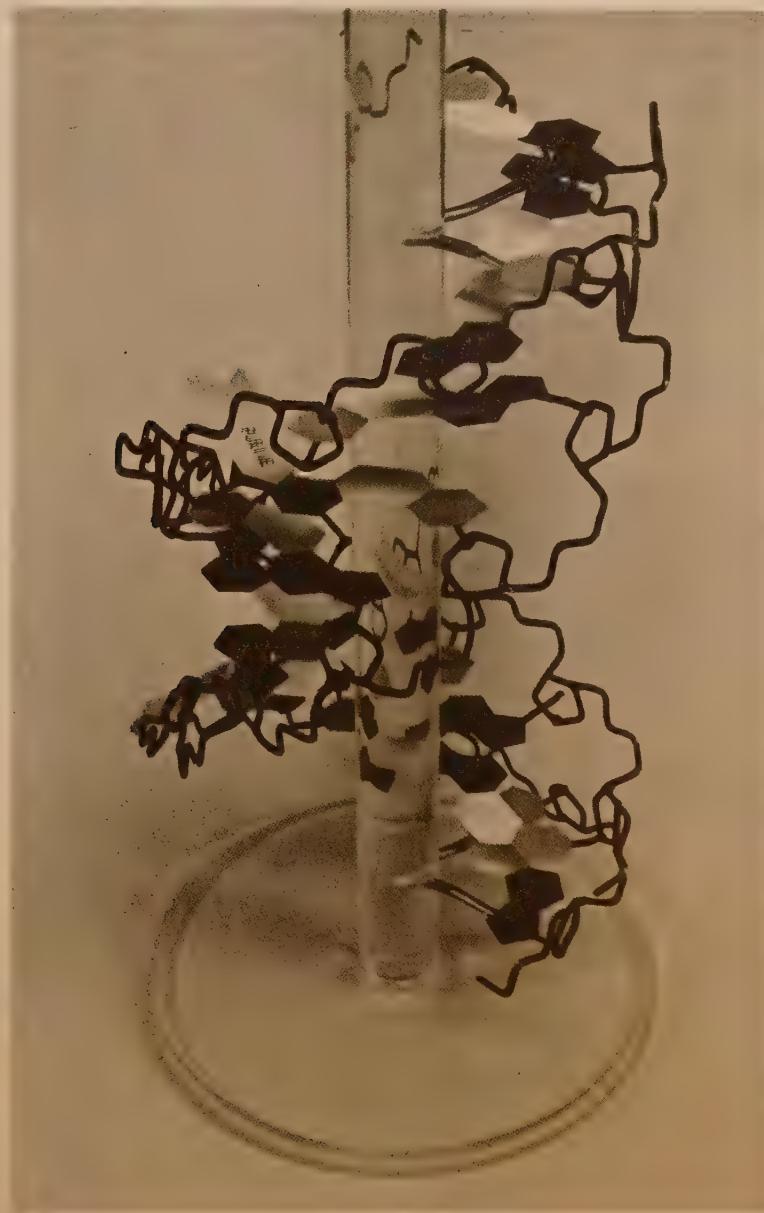
view along the  
DNA spiral axis



deoxyribose phosphate chains form the antiparallel spirals. No two-dimensional representation can be more than suggestive. Figure 18.5 is a photograph of a three-dimensional model constructed with appropriate dimensional relations.

That DNA is characteristic of the cell nucleus has been recognized for many years, but its formation is not restricted to the nucleus. Small amounts are found outside the nucleus, and, in any case, there are cells without demonstrable nuclei that obviously make the polymer. Almost continual

**FIGURE 18.5**  
photo of three-dimensional DNA model



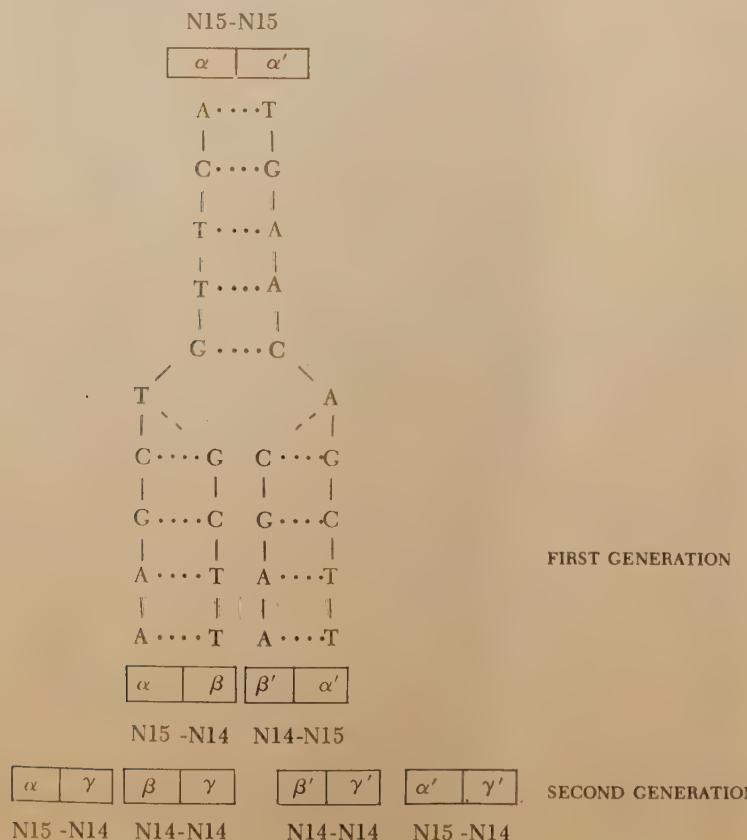
synthesis of DNA occurs in bacteria (no nucleus), but many other types of cells synthesize DNA only during a part of the intermitotic period. For convenience bacteria can be used as a prime experimental material if the assumption is made that the fundamental processes of all cells are identical. Much of our current knowledge of DNA synthesis is derived from experiments with bacteria, but it is unlikely that the process in other cells will prove to be identical in all respects. As stated before, bacteria are organized for rapid and continual growth. In more specialized cells it may be anticipated that the DNA synthesis mechanism is closely coupled with complex metabolic controls.

### replication

The regnant model of DNA is compatible with, but does not demand, a *semiconservative* mechanism of replication. Assuming DNA to be *the* genetic substance of the cell, we may conceive of a replication process in which the DNA monomers<sup>o</sup> are conserved from one generation to its successor. The monomers would be complemented with new copies formed as on a template. The trivial model in Fig. 18.6 illustrates the elementary features of such a

<sup>o</sup> The single chain as differentiated from the paired-chain species.

**FIGURE 18.6**  
semiconservative  
replication of  
chains



process: that is, semiconservation and the base pairing that restricts the possibilities during replication. This hypothesis was tested in 1958 by Meselson and Stahl. They labeled bacteria, and their DNA, with  $^{15}\text{N}$ . After one generation, or one doubling, the resultant DNA, when examined by density gradient centrifugation,\* was found to contain equal amounts of  $^{14}\text{N}$  and  $^{15}\text{N}$ , as would be expected from the above mechanism. DNA was isolated from a second generation also and was found to consist (density gradient centrifugation) of 50% hybrid dimers ( $^{15}\text{N}$ - $^{14}\text{N}$ ) and 50%  $^{14}\text{N}$ -containing dimers. If the  $\alpha$  chains in Fig. 18.6 contain only  $^{15}\text{N}$  and the  $\beta$  and  $\gamma$  chains only  $^{14}\text{N}$ , then the model corresponds to the data if we assume the antiparallel chains separate completely at each replication. Unfortunately, it remains very difficult to picture the process that leads to this separation. What causes the abolition of the base pairing of the parent strand? Does replication begin at one end or at both ends? How can the parent strand unwind and the daughter strands wind up simultaneously? There has been speculation, but there are no satisfactory models.

\*In the analytical ultracentrifuge (Spinco Model E is the dominant instrument) salt solutions can be spun at speeds high enough to create a density gradient. If a high-density salt such as CsCl is used, DNA or other very large polymers can be added, and after many hours spinning, the polymer will be found in a discrete band. At equilibrium, sedimentation forces and diffusion forces balance each other, the salt forms a density gradient, and the polymer concentrates in the region corresponding to its density. The technique allows the differentiation of  $^{14}\text{N}$ - and  $^{15}\text{N}$ -labeled DNA molecules and hence is very sensitive to changes in density when the molecule is very large. It is much less discriminating for smaller molecules, even proteins, because of diffusion.

**FIGURE 18.7**  
bacteriophage  
 $\phi$ x-174 [5]  
small particles are  
phage. Large par-  
ticles are polystyrene  
latex.



The existence of single-stranded DNA was first demonstrated in a bacteriophage ( $\phi$ X-174). Since then, many studies have been devoted to the question of the "replicative form" of DNA. In  $\phi$ X-174 it has been found that a double-stranded intermediate appears during the replication of DNA. In bacteria there appears to be only one DNA molecule per cell. Evidence has been presented that this is a single gigantic circular macromolecule that, when broken, would stretch out over nearly a millimeter. The weight is about  $2 \times 10^9$  molecular-weight units. This very fragile structure corresponds to the bacterial chromosome. In animals there are several chromosomes per cell and perhaps more than one DNA molecule per chromosome. If all DNA molecules are circular, then the replicative process is even more difficult to visualize.

### **synthetic DNA**

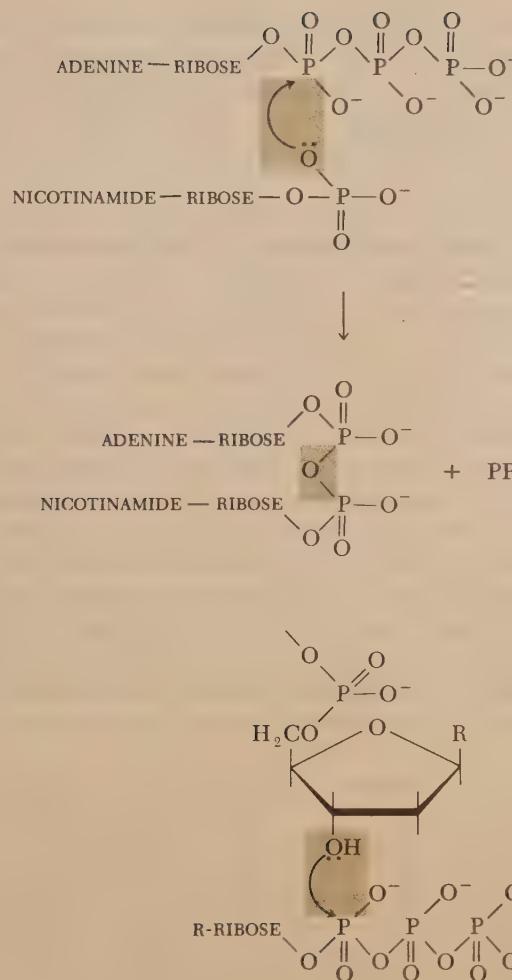
Despite our very imperfect understanding of the mechanism of DNA replication, there have been attempts to obtain chemical evidence about the mechanism for hetero polymerization of deoxy nucleotides. The work of Kornberg and his associates looms large in this effort. Initially it was thought likely that the mechanism of biosynthesis of NAD and other coenzymes might provide a clue (Fig. 18.8) and that nucleotide addition was effected by a nucleophilic attack in the enzyme-substrate complex. Since the biosynthesis of purines and pyrimidines yields the 5'-nucleotides, it was reasoned that the 3'-OH of the DNA chain (to be lengthened) would be involved in a nucleophilic attack on a purine or pyrimidine triphosphate as in Fig. 18.8. Thus the result would be



In order to test this, incorporation experiments were carried out. Reaction mixtures contained an *E. coli* extract (enzymes + DNA) and triphosphates of high  $^{14}\text{C}$  specific activity. Those added were dATP, dGTP, dCTP, and dTTP. Upon incubation it was found that almost total destruction of DNA by nucleases and diesterases occurred. About 50 counts out of  $10^6$  added were incorporated into a form that might be DNA. The key to progress in this research effort was enzyme purification (REF. 9). When DNA POLYMERASE (2.7.7.7) was highly purified, it was found to catalyze the net synthesis of DNA from the triphosphates provided a DNA primer was present. It is believed that the primer acts as a template for a complementary chain and forms hydrogen bonds sequentially with the various nucleotides in the proper order. The resultant product possesses properties similar to those of the "native" DNA and behaves like a long nonflexible molecule having a molecular weight of about  $6 \times 10^6$ . When heated to a temperature of  $50^\circ$ , flexibility ensues and the molecule is said to "melt."<sup>\*</sup> It has been proposed that the hydrogen bonding between the nitrogen bases is destroyed so that a random configuration of single strands or monomers results. These monomers serve as excellent primers.

<sup>\*</sup>The "melting" of a polymer refers to loss of some structure just as the more familiar melting of small molecules concurs with the loss of crystal structure.

**FIGURE 18.8**  
mechanism of  
dinucleotide  
synthesis



Some substitution of bases other than those noted above has been found to be possible. A comparison is shown in Table 18.1. When only the four usual bases are used, the A-T and G-C ratios of the product are those of the

**TABLE 18.1**  
pyrimidine and  
purine analogs  
incorporated into  
synthetic DNA

analog triphosphate	uptake, in percent of control		
	dTTP	dCTP	dGTP
uracil	54		
5-bromo uracil	97		
5-fluoro uracil	32		
5-methyl cytosine		185	
5-bromo cytosine		118	
5-fluoro cytosine		63	
hypoxanthine			25

primer. Moreover, if a dAT synthetic\* copolymer is used as a primer, only A and T are found in the final product even though G and C are in the medium as triphosphates. It has been concluded, therefore, that the polymerase catalyzes the formation of the secondary phosphate bonds but that the sequence is specified by the primer.

### sequence patterns

The base-sequence pattern in DNA preparations, synthetic or natural, has not been determined satisfactorily. For *oligo ribonucleotides* the following technique is available: If the trinucleotide pApGpC† is treated with periodic acid ( $\text{HIO}_4$ ), the ribose, bearing free 2' and 3' hydroxyl groups will be oxidized. The addition of an amine (such as glycine) results in a group transfer and the production of pApGp and cytosine. Thus it is known that cytosine is the base at the "right-hand end" of the chain. The dinucleotide can be exposed to a PHOSPHOMONOESTERASE (3.1.3.6) that is specific only for the 3' bond, and this hydrolysis yields pApG which now contains two adjacent hydroxyls oxidizable by  $\text{HIO}_4$ . Repetition of the process yields the penultimate base of the original trinucleotide. Although in theory this could be applied to long chains of nucleotides, in practice it is not, because small losses occur at each step and the accuracy decreases rapidly. Obviously, it can not be applied to the determination of deoxyribonucleotide sequences, short or long.

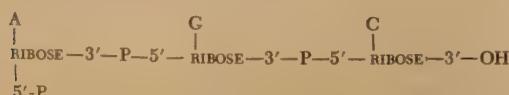
A technique termed "nearest-neighbor base sequencing" (REF. 8) has been applied to synthetic DNA. If a primer DNA is incubated with enzyme, dATP, dGTP, dCTP, and dT<sup>32</sup>PPP, an event such as the following might be visualized.



If the product is hydrolyzed with micrococcal (DEOXYRIBO)NUCLEASE (3.1.4.7) and spleen PHOSPHODIESTERASE (3.1.4.1), a mixture of 3'-deoxyribonucleotides is produced: adenine deoxyribose-3'-P, guanine deoxyribose-3'-P, cytosine deoxyribose-3'-<sup>32</sup>P, and thymine deoxyribose-3'-P. The labeled P is now associated with the base that was "to the left" of the thymine which originally bore the <sup>32</sup>P. This allows an identification of the base neighbors that occur in the linear sequence. Determination of the specific activity would provide a measure of the frequency of neighboring. Further experiments in which the other triphosphates were labeled would provide data for the frequencies of all 16 possible neighboring couples. (The word *pair* is avoided here because base *pairing* has another connotation.) The results of such experimentation support the Watson-Crick hypothesis of base pairing and indicate nonrandom sequences that correspond to the primer.

\* If DNA polymerase is incubated with dATP and dITP, a dAT copolymer slowly forms. The reaction seems self-primed, and the product is a double-stranded helix. The sequence of each strand is A-T-A-T-. In contrast, if the enzyme is incubated with dGTP and dCTP, another double-stranded copolymer is formed in which one strand contains only C, and the other contains only G.

† This is the conventional abbreviation for



As a model system, *E. coli* and *B. subtilis* DNA polymerases have been valuable. It has helped to substantiate the Watson-Crick concept of hydrogen bonding between A-T and G-C pairs and to confirm this phenomenon as a specific ordering force necessary to the production of a replicate. It is *assumed* that a complete nucleotide is added at each chain-extending event and that this is, in fact, the nature of the polymerization in the cell. Actually, until some synthetic DNA preparation is shown to have biological activity, final evaluation of this work must be held in abeyance. Examination of synthetic DNA preparations with the electron microscope has revealed branch points which do not occur in native DNA. This may mean that the DNA polymerase studied is a repair enzyme and that the discovery of the mechanism of DNA synthesis lies in the future.

Unhappily, as yet mammalian DNA polymerase preparations do not permit *net* synthesis as do the bacterial enzymes. The mammalian enzyme can act only on a single-stranded primer, whereas the bacterial enzyme yields a product with either single- or double-stranded primer. This may mean that the mechanism of DNA synthesis in mammals differs from that in bacteria, or it may only reflect our elementary knowledge. Despite all that has been said, printed, and published, the synthetic DNA preparations that have been produced so far are models and nothing (biologically) more.

### bacterial transformation

The student may well wonder what sort of a biological test could determine the activity of a synthetic DNA preparation. The answer is a phenomenon known as "transformation" in bacteria. Two related but nonidentical bacteria, such as type II and type III pneumococci, may be used. Experimentally it is useful that each type can grow encapsulated in a complex carbohydrate gel. The carbohydrate not only lends the bacterial colonies a characteristic *smooth* appearance but also is immunologically\* singular to this type. On the proper medium *rough* colonies of both types of bacteria can also be obtained, and the bacteria of these colonies lack the carbohydrate coating.

If a suspension of type II (rough) pneumococci is made and anti II (rough) serum is added, no growth will occur, because the bacteria will be clumped by the antibody to the so-called "somatic" antigen (the noncarbohydrate portion of the cell). If a heat-killed type III (smooth) culture is added to the suspension of type II (rough) bacteria and anti II (rough) serum, and the entire mixture is incubated, a turbidity will appear. Examination will show *live* type III (smooth) cells. How can this be? The conclusion reached after exhaustive testing was that something from the heat-killed cells penetrated the live ones and transformed them from type II (rough) to type III (smooth). This conclusion has been widely accepted. The material causing transformation is DNA, and enough of it can penetrate the clumped type II cells to act as a primer for synthesis. During the growth of new cells, synthesis of type III

\*If carbohydrate derived from these bacteria is injected into certain animals under the proper conditions, the animal will respond by synthesizing a unique protein. If the blood serum of injected animals is added to a solution of the carbohydrate, usually a precipitate will form. In such a case the carbohydrate is called an *antigen*, the protein liberated into the blood is an *antibody*, and the formation of a precipitate is called a *precipitin reaction*.

DNA and DNA-directed mRNA results in enzymes that now effect the synthesis of type III carbohydrate. This does not interact with the antiserum, and hence the new cells, "transformed" cells, are free to grow.

Whether this phenomenon is restricted to microorganisms remains uncertain. Some experiments that have been reported were thought to show a similar phenomenon in higher animals, but the work has not been widely accepted. If it is true that modified DNA can be introduced into a mammalian egg and that normal cell division will follow, we have need for a most explicit control of such experimentation. As implied above, no artificial DNA has as yet shown any transforming capability.

**comment** DNA must be one of the most intensely investigated substances ever to receive scientific attention. Those inclined to do so will find that admirable and successful work was done on nucleic acids in the early years of the twentieth century. Such was the impact of the Watson-Crick hypothesis with its charismatic symbol that an enormous amplification of research of DNA ensued. In the face of such a mass of results, data, and speculation the nonspecialist can only continue to search for the generalizations that evolve.

The Watson-Crick model of DNA correlated much of the body of data at hand when it was proposed, and many studies in the interim have tended to validate it. Some physical questions remain, however. Recent interpretations of spectral data have focused attention on the importance of the stacking of the bases. Thus there is a growing conviction that intrachain interaction of the bases may be of importance in addition to the interchain hydrogen bonding recognized by Watson and Crick. It will not be surprising if modifications of the present model are forthcoming in the near future. Moreover, in contrast to our knowledge about protein we know almost nothing of the (base) sequences.

The bulk of the probing has been done with bacterial DNA. Nothing, so far, indicates a difference between bacterial and metazoan DNA, and tests on cells from higher organisms indicate a semiconservative replication. Despite this, results obtained with bacterial systems cannot be assumed to be those expected from the cells of higher organisms. In a word, the student must understand that there remain very fundamental questions to answer concerning the chemistry, the biochemistry, and the biology of DNA. We have a good structural model, but all the functional models currently available are seriously lacking.

One of the purposes of this book is to relate function to structure, and in preceding sections the anatomical sites of reactions have been stipulated as often as possible. In the case of DNA we encounter a quite unclear situation. In bacteria, DNA is not confined to a subcellular structure. In other cells the major percentage occurs inside the nucleus and only very small amounts are found in other organelles. Wherever it is found, DNA participates in two major reactions only: it replicates and it serves as a pattern for the synthesis of ribonucleic acids that possess a portion of the genetic code.

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# 19

## the ribonucleic acids

### mRNA

The work of Caspersson (1940–1950) on the ultraviolet microspectrophotometry<sup>o</sup> of individual cells provided good evidence that most of the DNA is in the nucleus and that protein synthesis is probably extranuclear. As experimentation continued there was an accretion of evidence that RNA synthesis is coupled to protein synthesis. When it was proposed that base sequences, in DNA, specify amino acid sequences in proteins, there was more than ever a need to discover the substance that couples the directions of the nucleus to the syntheses in other parts of the cell. Good evidence may now be found in the literature to support the proposal of a *messenger* RNA (mRNA) for this role. In rat liver, mRNA is polydisperse and the molecular weights range from 30 to  $800 \times 10^3$ ; in *E. coli* the range is from 2 to  $20 \times 10^5$ . According to recent evidence, the mRNA in early sea urchin embryos is present in several size classes. At the moment the preparation of mRNA in anything but very small quantities is impossible and represents a barrier to chemical investigation. Part of the difficulty is due to the short half-life of mRNA molecules in most cells. It varies but is of the order of a few minutes in bacteria.

A polymerase (2.7.7.6) present in bacterial cells (as well as in rat liver) catalyzes the assembly of ATP, GTP, UTP, and CTP into RNA, *but only if DNA is present as a primer*. Recent evidence suggests that only one strand of the DNA dimer has a template function. That the primer controls the sequential assembly of nucleotides is suggested by the following experimental knowledge:

- 1 The DNA used can be prepared from a source different from that of the enzyme. In such a mixed system the product is found to reflect the sequences in the primer.
- 2 The A/U/G/C ratios of the RNA product are complementary to the A/T/G/C ratios of the DNA primer.
- 3 Use of a synthetic polynucleotide<sup>†</sup> containing only T as a primer results in a product containing only A.
- 4 Hybrid molecules containing one monomer of RNA and one monomer of its primer DNA can be produced by “melting” the polymers, mixing, and “annealing” (that is, cooling slowly).

That RNA couples the genetic directive to the extranuclear synthetic apparatus was proposed twenty years ago, but it was not realized then that more than one type of RNA exists.

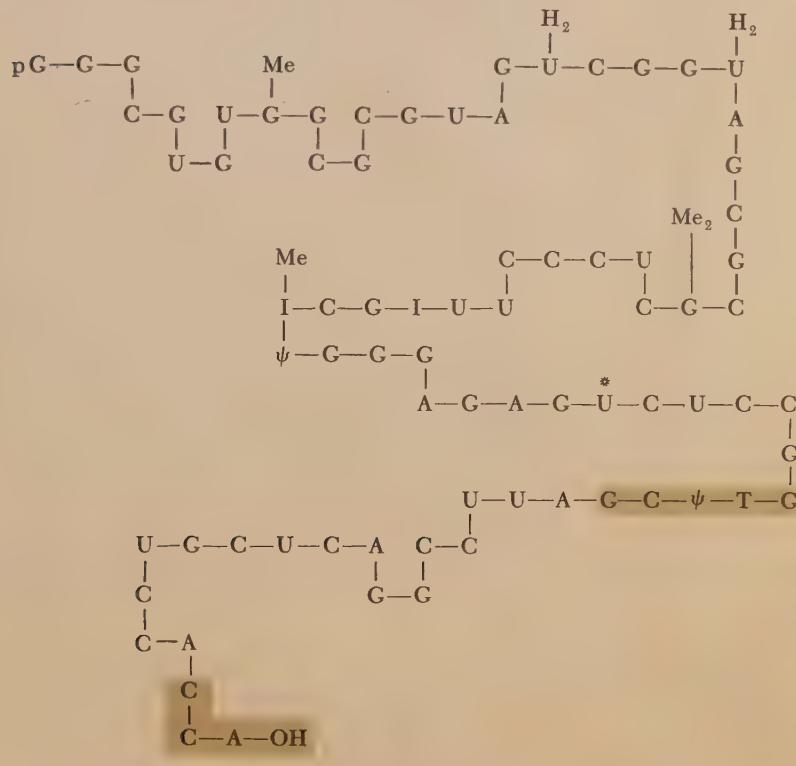
<sup>o</sup>The extinction coefficients, absorptivities, or absorption coefficients, of purine and pyrimidine bases in the ultraviolet (250 to 270 m $\mu$ ) are very high. By using quartz optics, Caspersson developed an elegant technique of microspectrophotometry that allowed quantitative estimations to be made on subcellular structures.

<sup>†</sup>Prepared with polynucleotide phosphorylase—see following pages.

**sRNA**

Another type of ribonucleic acid found outside the nucleus is that termed transfer or soluble RNA (sRNA). Crude preparations of sRNA are mixtures of different molecular species of similar size having a molecular weight of about 25,000. Recently it has been shown possible to isolate a single species and to determine the nucleotide sequence (REF. 2) recorded in Fig. 19.1. This is the first successful sequence determination of a biologically active nucleic acid. Since sRNA has a sequence complementary to the DNA of the same organism, it appears that the locus of synthesis is DNA. If we assume

**FIGURE 19.1**  
nucleotide sequence  
of yeast alanine  
sRNA



Me  
|  
G = 1-Me GUANOSINE

I = INOSINE

H<sub>2</sub>  
|  
U = 5,6-DIHYDRO URIDINE

U\* = MIXTURE OF U + UH<sub>2</sub>

Me<sub>2</sub>  
|  
G = N-2-DIMETHYL GUANOSINE

T = RIBOTHYMIDINE

ψ = PSEUDOURIDINE

MOLECULAR WEIGHT OF Na SALT  
= 26,600

Me  
|  
I = 1-METHYL INOSINE

pG = GUANOSINE-5'- PHOSPHATE

A-OH = ADENOSINE -3'-OH

that the complementarity extends from beginning to end of the sRNA, we may claim to know the nucleotide sequence of the gene when the sRNA sequence has been determined. In addition to the ribonucleotides of A, G, C, and U, several minor bases are to be noted. Evidence that pseudouridine is formed after the nucleotide sequence of RNA has been assembled has been presented. Also, modification of the bases by methylation seems to occur after the incorporation of the bases into the macromolecule. Two areas of the sequence in Fig. 19.1 require comment. The —CpCpA-3'-OH terminal ending appears to be common to all sRNA molecules, and the pentanucleotide —GpTpψpCpGp— is probably common to all types of yeast sRNA.

The three-dimensional structure of sRNA remains a controversial issue, although it is agreed that the nucleotide sequence is helical and probably coiled upon itself. In Fig. 19.1 the shape of the chain has no significance.

A function of sRNA is to form an intermediate with an activated amino acid for which it is specific. The intermediate is the obligate donor of an amino acyl group to a polypeptide chain in the process of elongation at the polysome locus. It is believed that each amino acid is matched by a singular sRNA having a singular sequence. However, even highly purified sRNA preparations may show activity with amino acids other than the major one. Our knowledge is as yet insufficient to decide whether this is due to contamination or whether there is some type of overlap in specificity. Preliminary data indicate that a high specificity exists in the reaction between the activated amino acid and sRNA.

For each amino acid there is an ACTIVATING ENZYME that catalyzes formation of the amino acyl adenylate.

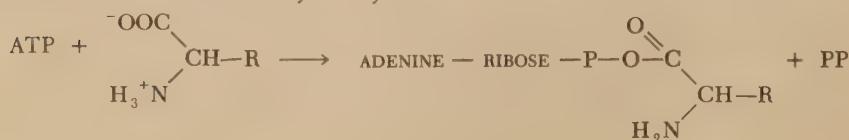
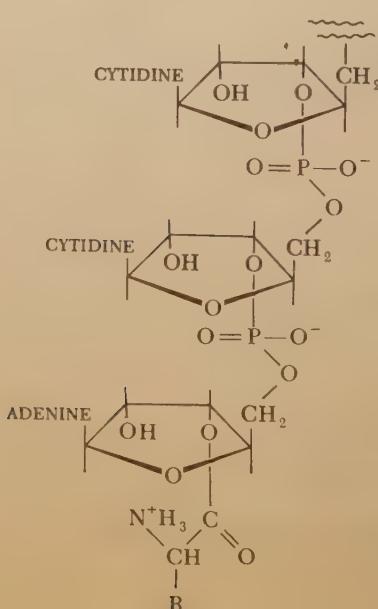


FIGURE 19.2  
portion of acyl  
sRNA



This unstable intermediate reacts with a suitable acceptor, in this case *transfer* or sRNA. To be specific, it is the 2'- or 3'-OH of the ribose, in the terminal adenylic acid, that appears to be the acceptor. In Fig. 19.2 the acyl group is shown attached to 2', but 3' may be correct. It is, then, this amino acyl sRNA that is the complex donor of the amino acid monomers for polypeptide synthesis.

The mechanistic details of sRNA synthesis are unknown. Since sRNA sequences are complementary to DNA, it is possible that the mechanisms for mRNA and sRNA syntheses are the same. In bacteria there are POLYNUCLEOTIDE PHOSPHORYLASES (2.7.7.8) whose substrates are nucleoside diphosphates. When one of these enzymes is incubated with ADP, GDP, UDP, or CDP, a variety of small RNA-like products can be produced. Such substances have been extremely useful experimentally, but it is doubtful that they are involved in the synthesis of RNA. The nucleotide sequences that form in a mixture of four nucleotide types have no regularity, and it is doubtful that such products are involved in protein synthesis. The doubt does not rule out an RNA that may be involved in some other process.

**rRNA** Most cellular RNA has a molecular weight of about  $10^6$  and is in the ribosomes (rRNA). Here the RNA is not small as in sRNA nor does it have a very short life as does mRNA. Ribosomes may be very plentiful in a cell. In *E. coli* during logarithmic growth they represent one-third of the cell mass and, as isolated, exist in several sizes. The size of such particles is usually expressed in Svedbergs,<sup>\*</sup> the unit of the sedimentation coefficient determined by using the ultracentrifuge. This is illustrated in Table 19.1.

\*See Appendix II.

TABLE 19.1  
*E. coli*  
ribosomes

<i>preparation conditions</i>	<i>s</i> $\times$ $10^{13}$	<i>molecular weight</i>
0.01 M phosphate pH 7.0		
0.001 M Mg <sup>++</sup>	30	$0.8 \times 10^6$
	50	$1.8 \times 10^6$
0.01 M TRIS*, pH 7.4		
0.001 M Mg <sup>++</sup>	70	$2.6 \times 10^6$
0.005 M Mg <sup>++</sup>	100	$5.2 \times 10^6$
<i>comparison of S values</i>		
ribonuclease	1.6	13,685
hemoglobin	4.3	64,000
urease	18.6	500,000

\* Tris (hydroxymethyl) amino methane—often used for buffering near pH 7 as an alternative to phosphate. The ionic strength for a given concentration is much lower and specific ion effects may be avoided.

A glance at the table reveals that, in terms of molecular weight,  $30\text{ S} + 50\text{ S} = 70\text{ S}$  and that  $70\text{ S} + 70\text{ S} = 100\text{ S}$ . Note that the size of a large protein is not far below that of a small ribosome. (Not all ribosomes react with  $\text{Mg}^{++}$  as seen in Table 19.1.)

In almost all types of cells the ribosomes can be isolated as 70- to 85-S particles and can be shown to consist of 30-S and 50-S subunits. Moreover, ribosomes appear to be present as much larger aggregates up to about 600 S. For such aggregates as these, whose density and shape would be similar,  $S^2 = kM$ , a straight-line function. Particles of 73, 113, 147, 178, . . . , 280 S isolated from rat liver are found to represent 1, 2, 3, 4, . . . , 8 repeating molecular-weight ( $M$ ) units. The larger aggregates, above about five repeating units, are those involved in the bulk of protein synthesis.

Both ribosomal RNA and sRNA evince some complementarity to DNA. What proportion is complementary is quite unknown, and substantial portions of these RNA types may show no species specificity. There is some evidence that ribosomal RNA is produced in the nuclear region and that it can hybridize to some extent with one strand of its homologous DNA. Moreover, it has been reported that the loci for sRNA and rRNA have been identified on the chromosomes of *B. subtilis*. Despite this evidence, it is as yet too early to designate ribosomal RNA, or soluble RNA, synthesis as "DNA-directed."

**comment** A perusal of Allen's book (REF. 4) will recall to the mature biochemist the welter of questions that have permeated research on the ribonucleic acids. Most of the published research on these substances was done at a time when we had very little idea of their function. Ribonucleic acids were shown to exist in the nucleus and particularly the nucleolus (Sec. 25), in the microsomal fraction, in the supernatant fraction, and even, in traces, in the mitochondria.

At present mRNA must be considered more of a promising working hypothesis than a well-characterized substance. Despite our lack of detailed knowledge, the *idea* of a messenger RNA has been distinctly productive. The use of *model* mRNA, as described in the next section, has made possible the experimental testing of the genetic code. It is also known that the RNA of some viruses also behaves as a template. The recent work on sRNA has made possible an exploitation that will probably greatly amplify our knowledge of this biopolymer. The problems with regard to the chemistry of rRNA are again more serious. This is a very large polymer, and the "sequencing" problems are formidable. As you will see in the next section, the function of the ribosome is a most intriguing one, but again, the details are shrouded.

To the biochemist the ribonucleic acids have furnished more than one surprise. Until recently it seemed unnecessary to consider as structural bases any aside from A, G, C, and U. There is now excellent evidence that the minor bases are very much a part of the structure. More than that, they seem to arise by the modification of the bases already in polymeric form (REF. 3). The findings stimulate the imagination. How is such modification controlled?

Can environmental factors participate in the control? Are all polymers subject to modification or is there a size limit? In principle, the results imply that the DNA-RNA relation may produce a genetically determined structure but that fine details may be altered by metabolism.

Although the majority of investigations on the ribonucleic acids is devoted to the function of the acids in protein synthesis, other functions are gaining attention. The neuron is notable for its ability to produce RNA in quantity and with a composition that varies with stimulation. It seems certain that RNA metabolism will form an interesting chapter in the biochemistry of the neuron and its response to the environment.

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# 20

## protein synthesis

In the cell, protein synthesis occurs at several loci, but in each instance the synthetic complex seems to involve aggregates of macromolecules. These aggregates of ribosomes, or monosomes, composed of rRNA are believed to be connected by a filament of mRNA that supplies the synthetic directive. (This is as yet a working hypothesis.) The units from which the polypeptide (protein) chain is assembled are supplied as amino acyl sRNA complexes. Presumably there is a sequence of bases in sRNA complementary to the "message" sequence of mRNA. In short, this is a process unlike any other and involving complex interactions of three kinds of macromolecules. The monosome presumably provides a chemical and physical environment for the sequential formation of the covalent peptide bond. The mRNA must provide a translatable code (unidirectional from the 5' end to the 3' end); a sequence of purines and pyrimidines that somehow dictates the corresponding sequence of amino acids.

Since many monosomes may be associated with a single molecule of mRNA, it is likely that the assembly of several polypeptide chains occurs simultaneously. This complex must be supplied with amino acyl sRNA complexes of all the amino acids simultaneously. A process of such complexity might be expected to be slow when compared with the reactions of small molecules characteristic of metabolism and usually occurring in microseconds or less. Current guesses are that polypeptides of about 20,000 molecular-weight units are assembled in about five seconds in bacteria. In higher organisms the process may require a few minutes. Other estimates have been stated as 100 amino acid residues per second in bacteria and 2 residues per second in hemoglobin synthesis. Labeling experiments have shown that, in hemoglobin synthesis, the polypeptide sequence is started at the amino end.

### **coding**

The deciphering of the "RNA codewords" has been a fascinating task. As Perutz has remarked (REF. 11, Sec. 19), "The entire edifice of molecular biology is based on the hypothesis of the sequence of bases in a nucleic acid chain forming a code which determines the sequence of amino acids in a polypeptide chain." In 1961, Nirenberg and Matthei made the seminal experimental observation that the use of an artificial mRNA, polyuridylic acid (poly U), added to a protein-synthesizing system from *E. coli*, yielded a polypeptide containing only phenylalanine. The polynucleotides used as mRNA in this and many subsequent experiments were products of polynucleotide phosphorylase noted in Sec. 19. It was found that the activity of the mRNA was nearly proportional to its length and that only single-stranded polynucleotides were effective. Thus monosomes, inactive alone, became synthetically potent as soon as they came into contact with a single-stranded polynucleotide

of a suitable length. Although poly U alone potentiated the synthesis of only polyphenylalanine, mixtures of polynucleotides were found to allow the synthesis of mixed polypeptides.

Ten years earlier Dounce had reasoned that the relation between 4 bases and 20 amino acids must involve base sequences. If pairs were involved, only 16 permutations could result, and it was speculated that triplets of bases must code for each amino acid. But there are 64 triplets and only 20 amino acids, and it was therefore speculated that the code is "degenerate"—a term meaning that there is more than one triplet per amino acid. Genetic data are thought to contraindicate overlapping. It was found that poly U (one triplet possible) allowed polypeptide formation for only one amino acid, poly UC (8 triplets possible) catalyzed or directed polypeptide formation for 4 amino acids, poly ACG (27 triplets) widened the possibility to 9 amino acids. These polynucleotides were roughly equal in effectiveness with respect to the quantity used.

Deciphering of the code has been facilitated by the recent observation that trinucleotides can specify the binding of amino acids to ribosomes. If an mRNA model such as polyuridylic acid, or even a trinucleotide, is added to ribosomes, a complex forms. This complex further binds specific sRNA

TABLE 20.1  
codon assignments from polypeptide synthesis and stimulation of aminoacyl-sRNA binding to ribosomes\*

		second				
	first	U	C	A	G	third
U		Phe	Ser	Tyr	Cys	U
		Phe	Ser	Tyr	Cys	C
		Ser				A
		Leu	Ser		Try	G
C			Pro	His	Arg	U
		Leu	Pro	His	Arg	C
			Pro	Gln	Arg	A
		Leu	Pro	Gln	Arg	G
A		Ileu	Thr	Asn	Ser	U
		Ileu	Thr	Asn	Ser	C
			Thr	Lys	Arg	A
		Met	Thr	Lys		G
G		Val	Ala	Asp	Gly	U
		Val	Ala	Asp	Gly	C
		Val	Ala	Glu	Gly	A
		Val	Ala	Glu	Gly	G

\* The assignments not underlined are on the basis of binding experiments only. The assignments singly underlined are on the basis of copolyptide and/or homopolyptide synthesis and gave essentially no binding. The assignments doubly underlined are derived from both polypeptide synthesis and binding experiments.

in the absence of protein synthesis. The specificity of the sRNA can be determined by using  $^{14}\text{C}$ -labeled aminoacyl sRNA, so that the ribosomes become labeled only if the mRNA model codes for the labeled amino acid. The method is flawed in that not all trinucleotides are complexed with the same firmness. Despite this shortcoming, the technique permits the identification of many triplet "codons" and, most important, the sequence of bases within the codon (REF. 3). The use of either pUpUpU or pUpUpC serves as an mRNA codon<sup>\*</sup> for Phe. For Ser the codon nucleotides are pUpCpC and pUpCpU. No text can hope to do justice to a subject developing at the rate of this one.<sup>†</sup>

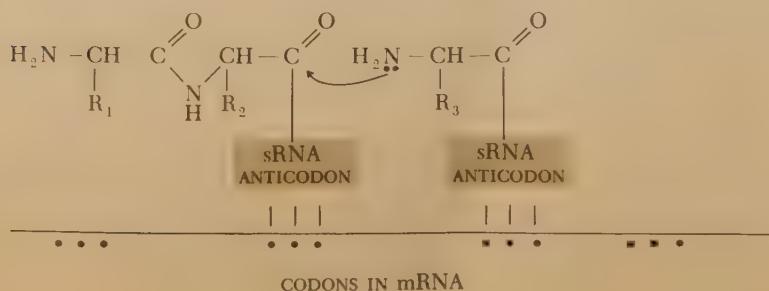
It is obvious that many questions about the mechanism of protein synthesis want answering. For example, it is necessary to add GTP to experimental systems but its function in the synthetic process is unknown. Proteins cover a wide variety of polypeptide chain lengths. Shall we assume the same variety of mRNA lengths? By itself mRNA does not bond aminoacyl sRNA. How is the latter bound to the ribosome? The addition of organic solvents to *E. coli* systems not only increases the activity in incorporation of amino acids in peptide linkage but appears to change the coding. The level of  $\text{Mg}^{++}$  also appears to change the coding specificity. Do such experimental observations have biological significance? Finally, despite the very considerable experimental coups achieved, no net synthesis of protein in a truly cell-free system has yet been achieved. It is likely that polypeptide chains, unfinished at the time of cell breakage, may be completed *in vitro*. Initiation of new chains requires reattachment of the ribosomes to mRNA and, after cell breakage, this occurs at a much lower rate. It is known that  $\text{Mg}^{++}$  and an ATP-synthesizing system are requisite for attachment, but unknown factors must dictate that detachment prevail *in vitro*.

For the chemist, any adequate discussion of polymer formation must include a description of chain initiation and a mechanism for chain termination. Some recent genetic and chemical evidence suggests that termination involves an sRNA having a "nonsense" anticodon. In Fig. 20.1 the mechanism depicted would require something of this kind to release the completed poly-

<sup>\*</sup> Codon sequences are in mRNA; anticodon (complementary) sequences are in sRNA. mRNA "reads" 5' → 3', as noted on the first page of this section.

<sup>†</sup> At present Khorana and his coworkers are testing the coding properties of a large number of synthetic oligonucleotides with sequences known from the course of laboratory synthesis. The material includes not only trinucleotides but also oligonucleotides of repeating sequences (REF. 19).

**FIGURE 20.1**  
extension of a  
polypeptide chain



peptide chain from the synthesis complex. In regard to chain initiation there is the suspicion that a special sRNA that will accommodate an acyl-substituted amino acid may be required. Thus the free amino groups seen at the left in Fig. 20.1 may be formylated or acetylated initially. If this were methionine, for example, one sRNA for Met would facilitate insertion of the amino acid at every position except the initial one, whereas another sRNA would be specific for formyl-Met and the initial position.

### mutation

It is tempting to believe that we now have some substantial and revealing clues to the details of protein synthesis. A corollary is that the protein patterns of a cell reflect the fundamental repeating pattern of the cell. This epigenetic pattern is maintained over a much longer time scale than any metabolic activity, and it persists beyond the period of existence of a single cell. Enthusiasts are occasionally prone to speak of DNA as immortal, but this is poetic license usurped by nonpoets.<sup>o</sup> Any *specific* DNA is susceptible to change, and that change has long ago been termed a *mutation* by the biologist. If DNA is indeed *the* genetic substance, then it must be very stable through an extraordinarily large number of copies and yet provide the opportunity for change. Suddenly, then, a mutational event has now become a chemical concern and the biochemist must inquire into the nature of a rare, random, but observable occurrence. In fact, the production and control of mutation in microorganisms has provided experimentalists with a technique difficult to overvalue.

In bacteria there is evidence that a major portion of the DNA strand is given over to the coding of mRNA. Presumably, there are small segments devoted to sRNA and ribosomal RNA. Many agents can effect changes in the DNA strand: chemical agents of many kinds and radiation of various types. Such changes, if not lethal, will be discernible genetically or biochemically. Many workers are attempting to gather direct chemical evidence. Some kinds of changes are obvious. If an agent capable of causing deamination is added to DNA, it might be expected that a few cytosines would be converted to uracils. Were this to happen, the normal C-G hydrogen bonding would not occur.

There is also evidence that ultraviolet radiation of the proper wavelength can cause the formation of a dimer between adjacent thymine residues and between adjacent cytosine residues. Such dimers are usually lethal. In addition it may be speculated that a mistake may be made, chemically, during replication, with the incorporation of the wrong base or a derivative of the right one. Finally, the suggestion has been made that "tunneling"<sup>†</sup> in DNA

<sup>o</sup> A bemused reviewer has reminded me that the roach has been on earth  $5 \times 10^8$  years. It is difficult for me to imagine anything less poetic.

<sup>†</sup>The rate of deuteration of sodium DNA is extremely fast; thus the hydrogens involved in hydrogen bonding exchange readily with protons or water molecules. The rate for  $^1\text{H}$  is six times that of  $^2\text{H}$  which, it is conjectured, means that quantum-mechanical tunneling may occur (REF. 14). Such an observation is congruent with the random nature of the mutation process, since neither process is affected by temperature, pressure, concentration, and so on. It does not explain, however, how DNA molecules can remain stable for so long.

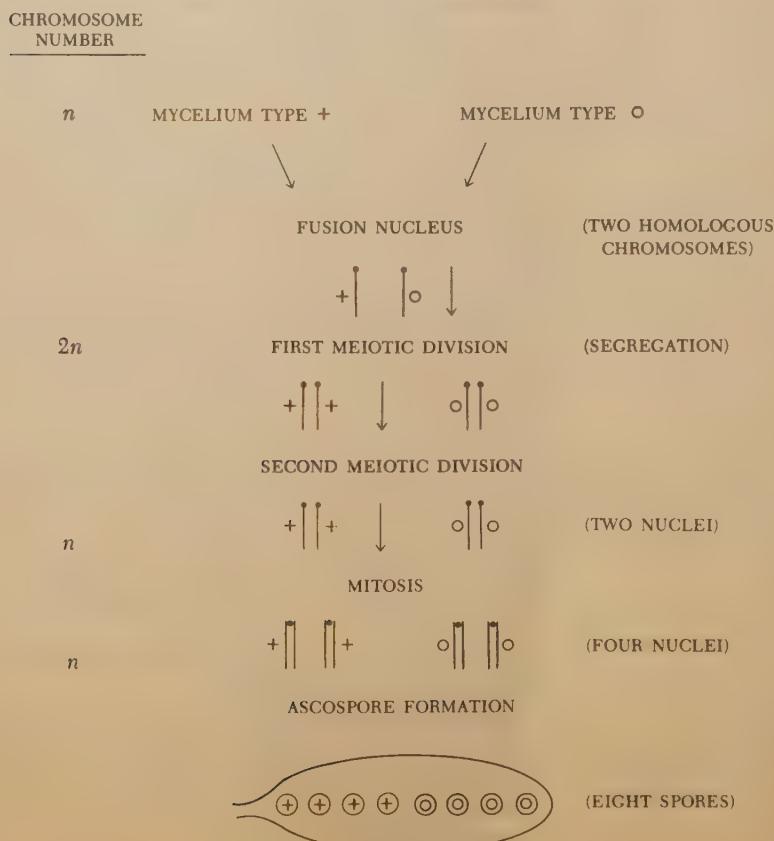
hydrogen bonds could lead to tautomerization which would, of course, produce a sequence aberration.

In addition to these changes are the reversions. For example, radiation damage that causes dimer formation can be reversed both by enzymes, which cause monomerization, and by radiation differing in wavelength, which can also cause dimer instability.

### gene-enzyme relations

During the late 1930s, Beadle and Tatum formulated a concept that became a challenging and productive working hypothesis. It developed out of a series of investigations on *Neurospora crassa* and was referred to as the "one gene-one enzyme" hypothesis. *Neurospora* has biological properties that make it a desirable experimental material. It is a fungus whose sexual spores are produced in a case-like ascus, and their position reflects the biological events of meiosis and mitosis. In Fig. 20.2 is represented the sequence of events that occur for each chromosome, which represents a multiplicity of genes. With care the spores can be separated sequentially and each spore used to inoculate a tube of media. Each spore is haploid, has seven chromosomes, and has only one gene of each type. The procedure thus results in a rapid sensing of genetic events.

**FIGURE 20.2** spore formation in *Neurospora crassa* after sexual fusion



It was found possible to culture wild-type *Neurospora* on a chemically defined medium containing inorganic salts, sugar, and biotin. This was termed a "minimal" medium. Cultures were exposed to mutation-inducing radiation, and the spores were sorted out and sown on a *complete* medium, containing several vitamins and nitrogen-containing compounds. Each culture resulting was then tested for the ability to grow on *minimal* medium. Some cultures were found to lack the ability of the wild type to grow, a sign of mutational alteration. The growth dependence of each derivative culture was then determined to identify the nutritional need: amino acid, purine, pyrimidine, or vitamin.

The results of this kind of experimentation showed that mutation resulted in the loss of an enzyme in a metabolic reaction sequence. Moreover, the correspondence appeared to be 1/1. This simplistic view was later modified when it was recognized that more than one gene could be involved in one enzyme. Hence, at present, the working hypothesis is "one gene—one polypeptide chain." An alternative statement that was also made at one time implicated a mechanism—this was the "one gene—one ribosome—one enzyme" hypothesis. The following observations have not favored the latter alternative.

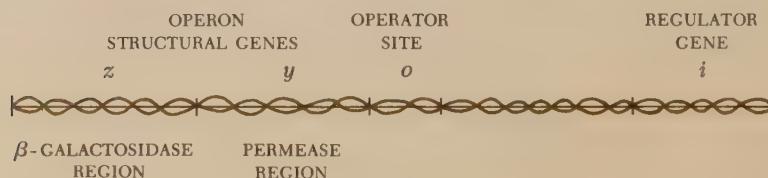
The nucleotide composition of ribosomal RNA does not correspond to that of the DNA congener *in toto*, and it is doubtful that ribosomal RNA contains a large section of the purine-pyrimidine code. The molecular weight of ribosomal RNA varies little and probably includes only a small number of sequence types. If so, it can hardly correspond to polypeptides of varying length. If there were a special ribosome for each polypeptide, there should be more than a thousand kinds per cell. This would mean a very low synthetic rate for any one ribosome.

The RNA of ribosomes, in bacteria at least, is quite stable and does not form or disappear rapidly. It has been found that 5-fluorouracil can be incorporated into RNA in place of uracil. The effect is almost immediate ( $\beta$ -GALACTOSIDASE synthesis in *E. coli* is blocked, for example) when the analog is added to the medium, and therefore it seems unlikely that the analog is being incorporated into ribosomal RNA. These observations support the idea that the "one gene—one polypeptide" relation is mediated not by a stable ribosome, but by a labile mRNA.

There is now substantial evidence for the following working hypothesis, or model: that a section of DNA is a *structural gene*,<sup>9</sup> that the linear base sequence of DNA is converted to an amino acid sequence via mRNA, that a point mutation in a structural gene affects the synthesis of only one kind of polypeptide chain, and that this effect may be restricted to a single amino acid.

<sup>9</sup> Structural genes define the sequence of amino acids in the synthesis of a polypeptide chain. At present it is thought that sequence also dictates three-dimensional relations in proteins. Further, there has been postulated a *regulator* gene that governs the rate of protein synthesis, perhaps by control of cytoplasmic factors. The term *operon* refers to several linked structural genes. When the rate of synthesis of any protein coded by a gene in the operon is affected by the regulator, each of the other genes in the operon is affected similarly.

Consonant with this model is the relation "one *structural* gene—one polypeptide chain." Work on several proteins has now developed into a corroboration of this view, but the most intensive investigations have focused on the enzyme  $\beta$ -GALACTOSIDASE (3.2.1.23). The ability of *E. coli* to utilize  $\beta$ -galactosides, such as lactose, as energy sources is dependent on the presence of both a  $\beta$ -galactosidase (for hydrolysis) and a "permease" that potentiates transfer of the substrate into the cell. The application of refined genetic mapping techniques has revealed the close relation of the structural genes for  $\beta$ -galactosidase and permease in the operon and another adjacent locus termed the *operator* gene. This gene presumably affects the "transcription" of the structural genes, the process by which the base sequence is translated or transcribed into the amino acid sequence. The control of polypeptide



chain synthesis by the regulator gene is thought to be mediated by the operator. A more detailed discussion is to be found in Sec. 31.

Presentation of further evidence in support of the one gene—one polypeptide concept requires some detailed knowledge of protein structure, and this will be our next concern.

#### comment

Of all the sections in this book, this one has the highest content of molecular biology. The development of molecular biology demanded that attention be given to the synthesis of macromolecules, that more effort be expended on the epigenetic process. At this point biochemistry and biology become inextricably interwoven, and we have seen that the boundaries of disciplines are occasionally better ignored than recognized.

Several good model systems for studying protein synthesis are now at hand. By work with preparations derived from erythrocytes, from *E. coli*, and from thymus nuclei, much has been accomplished in assembling workable and reproducible systems. It is clear and nondebatable that a "message" passes from DNA through RNA to a complicated encounter of macromolecules that finally results in protein. It is not established that synthesis is identical in all cells, nor has true cell-free synthesis been achieved. Indeed, almost all studies have been made with systems that produce enzymes or proteins with a special function, such as hemoglobin, and therefore it is by no means certain that only one type of protein synthesis exists.

It is true that it is the DNA-RNA-protein relationships that furnish the cell with its complement of catalysts, and the complex of the catalyzed reactions is the concern of classical biochemistry. But it is also true that no macromolecular synthesis can occur outside, or independently of, the matrix of reactions. Hence the study of protein synthesis, indeed of all macromolec-

ular synthesis, like the study of metabolism, involves the study of models. In order to gain some judgment concerning the validity of these models, other facts and ideas must be assessed. Protein synthesis will be considered again in Sec. 23.

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# 21

## fibrous proteins and X-ray diffraction

The discussion thus far has not referred to a diversity of protein types, but if anything meaningful is to be said further about protein synthesis, we must pause to consider protein structure. Some of the least ambiguous data for protein structure are derived from X-ray diffraction studies. But the diffraction patterns easiest to interpret derive not from the globular proteins, many of which are enzymes, but from the fibrous proteins. From a purely pedagogic impulse, then, I have decided to discuss these proteins first.

In multicellular organisms cohesiveness and form depend to some extent on fibers containing protein. Not only are the structures fibrous when viewed with the microscope, they also are formed from "fibrous" proteins that are highly asymmetric. The most prevalent protein of this type in animals, termed collagen, is characteristic of skin, tendon, and other forms of connective tissue. Presumably it is synthesized within cells called fibroblasts, somehow moves into the intercellular spaces, and finally aggregates to form fibers of several classes. *Fibers* are several micra in diameter and are easily visible. *Fibrils*, clearly demonstrable with the electron microscope, are about  $10^3$  Å wide. *Protocollagen* is the name given to monomeric units of a protofibril. These units are about 14 by 2,800 Å and have a molecular weight of about 300,000.

The collagen group of proteins is characterized by amino acid composition, X-ray diffraction, and the patterns observable by electron microscopy. The composition shown in Table 21.1 is unusual in that three amino acids constitute more than 50% of the total, and it is noteworthy that both cysteine and tryptophan are absent. The collagen content of different tissues varies, but for mammals, it forms about 25% of the protein. Collagen-like molecules are also found in less highly evolved creatures.

TABLE 21.1  
amino acid  
composition  
of collagen  
(human tendon)  
expressed as  
residues amino  
acid per 1,000  
residues

Gly	324	Arg	49	Met	6
Pro	126	Asp	48	Tyr	4
OH—Pro	92	Leu	26	CysH	—
Glu	72	Ser	37	Try	—
Ala	111	Lys	22		
		Phe	14		
		Thr	18.5		
		Val	25		
		Ileu	11		
		OH—Lys	9		
		His	5		

**FIGURE 21.1**  
X-ray diffraction  
pattern of collagen

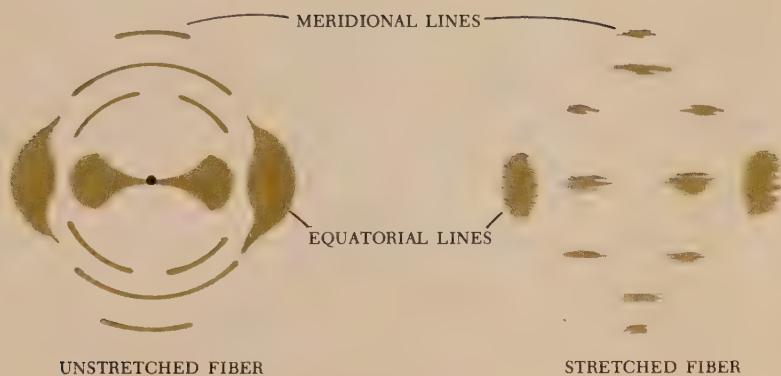


Figure 21.1 is a sketch of X-ray diffraction patterns obtained with collagen fibers. There is not as yet complete agreement on the interpretation of these patterns for collagen, but some conclusions can be drawn. In order to present them, an elementary discussion of X-ray diffraction is first necessary.

Many of us learned early in our scientific education that X rays are diffracted, reflected, and scattered by atoms in a manner somewhat similar to that in which plane surfaces reflect light. When a crystal is interposed between an X-ray source and a sensitive film, the pattern obtained is seen to change with the orientation of the crystal. This pattern is due to constructive or destructive interference. Consider a beam of X rays striking layers of atoms in a crystal as in Fig. 21.2.

The diagram is grossly out of scale, of course; it is intended that the distance from  $A$  to the film is essentially the same as the distance from  $B$  to the film, since  $AB'$  is very small in comparison to the distance to the film. The scattering from  $B$  may be out of phase with that from  $A$ . At the film, scattering from  $A$  is out of phase by  $2\pi(AB'/\lambda)$  and, depending on the incident angle and the wavelength of the X-ray beam, there may be additive or destructive inter-

**FIGURE 21.2**  
additive or destruc-  
tive interference in  
X-ray diffraction

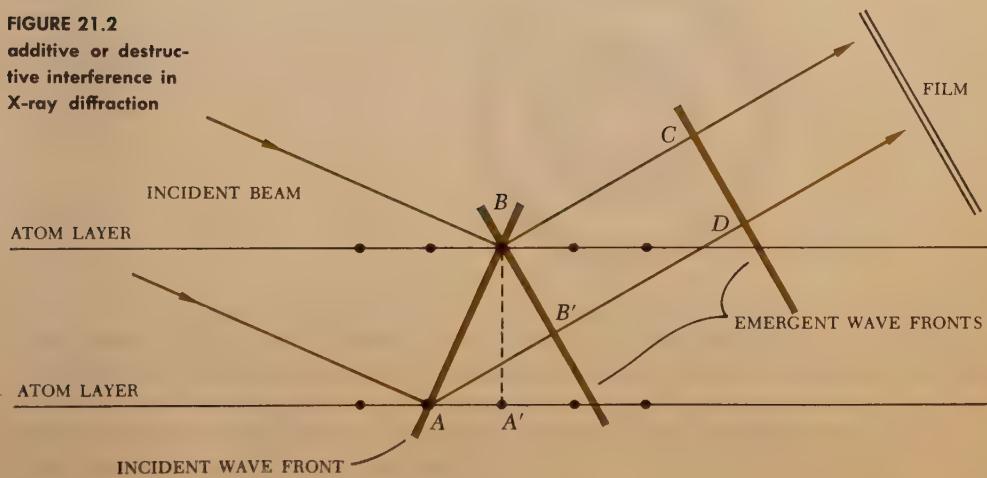
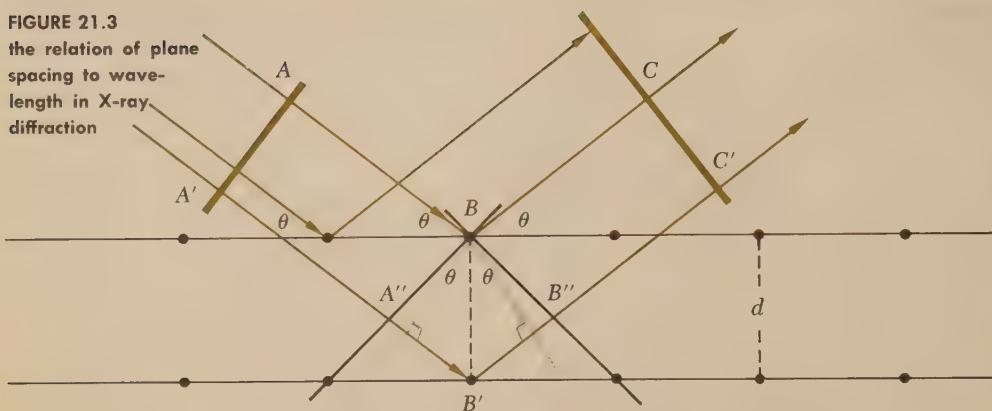


FIGURE 21.3

the relation of plane spacing to wavelength in X-ray diffraction



ference. For our purposes maximum constructive interference is desirable, and this occurs when the emergent rays are exactly  $2\pi$ , or a multiple of  $2\pi$ , apart. This condition varies with both wavelength and the distance  $A'B$ . A two-dimensional plane lattice of atoms would yield specular reflection as from a mirror.

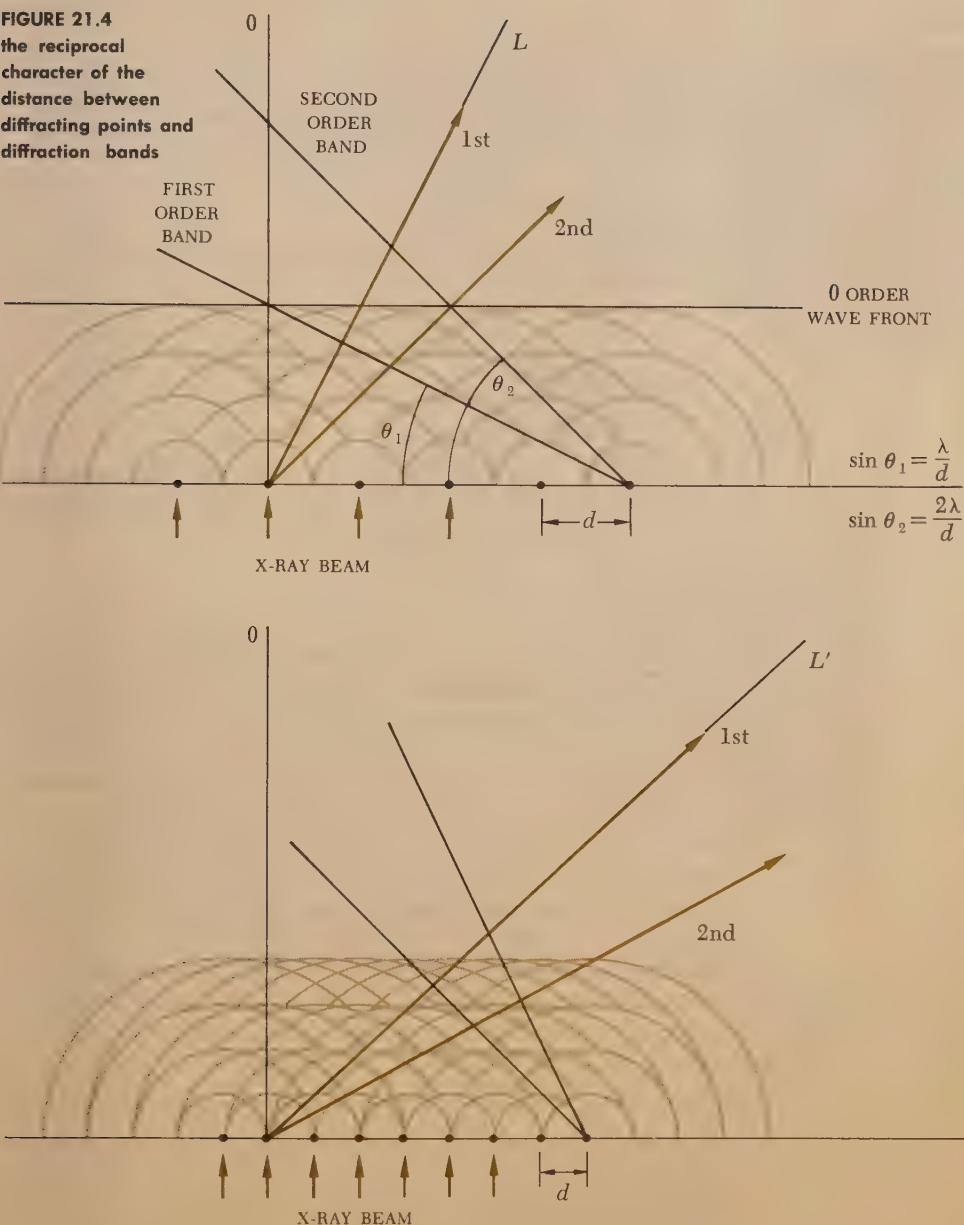
A study of diffraction spot intensities allows a calculation of plane spacings as indicated by the construction in Fig. 21.3. Considering the intensity of the two beams at plane  $C-C'$  the difference in path length is  $(A'B'C' - ABC)$ . The difference,  $A''B'B''$ , is  $2d \sin \theta$ . It follows that  $2d \sin \theta$  should have a value of  $n\lambda$  if the rays are to reinforce each other (Bragg equation). Note that  $2\theta$  is the angle between incident and emergent rays.

If a beam of X rays is directed at a substance having very little ordered structure, such as a glass or certain carbon preparations, then the following pattern is seen. The halos are diffuse but distinctive, and they are interpreted to signify that atoms cannot approach closer than the sum of the radii. The patterns thus reflect atom sizes.



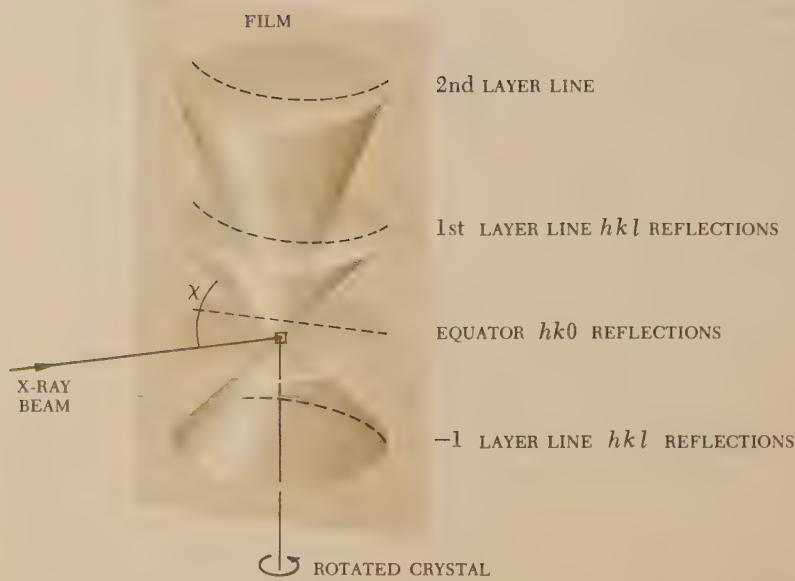
If X rays impinge on an array of atoms equidistant from each other, and if the incident beam is at right angles to the array, the diffraction pattern will be a pattern of light and dark bands. In Fig. 21.4 the first-order diffraction band is shown to be due to reinforcement of wave fronts in phase. A weaker second-order band is also shown. The relation  $\lambda = d \sin \theta$  is obvious on inspection, and  $n\lambda = d \sin \theta$  is thus valid for any band order. The equation states that  $d$

**FIGURE 21.4**  
the reciprocal  
character of the  
distance between  
diffracting points and  
diffraction bands



and  $\lambda$  have a reciprocal relation, but the construction illustrates the reciprocal relation between  $d$  and  $OL$ . If  $OL$  is the distance between the undiffracted beam and the position of the first-order beam at the detector, then diffracting atoms close together will produce bands distinctly separated. If the atoms are relatively widely separated, then the distance  $OL$  will be short. An X-ray diffraction pattern obtained from a three-dimensional array has the same recip-

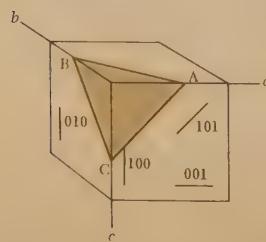
**FIGURE 21.5**  
X-ray scattering by  
rotating crystal



rocal nature, and the diffraction pattern results in layer lines on the film as shown in Fig. 21.5. In this case there is scattering from planes of atoms and rotation of a single crystal generates a series of cones of reflections. The figure is intended to illustrate that the layer lines will be cone sections if the film is flat. Straight lines can be obtained with a cylindrical film. Measurement of the distance between the layer lines allows calculation of the "unit-cell" size, since  $\sin \chi = 1\lambda/c$ . The angle  $\chi$  is designated in the diagram. The quantity 1 is one of the Miller indices  $h$ ,  $k$ , and  $l^*$ , and  $c$  is the unit-cell edge on the  $z$  coordinate. The distance of the layer line from the equator,  $s$ , and the radius of the camera,  $r$ , furnish the data needed, since  $\sin \chi = s/(r^2 + s^2)^{1/2}$ .

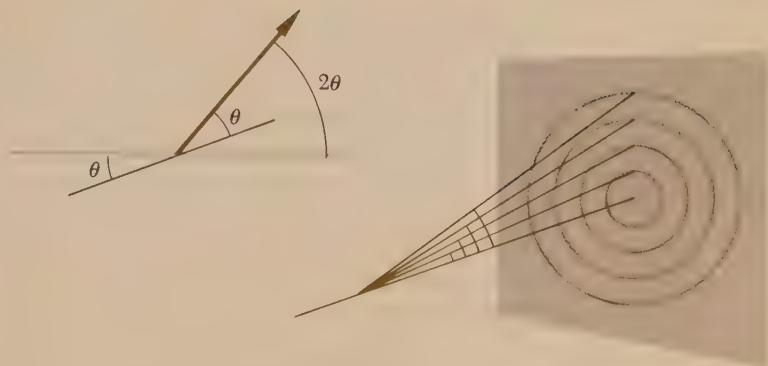
The layer lines above are formed from a linear array of reflections. It can be shown that the positions of the lines and spots are a function of the geometry of the crystal lattice. The intensities depend on the kinds of atoms in the lattice.

\* Let any crystal face such as  $ABC$  be related to a reference plane parallel to a crystal axis with normal intercepts of 1 on  $a$ ,  $b$ ,  $c$ .

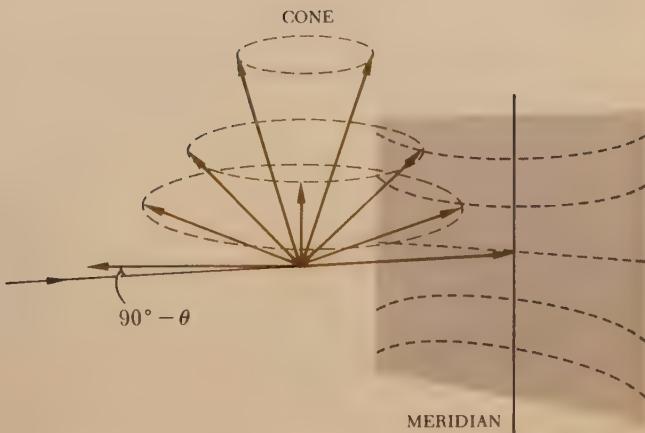


Suppose  $ABC$  has intercepts of 2.7, 4, 2. Multiply the reciprocals by a factor which will yield an integer. In this case multiplication by 8 will yield 3, 2, 4, which are  $h$ ,  $k$ ,  $l$ . Intervals between adjacent planes are  $a/h$ ,  $b/k$ , and  $c/l$ .

**FIGURE 21.6**  
X-ray diffraction pattern for powder.  
Each angle of  $2\theta$  is  
for a given set of  
crystallographic planes



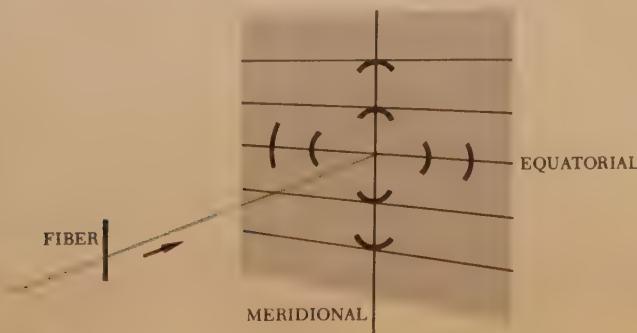
**FIGURE 21.7**  
X-ray diffraction diagram for crystal



If a beam of X rays is directed at a crystalline powder, a series of distinct rings forms the diffraction pattern in Fig. 21.6.

For the case of the single crystal, a much more explicit and useful pattern is obtained. In Fig. 21.7, the crystal is meant to be rotating about an axis, and this generates a cone of reflection. The layer lines on the film represent intersections of these cones. The pattern varies with the crystal system; that is, a tetragonal crystal yields a square pattern.

**FIGURE 21.8**  
fiber X-ray  
diffraction  
pattern



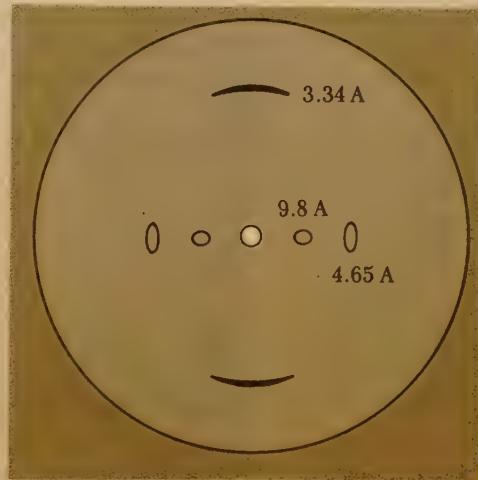
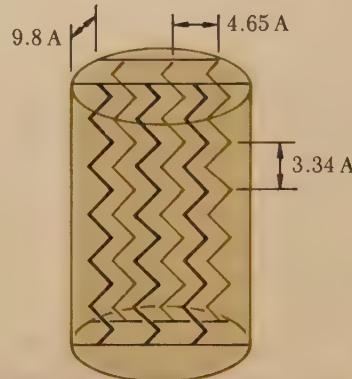
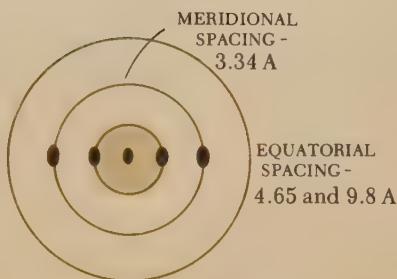
In the study of fibrous proteins, fiber diffraction patterns are obtained. Again there is obtained a pattern of spots similar to that of the rotating crystal above, since there is random orientation of crystalline areas within the fiber. Equatorial spots correspond to diffraction from planes parallel to the fiber axis; meridional spots, to repeating patterns along the fiber.

The simplest X-ray diffraction patterns of the protein class are generated by the relatively insoluble proteins of epidermis, hair, horn, silk, and feathers (keratins). Two different patterns are seen: those from the mounted sheet or fiber that might yield an " $\alpha$ " or a " $\beta$ " keratin pattern and those from the stretched fibers which are more often  $\beta$ . Thus it was noted that:



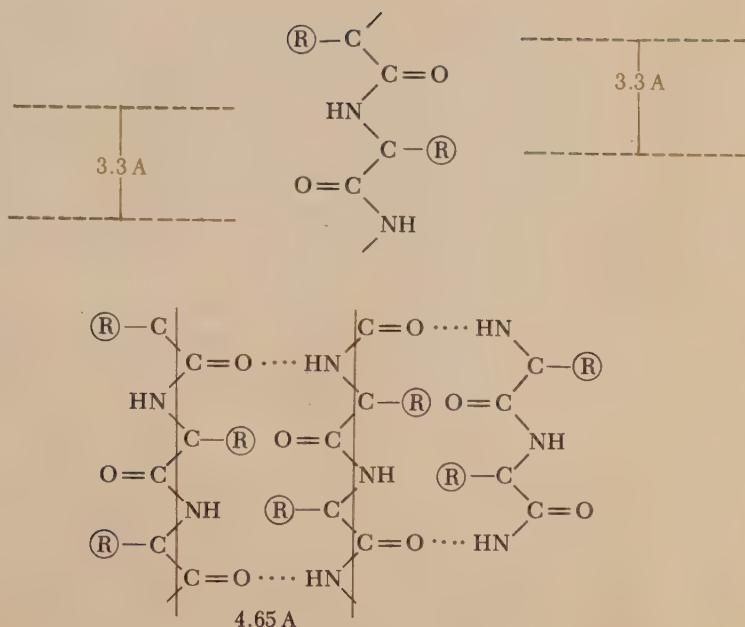
The  $\beta$ -keratin patterns proved the simplest to interpret and resembled the pattern of Fig. 21.9.

**FIGURE 21.9**  
X-ray diffraction  
pattern for  
 $\beta$ -keratin and the  
corresponding planes  
in a fiber [6]



$\alpha$   $\longleftrightarrow$   $\beta$

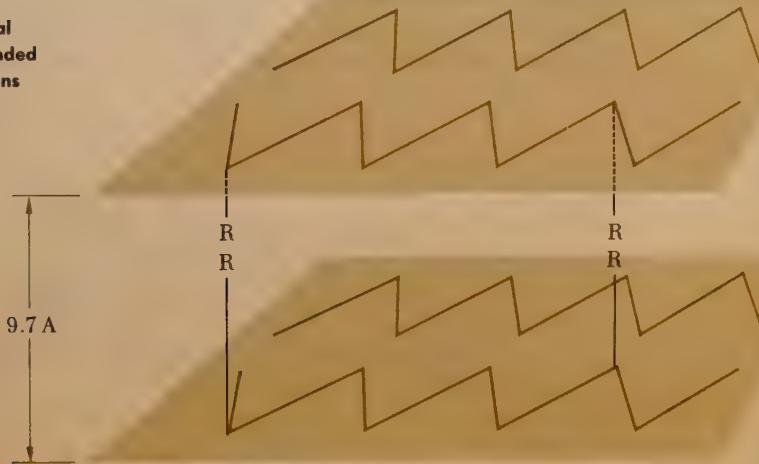
**FIGURE 21.10**  
extended  
polypeptide chain  
conformations (see  
also Fig. 11.1)



When a model of a fully extended polypeptide chain is assembled, it is found that the distance between identical points corresponds to the 3.3-Å repeat distance of the diffraction pattern. The repeating planes in the fiber were deduced to be those in the figure. The corresponding polypeptide chain conformations that are related to the 3.3- and 4.65-Å repeat distances are diagrammed in Fig. 21.10.

In both figures the R groups should be envisioned as alternating above and below the plane of the paper or the plane of the peptide bond. The lower figure is intended to show that the proper register of two or more extended chains permits side-to-side hydrogen bonding. A three-dimensional array of extended chains may be imagined to appear as in Fig. 21.11. This drawing is

**FIGURE 21.11**  
three-dimensional  
relation of extended  
polypeptide chains



obviously incomplete and is intended only to illustrate that two-dimensional arrays cannot be packed too closely. The amino acid residues will prevent packing closer than 9.7 Å. The possible residue interactions represent various types of bonding and include disulfide bonds and ionic and hydrophobic bonding. To sum up, the 3.3-Å repeat distance represents the organization of covalent peptide bonds and the 4.65-Å spacing is due to hydrogen bonding between peptide nitrogen and carbonyl oxygen. The 9.7-Å repeat distance relates to architectural features involving several types of bonding but probably only those of residues.

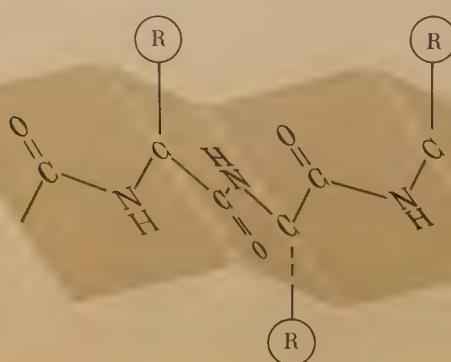
The 3.3-Å dimension is significantly smaller than the 3.6-Å distance obtained from model studies. It was proposed by Pauling and Corey that the polypeptide chain is not fully extended but forms a puckered structure which, when hydrogen-bonded to others, forms a pleated sheet as pictured in Fig. 21.12.

Proteins, bonded as shown in these sketches, are quite insoluble and form very compact structures. Obviously, the concept of molecular weight is no more apropos here than for very extended polysaccharide chains such as exist in cellulose. Often, molecular-weight measurements reflect the preparative procedure more than the true nature of the substance. Certain reagents such as  $\beta$ -mercaptoethanol, concentrated urea, and guanidine hydrochloride solutions can labilize some of the bonds so that it is possible to measure the molecular weight of a structural monomer. It must be assumed that these proteins have a repeating polypeptide chain unit of a definite length.

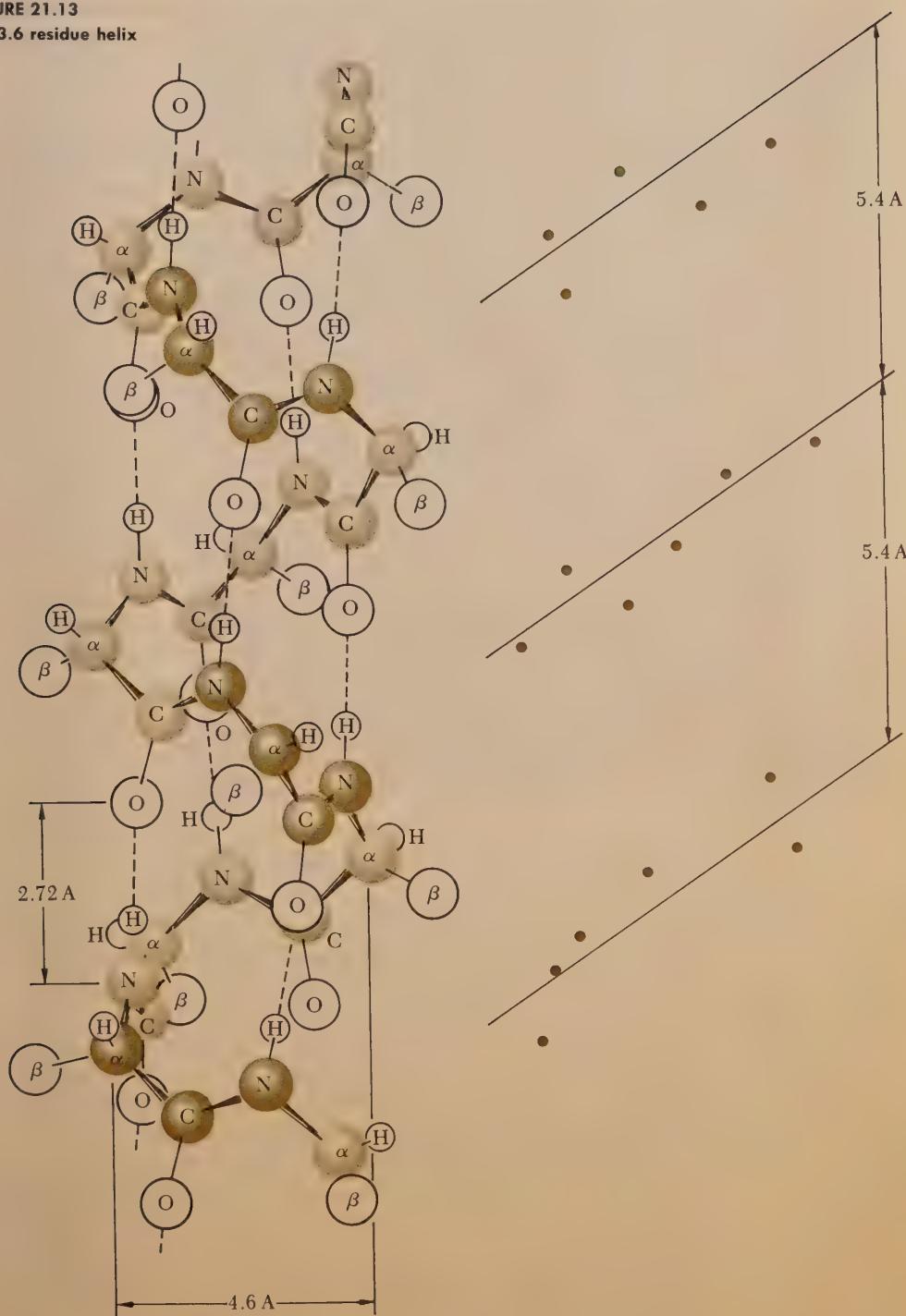
Hair and wool consist largely of characteristic proteins of this type. They are characterized chemically by having a very high sulfur content. Silk fibroin is the protein that most probably possesses a pleated-sheet structure. About 44% of the amino acid residues are glycyl and, of the remainder, alanyl and seryl form the bulk. Clearly, in amino acid content it is anything but a typical protein.

Fibrin is another example of structural complexity. The soluble plasma protein fibrinogen is the substance that permits the clotting of blood. The meshwork of the blood clot is a polymer named fibrin. The structural unit of this polymer has a molecular weight of 330,000. This unit is probably a dimer of a smaller structural subunit which, in turn, can be dissociated into

**FIGURE 21.12**  
pleated-sheet  
conformation



**FIGURE 21.13**  
the 3.6 residue helix



three polypeptide chains. The ultimate chain size seems to be about 55,000. Just how these chains are arranged and how the subunits are related has not yet been worked out.

### **$\alpha$ -keratin pattern and the $\alpha$ helix**

As noted, the  $\beta$ -keratin X-ray diffraction pattern is not very complicated, and it proved relatively simple to interpret. The  $\alpha$ -keratin pattern, not far different at first glance, provided a greater challenge. Measurements of the meridional arc yielded the value 5.15 Å. Equatorial reflections correspond to 4.65 and 9.8 Å. Later measurements also revealed a 1.5-Å meridional spacing.

A satisfactory model, validated by several criteria, was proposed by Pauling and Corey. It is a helical model, often referred to as the 3.6 residue or  $\alpha$  helix, with 3.6 residues per turn and a pitch such that the rise per turn is 5.4 Å. It is a property of helices that constructive interference results in layer lines corresponding to planes with the pitch of the helix, as pictured in Fig. 21.13. Hence the helix does not yield a repeating pattern of atoms such as that in Fig. 21.10. A model of such a helix shows very little unfilled space inside, not even space for a hydrogen atom, and the residues of the amino acids are seen to bristle outward. Rigidity of the helix is due to multiple hydrogen bonding between CO and NH groups that are adjacent in the helix but are not on adjacent amino acids. This bonding is illustrated in Fig. 21.13. Unfortunately, no two-dimensional drawing is adequate, and the student is urged to seek a three-dimensional model and to examine it closely.

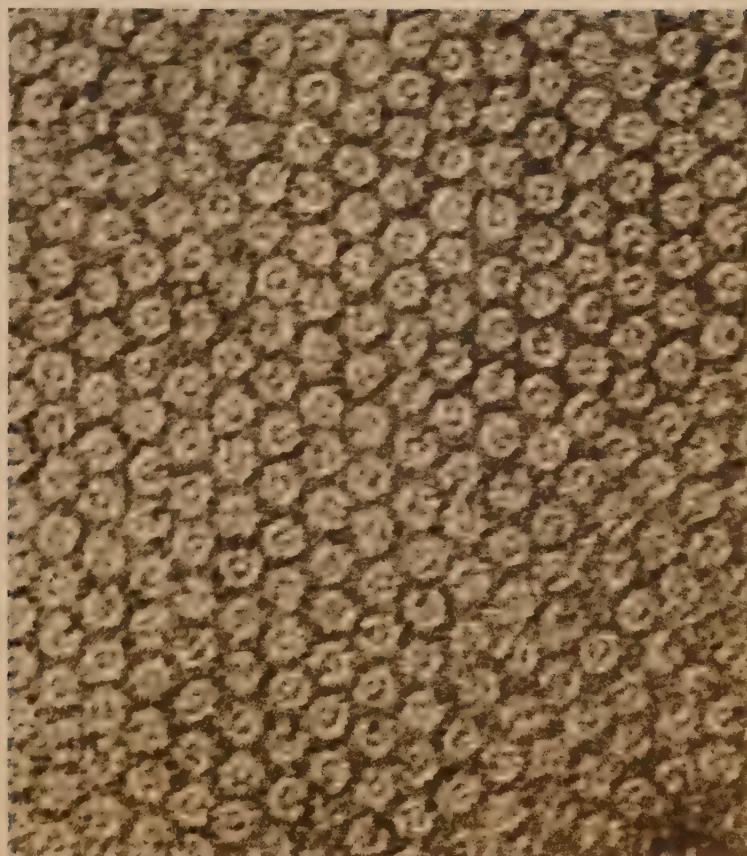
The discrepancy between the *observed* spacing, 5.15 Å, and that of the model, 5.4 Å, has been ascribed to a still further tightening or linear contraction owing to a helical twist of the helix. This secondary twist would have a much longer period. The diameter of the 3.6 residue or  $\alpha$  helix is 4.6 Å, and that of the secondary helix is 10 Å.

It has been proposed that hair and certain flagellae possess an eleven-stranded structure  similar to a cable that would have a diameter of 80 Å (Fig. 21.14). Corresponding layer lines have been described.

Further, each amino acid residue is seen at a 1.5-Å (5.4/3.6) translation along the axis of the  $\alpha$  helix. This reflection spot can be discerned in all natural protein fibers consisting of keratins, epidermin (noncollagen protein of skin), myosin (from muscle), and fibrinogen.

Myosin, actin, and tropomyosin form part of the complex contractile structure of muscle. All are fibrous proteins but are soluble to some extent. In a sense, myosin shares some of the properties of both fibrous and globular proteins. The molecule, having a molecular weight of 5.2 to  $6 \times 10^6$ , appears to consist of three structural subunits, three probably identical polypeptide chains, interwound in a rope-like fashion. Each chain appears to possess an  $\alpha$ -helical conformation. This assembly has a curiously susceptible area easily hydrolyzed by trypsin. The hydrolytic process liberates heavy and light meromyosins, each of which is dissociable into three chains. The fine details of myosin structure (and of myosin interaction with actin to form actomyosin) are uncertain enough to be debatable at present. Electron micrographs reveal a rod-like structure with a globular aspect at one end.

**FIGURE 21.14**  
 evidence for eleven-stranded structures [7]  
 cross section of part  
 of a wool fiber.  
 Each microfibril con-  
 sists of an array of  
 20-A protofibrils



In connection with such studies of protein structure there has been extensive investigation of synthetic polypeptides. They have been used as models in order to study the effects of charges, residue types, solvents, and other factors on the three-dimensional conformations of polypeptide chains. Some synthetic polypeptides have been found to yield diffraction patterns interpreted as evidence of the  $\alpha$ -helix structure (Sec. 22).

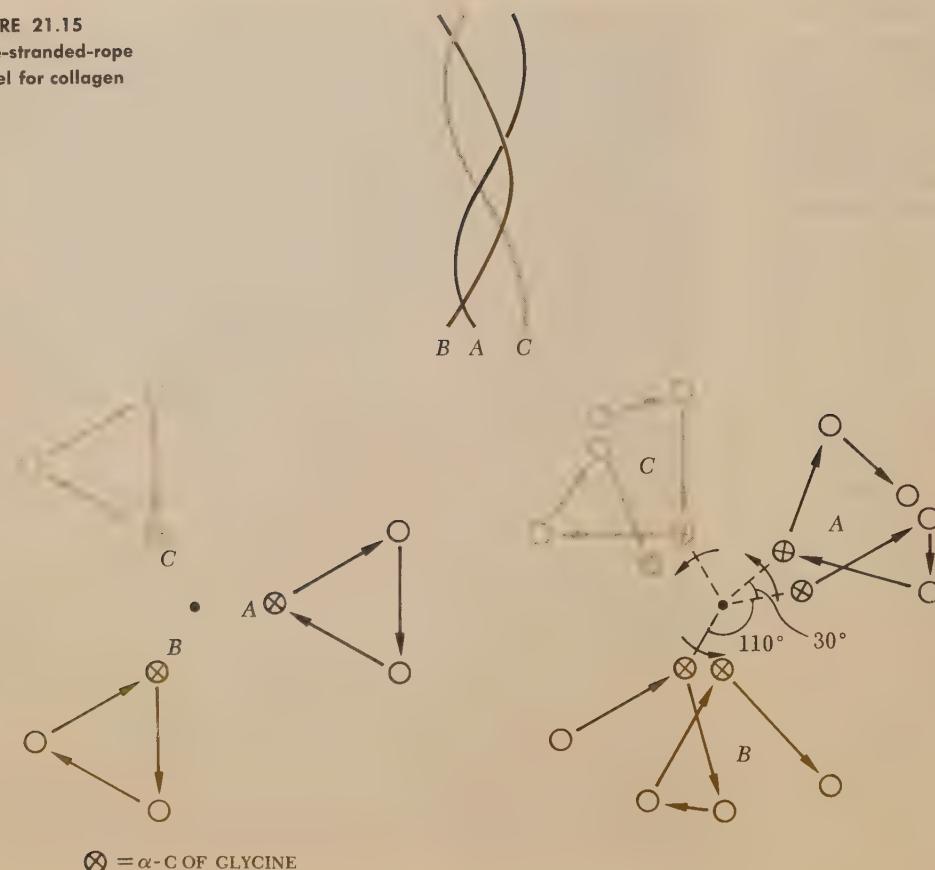
Returning to the collagen structure, we find that the diffraction patterns exhibit meridional spots indicating a 2.95-A spacing (unstretched) and equatorial reflections of 10 to 15 A (depending on hydration) and 4.4 to 4.6 A.

Any model of collagen must account for the X-ray spacing data, but it must also be consistent with solvation, asymmetry, and viscosity data. The extraordinary values for collagen are contrasted with the more representative values for serum albumin in Table 21.2.

**TABLE 21.2**  
 hydrodynamic  
 properties of  
 collagen and  
 serum albumin

protein	M	Solvation, g H <sub>2</sub> O/g	asymmetry	viscosity, cc/g
serum albumin	65,000	1.07	6.5	3.7
collagen	345,000	218	300	1,150

**FIGURE 21.15**  
three-stranded-rope  
model for collagen



Since collagen contains high percentages of proline and hydroxy proline, and since these amino acids lack the usual  $\alpha$ -amino group, it is impossible to have the intensive intrachain hydrogen bonding of the  $\alpha$  helix that is possible in the more average polypeptide chain. Moreover the presence of much glycine, which lacks the usual  $\beta$  carbon,\* leads to configurations different from those of most of the other amino acids.

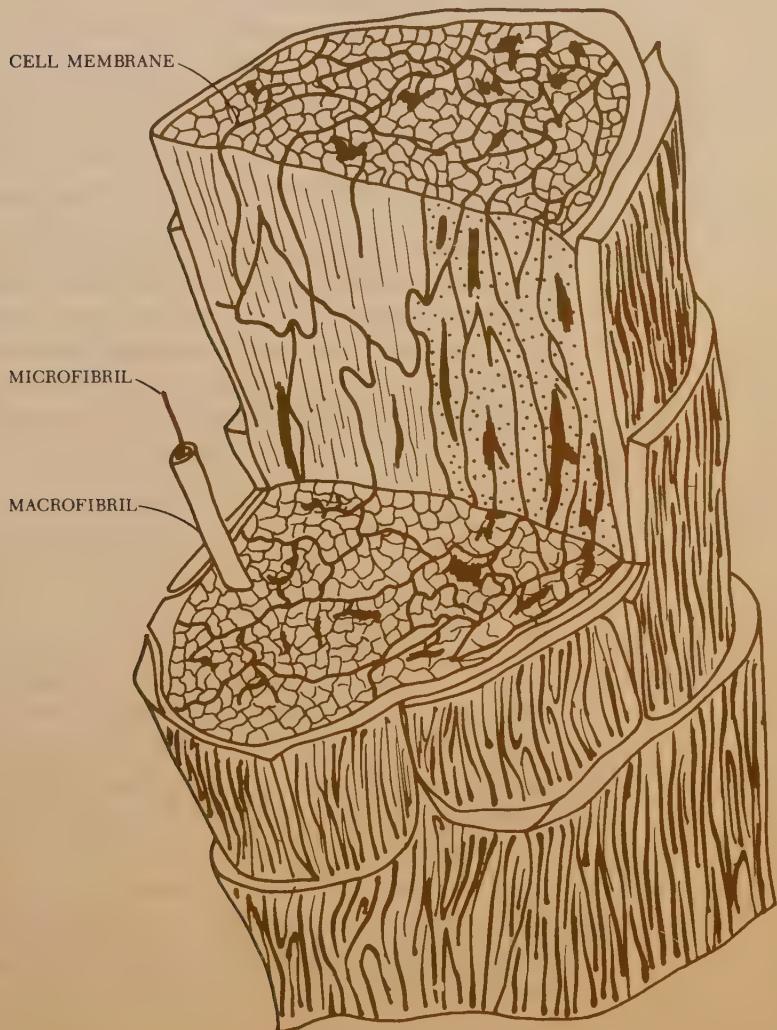
A model heavily favored at this time is that of a three-stranded rope in which each polypeptide chain is coiled about the others. Three noncoaxial helical chains are stabilized by interchain bonding. A sketch is shown in Fig. 21.15. Each polypeptide chain is believed to contain glycine at every third position. This arrangement would allow the formation of a triple-stranded array such as shown in the lower left of Fig. 21.15. However, the diffraction data show that a coiled-coil structure is more probable, as shown in cross section in the lower right of the figure. For every three residues each chain is twisted 30° about the common axis and the translation is 8.85 Å.

\*For polypeptide notation see the list of standard conventions and nomenclature for polypeptides *J. Biol. Chem.*, 241, 1004 (1966).

Each helix is characterized by 3.28 residues per turn. The B chain may be superimposed on the A chain by a clockwise rotation of  $110^\circ$  and a translation of 2.95 Å.

When collagen is dissolved in water and heated, it changes so suddenly that the viscosity plunges to about 2% of the original value. Coincidentally, the optical rotation changes from a very large negative value to a value near zero (Sec. 22). This collagen  $\rightarrow$  gelatin transition is interpreted as a "melting-out" of the helical configurations so that separated polypeptide chains result. They are no longer stabilized in a special conformation and are considered as "random coils." In this regard collagen resembles nucleic acids, that is, in the separation of the covalently bonded chains by heat alone. The phenomenon differs in that reassociation, during cooling, of such random chains is guided in the case of the nucleic acids but in collagen results in the "brush-

FIGURE 21.16  
stereogram of  
wool-fiber  
structure [8]



pile" random structure of gelatin. There is still a question concerning the stabilizing forces in the three-stranded structure. It has been suggested that water structure contributes to this stabilization. There is also experimental evidence for short "telopeptide" sequences or appendages responsible for the distinctive association behavior of the tropocollagen. Whatever the explanation, collagen has a great potentiality for forming large aggregates in a variety of shapes in biological tissues.

**comment** As stated at the beginning of this section, the fibrous proteins often yield rather simple but somewhat diffuse X-ray diffraction patterns. In a few cases it has been possible to deduce repeating patterns, such as the  $\alpha$ -helix, the pleated sheet, or the coiled coil, that present us with rather satisfying concepts. At the same time, there is much uncertainty about some aspects of the structure of fibrous proteins, especially the insoluble ones, because purification procedures are so unsatisfactory.

The globular proteins, structurally, are likely more complex, and certainly the X-ray diffraction patterns are noticeably more detailed. It is curious and admirable that the enormously difficult task of interpreting such data has been accomplished and that these interpretations can be compared with those obtained by other chemical and physical techniques.

It would be wrong to categorize proteins in a rigid manner, since proteins such as myosin have characteristics of both the fibrous and globular types. However, the two categories are useful for some types of discussion.

In Fig. 21.16 is shown the fine detail of a wool fiber, the microanatomy of a biological fiber. Those who seek to relate chemical events to biological structure will see in this a real challenge. As notable as our advances in knowledge of protein synthesis may be, it will be some time before we can describe, biochemically, the synthesis of such a structure.

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# 22

## globular proteins

The fibrous proteins include those most closely identified with the gross structures of animals. Collagen, as noted, is the most prevalent, and it occurs in all types of connective tissue as well as in bone. The keratins are the insoluble components of hair, horn, and skin. Other extracellular proteins such as the spider's web and the silky covering of cocoons are also fibrous proteins. In addition, fibrous proteins form the feathers of birds, the tendons of tails, the catch muscle of mollusks, and the flagella of microorganisms and of sperm cells. When the story of protein synthesis and protein structure is truly told, each of these will provide an entrancing chapter.

But this kind of protein is rare inside the cell, and almost entirely absent from bacteria. The studies of protein synthesis in the main have been restricted to soluble proteins, proteins that have enzymic functions and that are related to microstructures rather than macrostructures, subcellular structures rather than intracellular or polycellular structures. These proteins are grouped rather crudely as globular proteins. To the perceptive person it will be clear that we have here not two clearly distinct types of protein but a distribution with two nodes. The fibrous proteins exhibit an end-to-end polymerization that must occur if a fiber is to be formed, while the globular proteins show predominantly a polyhedral association. Not all globular proteins have catalytic activity in the strictest sense. Serum albumin does not, nor does tobacco mosaic virus protein. Yet the globular proteins do share some fundamental structural features, and these are frequently associated with catalytic activity.

There are a few proteins that can be described in respectable detail: hemoglobin, insulin, ribonuclease, lysozyme,  $\beta$ -lactoglobulin, myoglobin. But about most proteins almost everything remains to be learned.

Carefully prepared X-ray diffraction patterns from the crystals of globular proteins have many more spots than the fiber patterns of fibrous proteins. This complexity attests to a highly detailed structure and, it might be added, one befitting to the biological cell which, in its growth, decreases entropy. The hydrodynamic behavior indicates a compact structure; viscosity, diffusion, sedimentation, and electrophoretic measurements all favor a globular model rather than a fibrous, asymmetric one. Many of these proteins can be crystallized as well, indicating the existence of relatively compact units which are capable of fitting a crystal lattice. Protein crystals do differ from the crystals of smaller molecules in that contaminants are easily included, and thus crystallization is not the reliable method of purification for proteins that it is for simpler substances. Moreover, protein crystals tend to be quite small, to be very fragile, and to contain large amounts of solvent. It was discovered

long ago that the X-ray diffraction pattern of a dry protein crystal is very different from that of a crystal suspended in "mother liquor." Perutz<sup>\*</sup> has said that "protein crystals are no more than ordered gels." Nearly 55% of the volume of horse hemoglobin crystals is taken up by liquid of crystallization. With regard to crystal form it seems to matter little whether the liquid is pure water or concentrated ammonium sulfate solution. The hydration of the protein (water that is not available as solvent to electrolytes) is probably the same in solution as in the crystal. It is evident that neighboring protein molecules such as hemoglobin in a crystal touch each other at only a few points.

Thus protein molecules must have an architecture that will fit the order of the crystal, must be rather globular in solution, and must be highly hydrated. One must accept the idea that polypeptide chains can be, and are, folded in a characteristic and reproducible three-dimensional pattern.

### denaturation

Any model of the protein molecule must also be able to accommodate the phenomena of *denaturation*, a term that embraces such a variety of observations that a concise definition is impractical. As exemplification the effects of the following agents may be commented upon.

- Heat. The majority of soluble proteins become insoluble and precipitate if heated above 50°. There are exceptions, such as ribonuclease, which can be boiled without destroying its activity, and certain proteins, which are not soluble in the usual aqueous environment. However, one is so often confronted in daily life with the boiled egg that the coagulation that follows heat denaturation is a very familiar sight. In brief, the denaturation process is that of destroying structure and producing polypeptide chains in a state reflecting a low degree of order. The coagulation in this case is due to a reassociation of such chains in a fashion that differs from the original.
- Extremes of hydrogen-ion concentration. Proteins have groups which, when titrated, will result in a respectable charge density outside the region of pH 4 to 8. The development of charge on polypeptide chains may destroy the original order but allow another which is insoluble as such in the original solvent.
- Exposure to other forces. If a protein solution is spread to form a monolayer, either at an air-water or a solvent-water interface, structural changes that result in insolubility occur. Exposure to radiation will also produce structural changes. These may be due to the specific effects on atoms of the absorption of ultraviolet radiation, for example, or to less-defined effects of longer wavelengths such as ultrasound.

\*By permission of M. F. Perutz and the Elsevier Publishing Co., Amsterdam. From M. F. Perutz, *Proteins and Nucleic Acids*, 1962, p. 55.

When energy in any of a variety of forms is absorbed by proteins, or when the charge distribution is altered, there ensue changes that may be detected in a variety of ways. These ways embrace the determination of enzyme activity, of solubility, or of changes in the X-ray diffraction pattern. It was the latter that reinforced the notion that denaturation and the alteration of specific polypeptide chain folding are related. It is now commonplace to refer to processes once called denaturation as "chain unfolding," but this term also is imprecise and does not refer unambiguously to the best models available.

### dissociation- association

Yet another process that dictates certain features of the protein model is dissociation and association, which is most frequently revealed by molecular-weight studies. It has been shown that it is usually inappropriate to speak of *the molecular weight*\* of a protein. Dissociation or association may often be noted and reflected in the molecular-weight value by varying the ionic strength of a protein solution, changing the pH, or changing the concentration of the protein. For the most part dissociation phenomena represent changes in noncovalent bonding, although disulfide bonds may be involved in some cases. Recent experimentation indicates that the destruction of noncovalent bonding of any kind in protein structures may be reversible under proper circumstances, and hence it is more desirable to speak of denaturation-renaturation as well as dissociation-association processes.

The *primary* structure of proteins is a heritage from Emil Fischer, who employed the strength of nineteenth-century organic chemistry to demonstrate that proteins are polypeptides. *Secondary* structure was postulated for the fibrous proteins in order to rationalize the diffraction patterns by postulation of the pleated sheet and the  $\alpha$  helix. But globular proteins have hydrodynamic properties that imply a *tertiary* structure as well—a specific folding of polypeptide chains that usually already have a secondary structure. Even in the fibrous proteins a coiled coil was postulated, i.e., a third type of order. Finally, dissociation-association impels us to consider the particles containing polypeptide chains in characteristic primary, secondary, and tertiary arrangements as *subunits* of the final molecular complex.

\*The term molecular weight is often used carelessly. In the chemistry of small molecules the ideal, or theoretical, molecular weight is usually used, although experimental values are sometimes very different. Moreover, the molecular weight is most often determined by measuring a change in a colligative property and the number obtained is an  $M_n$ , a number-average molecular weight.

The molecular weights of macromolecules are usually  $M_w$ , or weight-average molecular weights, and involve measurements that are functions of the hydrodynamic properties. The experimental values are actually apparent  $M_w$  values, and it is not always obvious whether extrapolation procedures have been employed. In order to obtain molecular-weight estimates that reflect the true size of the macromolecule, it is necessary to determine whether there are nonideal departures due to concentration effects, charge effects, ion binding, and the like.

**X-ray  
techniques**

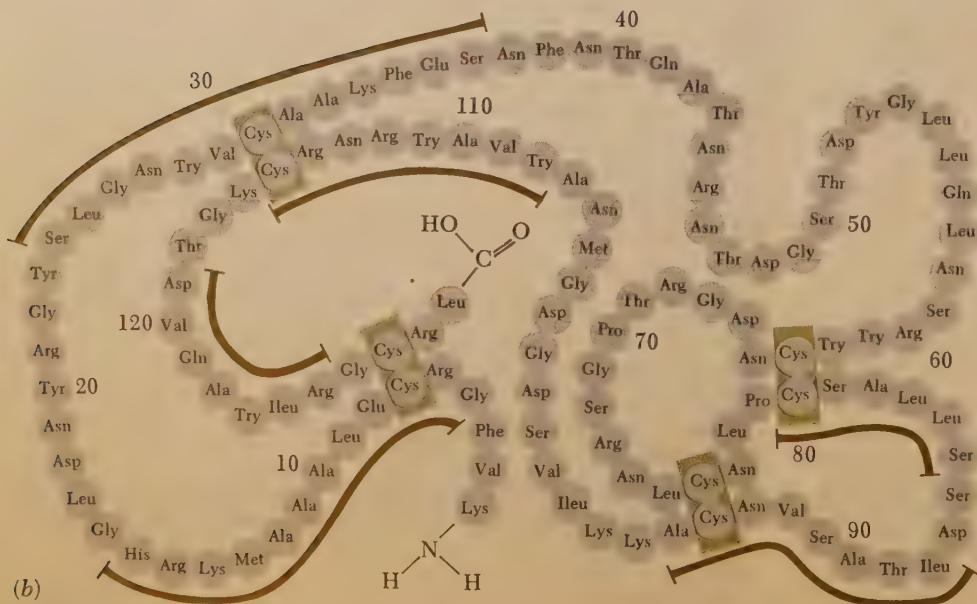
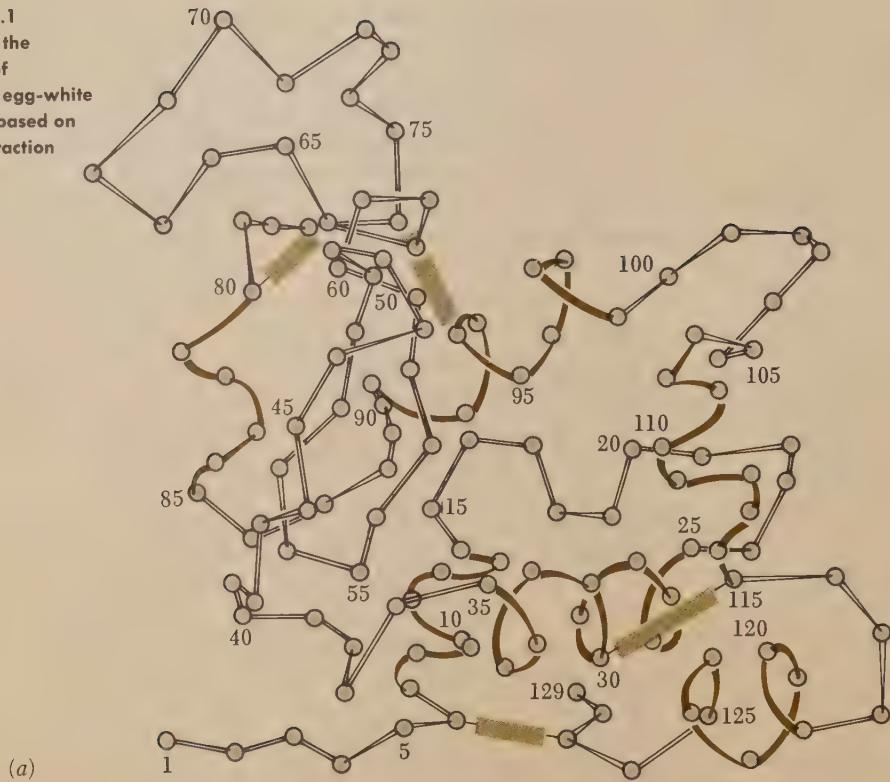
The investigation of such complicated structures must be pursued confidently and imaginatively but with humility. A notable conclusion about X-ray diffraction evidence was reached by Perutz around 1953. He stated that the seemingly insurmountable "phase problem" posed by this technique could be solved by using data from isomorphous crystals—crystals of protein and those of the same protein associated with a heavy atom. The protein "derivatives" were not those in which the metal atom was necessarily covalently bonded, but it was necessary that the metal atom have a definite place in the crystal lattice and that the crystal habit be identical with that of the original protein. The detailed exploration of this process should be undertaken by consulting other sources (REF. 1). Although the problem was solved in principle, there remained practical difficulties. The production of isomorphous crystals containing heavy atoms is more art than science; another problem is that some proteins have structures in which thermal vibrations are so severe that the patterns become indistinct.

Despite these problems, the work of Perutz and his coworkers on horse hemoglobin, and of Kendrew and his coworkers on whale myoglobin, has had a success that merits its acclaim. Recently the structure of lysozyme (3.2.1.17) has also been elucidated by similar techniques (Fig. 22.1). In order to use enough reflections to determine the positions of all the residues, it was necessary to assemble a considerable computer program. The data were interpreted in part by model building.

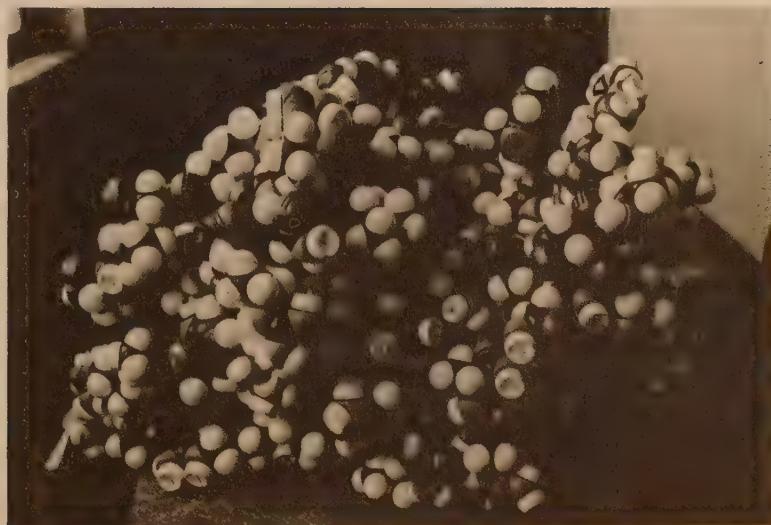
The work on myoglobin has been done with metmyoglobin, which differs from myoglobin in that the iron atom is in the ferric rather than the ferrous state. The polypeptide chain (17,000 molecular weight, 153 amino acid residues) has an  $\alpha$ -helical conformation throughout a major portion of its length. The tertiary structure is a folded figure of no particular regularity (Fig. 22.2). There are relatively straight sections of chains which are helical, but the turns, bends, and short sections are nonhelical. As would be expected, the proline residues (having an unusual  $\alpha$ -nitrogen) are at corners or in non-helical regions (Fig. 22.3), but there are also corners that do not involve proline. In general, the polar side chains tend to be embedded. It is estimated that 30% of the polar, and 45% of the nonpolar, residues are occluded in the chain matrix. Nearly all the polar groups, including peptide carbonyl and imino groups, interact with nearby groups, or water, or ions. The packing of the atoms is such that there is little room for water "inside." The position of the heme (or haem) group is clear, and it will be discussed in connection with its function (Sec. 28). There is no implication that the three-dimensional structure of myoglobin is closely similar to that of other proteins. Certainly proteins that possess little helical structure are known. Yet we can visualize the fundamental plan of myoglobin, and it will serve as a welcome standard for comparison.

As a check on conclusions, and as a guide to the informal guessing involved in formulating complex structures, it is appropriate to determine the amino

**FIGURE 22.1**  
**models of the**  
**structure of**  
**crystalline egg-white**  
**lysozyme based on**  
**X-ray diffraction**  
**data [9]**



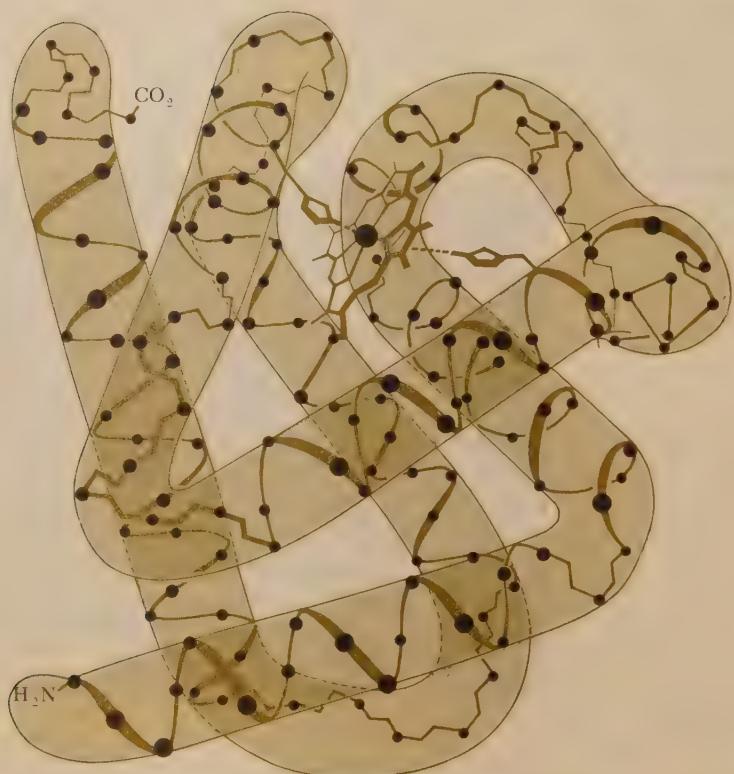
three-dimensional model of lysozyme constructed with "space-filling" models. Model shows cleft or trough that is catalytically active.



three-dimensional substrate model has been laid in catalytic trough. Paper separates substrate from enzyme model



**FIGURE 22.2**  
a model of the  
structure of  
crystalline myoglobin  
based on X-ray  
diffraction data [10]



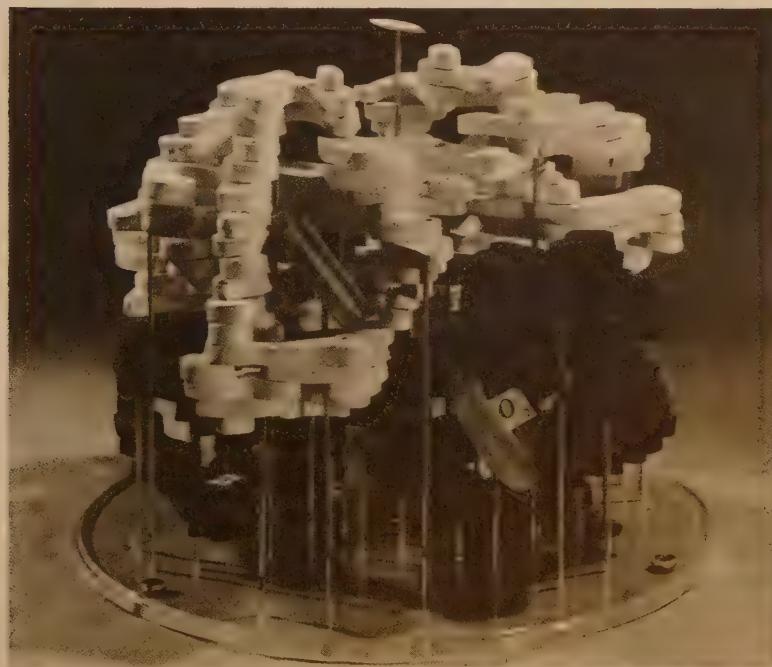
**FIGURE 22.3**  
effect of proline  
residue on direction  
of  $\alpha$ -helix axis [11]



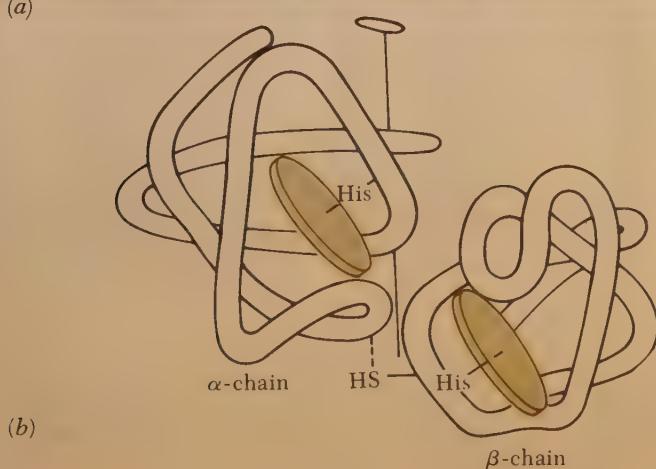
acid sequences. As noted before, some relation between sequence and structure exists; the sequence sets limits of structural possibilities.

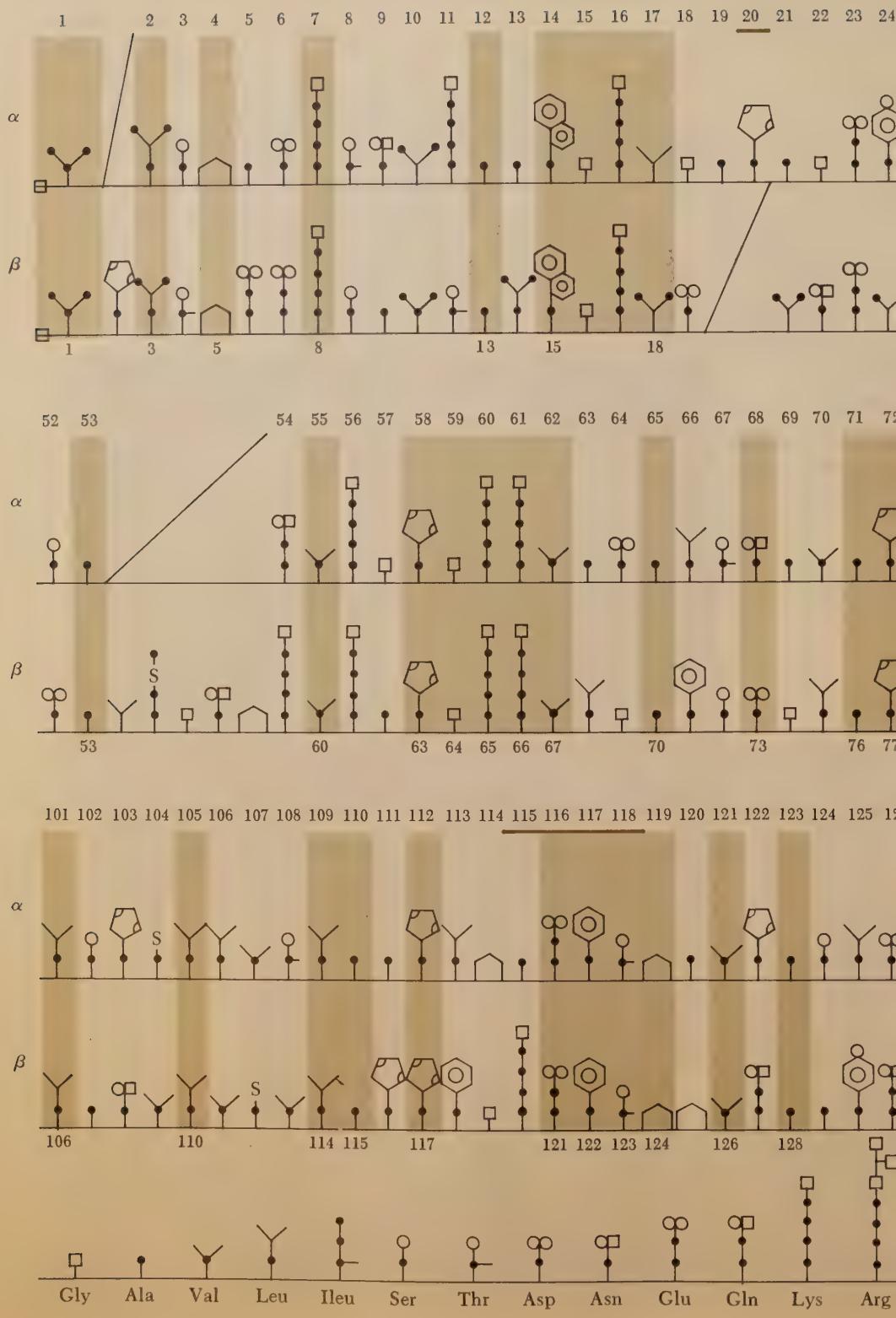
As a comparative standard, myoglobin has the useful attribute of existing as a one-polypeptide-chain protein. It is equally useful to have the hemoglobin structure for comparison, since it is a stable association of four subunits. In a molecular weight of about 64,000 are four polypeptide chains, four heme groups, and four iron atoms. There are two kinds of chains in hemoglobin, and from electrophoretic data these have been designated  $\alpha$  and  $\beta$ . Each pair of subunits is symmetrically related, but each type has a quite irregular shape (Fig. 22.4). When both pairs are in the proper inter-

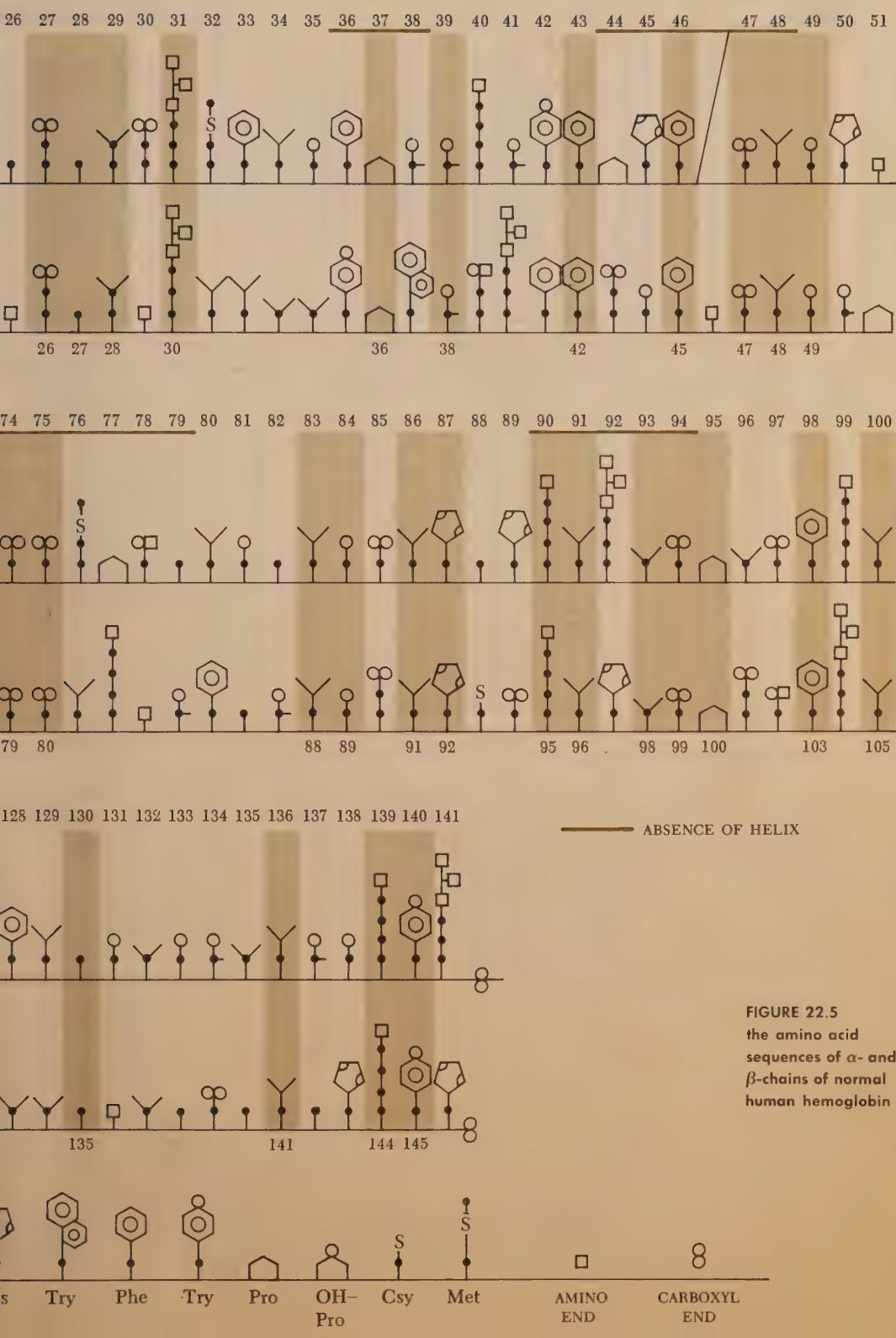
FIGURE 22.4  
a model of the  
structure of  
crystalline  
hemoglobin based on  
X-ray diffraction  
data [12]



(a)







**FIGURE 22.5**  
the amino acid  
sequences of  $\alpha$ - and  
 $\beta$ -chains of normal  
human hemoglobin

penetrating relation, they are complementary to the extent that there is little space left between them and the entire assembly is a spheroid with a rather bumpy surface. The folding of the polypeptide chains to form the structural subunit is similar to, but not identical with, that of myoglobin. Again there is evidence for a helical secondary structure. The amino acid sequences of the  $\alpha$  chain (141 residues) and the  $\beta$  chain (146 residues) have been compared (Fig. 22.5), and despite the similarity of tertiary structures for myoglobin and hemoglobin, the sequences have been observed to be rather different.

### hybridization of subunits

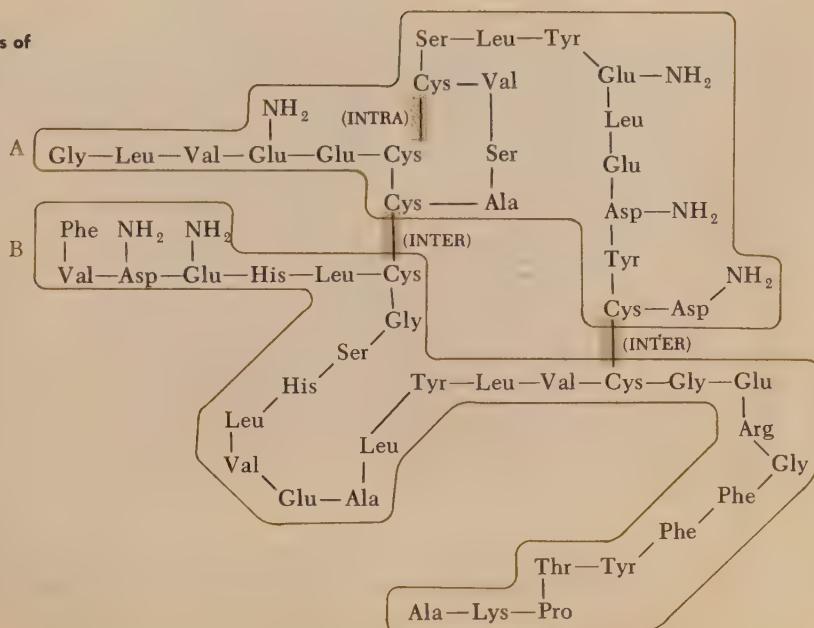
Although hemoglobin as usually prepared has good stability, dissociation of the subunits can occur and can be readily demonstrated by the ultracentrifuge or by electrophoresis. That species differences may be relatively slight can be demonstrated by hybridization experiments. Human hemoglobin A (HbA), designated as  $\alpha_2^A \beta_2^A$ , can be dissociated and mixed with canine hemoglobin  $\alpha_2^{\text{can}} \beta_2^{\text{can}}$ . Hybridization occurs to produce the new species  $\alpha_2^{\text{can}} \beta_2^A$  and  $\alpha_2^A \beta_2^{\text{can}}$ , each of which has a different mobility. The technique of hybridization in this way can be used to test for dissociation and for subunit complementarity.

In the case of myoglobin, and of hemoglobin, the pattern of polypeptide chain folding is known. That this pattern is a thermodynamically stable one is strongly suggested by recent experiments in which the major aspects of the three-dimensional structure of a protein are destroyed and then the resultant product is allowed to refold slowly. A variety of techniques have been used, and many enzymes that lose activity when unfolded regain their activity when "reconstituted." This observation has led to the belief that the folding in a "native" protein is the most stable arrangement of a particular amino acid sequence.

The use of X-ray diffraction data has helped to disclose what protein structure is, just as amino acid sequence studies help to narrow the possibilities of what it can be. A different type of experimentation is required to discover what forces stabilize the structure.

As noted, the disulfide link may contribute to stability both as an intrachain and an interchain bond. Insulin provides a good example of both, consisting of an A chain that contains the intrachain disulfide and the B chain bonded to the A chain by two interchain links (Fig. 22.6). In the figure the A and B chains are contorted in such a way that the cysteines are in juxtaposition, but the folding pictured has no meaning. Despite the fact that insulin has a molecular weight of only 5,733, the three-dimensional structure is not known. The analysis for both —SH and —S—S— groups in proteins presents unexpected problems. In many instances the groups are so integrated into the structure that they are rendered unreactive. In such cases the analytical data can be misleading, since they are a function of both chemical and structural factors. It is common practice to dissolve disulfide-containing proteins in concentrated urea or guanidine hydrochloride solutions to minimize structure effects before adding the reagents. In such solutions it

**FIGURE 22.6**  
The A and B chains of  
beef insulin



is thought that polypeptide chains are present in random conformations. Thus disulfide bonds may be reduced to sulfhydryl and then blocked by making a derivative, so that reformation of the original bonds is prevented. This provides a protein derivative for comparison with the original. Alternatively, if the analysis is for sulfhydryl groups, it is found that the maximum values will be obtained when the protein is dissolved in the urea or guanidine hydrochloride solutions.

The precise nature of the changes that occur in proteins dissolved in urea or guanidine hydrochloride has not yet been clarified. In most cases subunit association is grossly disturbed and the molecular weight drops to that of the smallest covalent-bonded structural subunit. The optical rotation may be observed to change in a way that indicates an absence of order in chain conformation. An increase in levorotation (for the right-handed helix) in which  $[\alpha]$  becomes more negative also accompanies other types of denaturation.

### optical-rotatory dispersion

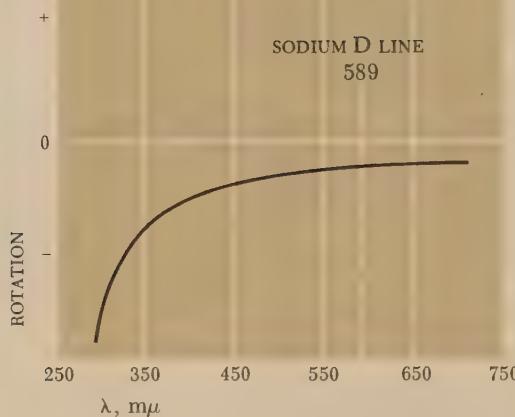
A simple (normal, plain) dispersion curve is graphed in Fig. 22.7. It may be described by the Drude equation (1900)

$$[m']_{\lambda} = \frac{A_c \lambda_c^2}{\lambda^2 - \lambda_c^2}$$

where

$$[m']_{\lambda} = \left[ \frac{3}{n^2} + 2 \right] \frac{M}{N} \frac{[a]_{\lambda}}{100}$$

**FIGURE 22.7**  
a simple dispersion  
curve



In these equations  $[m']_\lambda$  is the mean residue rotation corrected for the effect of the solvent refractive index  $n$ ,  $[a]_\lambda$  is the specific rotation at the wavelength  $\lambda$ ,  $a_c$  and  $\lambda_c$  are constants,  $M$  is the molecular weight, and  $N$  is the degree of polymerization.

The optical rotation of denatured protein at various wavelengths with the 250- to 650-m $\mu$  range is described by the simple equation, and  $\lambda_c$  is found to have a value of about 210 m $\mu$ .

When undenatured proteins are examined, a very different type of behavior is observed as in Fig. 22.8. Obviously, something interesting is occurring below about 240 m $\mu$ . Moreover, above this wavelength the rotation

**FIGURE 22.8**  
optical-rotatory  
dispersion of  
bovine serum  
albumin [13]

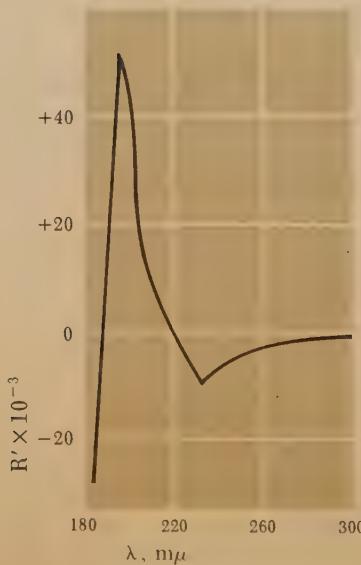
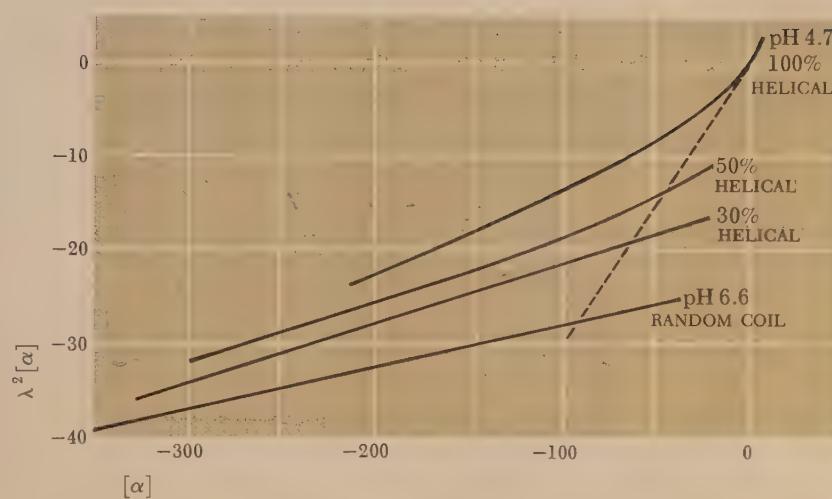


FIGURE 22.9

Dispersion data for poly-L-glutamic acid. These data are for the 300- to 750-m $\mu$  region. The polypeptide was dissolved in hexane-0.2 M NaCl (2) at various pH values. The dotted line runs through values at 589 m $\mu$ . At pH 6.6,  $\lambda_c = 212$ , but at lower pH values  $\lambda_c$  was higher, up to 250 m $\mu$ . The value of  $b_0$  at pH 4.7 was -630°.



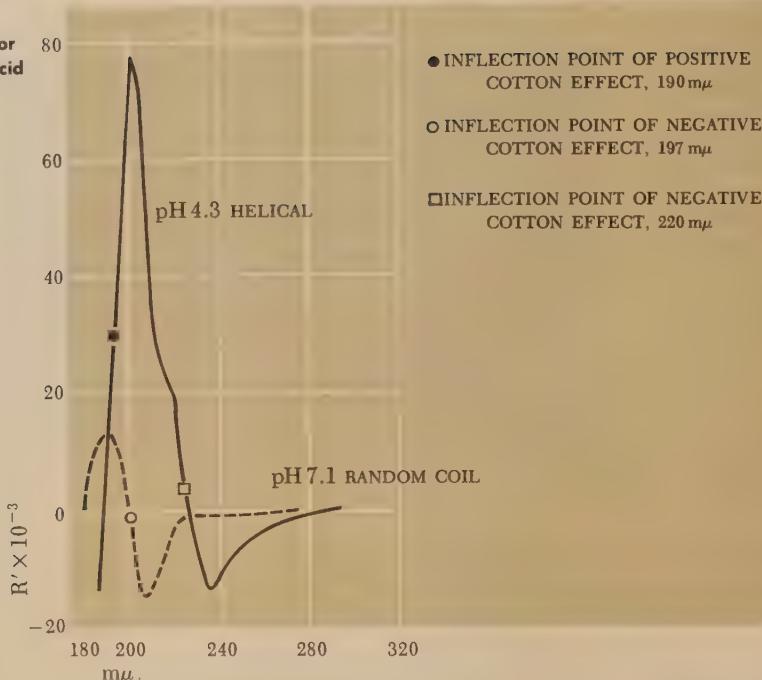
values tend to be more positive and many proteins yield values that require a modified equation suggested by Moffitt (1956).

$$[m']_{\lambda} = a_0 \left( \frac{\lambda_0^2}{\lambda^2 - \lambda_0^2} \right) + b_0 \left( \frac{\lambda_0^2}{\lambda^2 - \lambda_0^2} \right)^2$$

The symbols  $a_0$  and  $b_0$  are constants. Experiments yielded data showing that  $a_0$  is a function of the environment but  $b_0$  and  $\lambda_0$  are not. Studies were made on a model, the polypeptide poly-L-glutamate, which at low pH values is uncharged. This lack of charge would dispose toward a conformational change believed to be from right-handed  $\alpha$ -helix to random coil. Plots of dispersion data are given in Fig. 22.9. Further studies led to the proposal that if dispersion data for proteins (above 250 m $\mu$ ) were plotted and  $b_0$  evaluated, then the fraction  $b_0/600$  would be a measure of percent  $\alpha$  helix in the protein conformation. Estimates based on such data agree roughly with those based on X-ray diffraction studies. Left-handed helices such as in poly ( $\beta$ -benzyl-L-aspartate) yield  $b_0$  values that are positive, as do pleated-sheet conformations ( $\beta$  structures).

A related technique for ascertaining the presence and amount of helical conformation exploits the effect attributed to Cotton (1895). Figure 22.10 shows the rotatory dispersion of poly-L-glutamic acid at wavelengths lower than those in Fig. 22.9. Both random and helical conformations show negative Cotton effects of about the same magnitude, but a very large positive effect is observable for the helical form. Estimates of the helix content of proteins can be made by comparing  $[m']$  at 233 m $\mu$  for the native and denatured protein and are in reasonable agreement with those based on  $b_0$  values. Studies of the Cotton effect have revealed that the  $\alpha$  helix is indeed present in many proteins, that it is not universally present, and that the amount of the total chain length involved in a helical configuration may vary with conditions.

**FIGURE 22.10**  
**dispersion data for**  
**poly-L-glutamic acid**  
**[15]**



As you might predict, the charge patterns in proteins affect the three-dimensional structure. The effect of charge on poly-L-glutamate has been noted. Many such effects are electrostatic. We may also observe a situation in chains where alternating positive and negative charges interact. The interaction energy  $W$  for ideal dipoles in the most favorable orientation can be estimated if the dielectric constant can be evaluated properly.

$$W = \frac{-2\mu\mu'}{\epsilon D^3}$$

where  $\mu$  is the dipole moment,  $D$  the intermolecular distance, and  $\epsilon$  the effective ("microscopic") dielectric constant of the medium. For lipids  $\epsilon$  is about 2 and for water about 15.<sup>\*</sup> For two sets of charges energies of about 1 kcal/mole would be possible. Polarization or induction forces can also be present, but they would be two or three orders of magnitude less in strength.

Charge changes can also be used to dissociate proteins into structural subunits.  $\beta$ -Lactoglobulin exists as a dimer (molecular weight 36,000) at pH 5. When the pH is lowered to 2, there is a dissociation into monomers. Presumably, at pH 5 both negative and positive charges are present. At pH 2 negative charges are removed as the carboxyl groups are protonated; dissociation into monomers ensues because of repulsions among the positive charges. Many proteins have been found to dissociate, often reversibly, when the pH is lowered to 2 or raised to 11.

\*This has been estimated by measuring the acidity of acids,  $\text{NH}_2-(\text{CH}_2)_n-\text{COOH}$ , as a function of chain length.

In proteins, as in other large molecules, there are dispersion (London-van der Waals) forces, especially between nonpolar groups. These are due to the interaction of an instantaneous electric moment\* in one molecule inducing a moment in another molecule. Such forces are attractive, but they are a function of the sixth power of the distance separating the two groups. It has been estimated that the force between two CH<sub>2</sub> groups 5 Å apart would be about 0.1 kcal/mole. In a very large molecule the total force could be substantial because such forces are additive.

In the case of myoglobin and of hemoglobin there is much evidence for the opinion that the nonpolar residues are largely extended into the interior of the protein domain. Within the last few years protein chemists have been discussing the importance of hydrophobic bonding in such instances. Hydrophobic bonding is conceived to be due to interaction of nonpolar groups with the exclusion of water. Water is released, in this sense, to interact with other water molecules, and this process occurs with a  $-\Delta G$ . Only where there is a multiplicity of interactions is this effect likely to gain prominence. An unexpected consequence of the thermodynamic properties is that hydrophobic bonds are weaker at low temperatures and hence some proteins might dissociate into subunits or otherwise denature in the cold. Some do. (PHOSPHORYLASE and PYRUVATE CARBOXYLASE.)

When all of the evidence is taken together, it is clear that protein conformations are compromises among a variety of forces. There is ample opportunity for the reflection of both inherited sequences and the effects of the immediate environment. The groups of the residues in the inherited sequences dictate what is possible. The various environmental factors of ionic strength, temperature, concentration of protein, and the presence of nonpolar substances within the possible range of reaction.

**comment**

The riddles of protein structure have proved attractive to many competent men. Every sort of discipline must be called upon to furnish evidence concerning various aspects of structure. Whereas DNA has a relatively limited although highly necessary set of functions, proteins seem to be the very seat of diversity. The relation between protein structure and biological structures of various types has been mentioned, and the relation between protein structure and catalytic activity also was discussed briefly. But proteins have been shown to be transducers of chemical energy into mechanical energy as in muscle, they may be involved in the transduction of light energy into chemical energy, and it has been postulated that they may be the basis of the memory function. Such virtuosity of function can reside only in a substance that has variation in form.

At present we believe, or postulate, that the amino acid sequence of a

\*The electron cloud of one atom so distorts the electron field of a neighbor that a dipole is produced, i.e., the centers of positive and negative charge no longer coincide. The vector quantity corresponding to the direction and magnitude of this dipole is the moment.

protein dictates the stable conformation. Considering the number of combinations and permutations that can be exhibited by 20 amino acids,<sup>9</sup> this restriction does little to narrow the number of possibilities. But if we consider the shape of the polypeptide backbone and the rigidity of the peptide bond, there is a considerable increase in restriction. Some of the possibilities such as the  $\alpha$  helix and the pleated sheet can be considered as certainties. Other conformations may depend on particular sequences that are as yet unknown.

Some techniques, such as optical-rotatory dispersion, are sensitive to changes in hydrogen bonding and changes in helix formation. Others, such as ultracentrifugation or electrophoresis, are sensitive to size and shape changes such as occur in dissociation. X-ray diffraction techniques are limited at present to proteins that form isomorphous replacement crystals, although some data can be obtained from protein solutions by low-angle X-ray techniques. Sequence studies have provided invaluable guidance and confirmation of postulated structures.

The limitations of each technique and the difficulty of preparing substantial quantities of homogeneous enzymes provide us with many interesting, but biased, guesses about the shapes and the microanatomy of enzymes. So far, the X-ray diffraction studies have seemed the peerless victors in this struggle, but even so we can legitimately doubt that a model built from crystal data will suffice for visualizing the forms in solution. Indeed, we postulate that some proteins must change form in order to function.

It has not been possible to correlate either form or function with specific sequences. This failure should not be surprising, considering all that we expect from protein as a "hewer of wood and a drawer of water."

One generalization of importance is the relation of the structural gene to the polypeptide chain. We now know that a large number, perhaps a majority, of proteins contain one or more structural subunits. It is likely that these subunits are held together by all the types of noncovalent forces that exist. How these subunits affect each other functionally has been demonstrated in hemoglobin (Sec. 28) but not explicitly in other proteins.

It may be appropriate here to emphasize the contrast in the behavior of the nucleic acids and the proteins with respect to heat. Despite the much greater length of the polynucleotide chains, separated chains reunite when the solutions of nucleic acids are cooled slowly. Specific interchain bonding seems to be adequate for reconstitution. The behavior of collagen is similar in that heating alone can separate the chains of the three-stranded structure. Again, interchain bonding is definitive. Most globular proteins behave quite differently. Intrachain bonding is very significant, but beyond that the inter-chain relationships seem more complex. When proteins are heated in solution, new conformations and interchain relationships develop, and only rarely is reconstitution possible.

<sup>9</sup> This is the number of amino acids for which there seem to be corresponding sRNA species. Hydroxyproline is not included; asparagine and glutamine are.

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Note: The symbols used in Figure 22.5 are from D. Wellner and A. Meister, *Science*, **151**, 77 (1966).

# 23

## more about protein synthesis

The association of genetic events with changes in proteins or enzymes has been largely based on observation of biological growth or the determination of changes in activity. The latter type of evidence has always been somewhat suspect by biochemical criteria. Enzymic activity is not a reliable index to the presence of the protein that normally possesses that activity. For example, a protein, as a result of a mutational event, might be altered slightly instead of deleted altogether. Alternatively, a change might so occur that the activity is altered but the bulk of the protein is synthesized and present. With the notion in the air that the mutational alteration of one base results in the alteration of one amino acid, experimentation has spread to nonenzyme proteins. There is now an accumulation of data on the sequence of hemoglobins that bears out the working hypothesis.

The polypeptide chains of a number of hemoglobins have been separated and their amino acid sequences determined. This procedure has involved dissociation of the hemoglobin, with acid, for example, and separation of the subunit types by electrophoresis or column chromatography. That the subunits are separable by such procedure is, in itself, an indication of differences in sequence. The sequence of amino acids may be determined by methods identical with, or similar to, those developed by Sanger in his classic investigation of insulin. Very briefly, the protein sample is partially hydrolyzed (a variety of agents may be employed), and the resultant peptides are chromatographed. The complete hydrolysis of a protein to its component amino acids requires more than 24 hr boiling with acid or base, and hence there is adequate time to examine the peptides formed by random hydrolysis. Since the hydrolysis is random, there are substantial overlap sequences. Specific hydrolysis by proteolytic enzymes is also employed. Further hydrolysis allows deduction of sequence by matching procedures. For a small protein such as insulin the process is tedious; for larger ones it is heroic.

A variant of this procedure called "fingerprinting" is also commonly used to compare proteins. Digestion of the  $\alpha$  chain of hemoglobin with trypsin,<sup>°</sup> for example, results in a mixture of peptides that, when chromatographed in two dimensions on paper, results in a pattern of spots (when sprayed with ninhydrin). If the  $\beta$  chain is treated similarly, because the sequences are different, the patterns will be dissimilar. If there is a difference in only one or

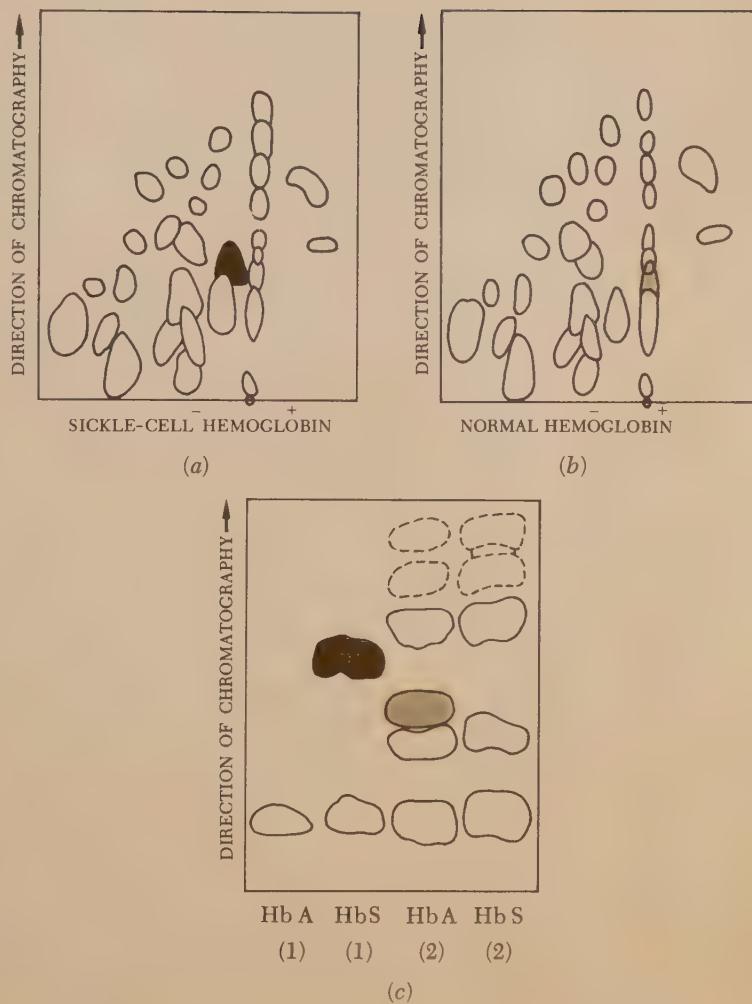
<sup>°</sup>In this type of work a number of enzymes may be used provided they can be obtained in a state of adequate purity. Trypsin catalyzes only the hydrolysis of peptide bonds involving the carboxyl groups of lysine and arginine; chymotrypsin is most effective for peptide bonds of tyrosine and phenylalanine. Hence, digestion mixtures will contain only the number of peptide types corresponding to the susceptible bonds in the polypeptide chain.

**FIGURE 23.1**

peptide maps.

(a and b) "Fingerprints" of human "sickle-cell" and normal hemoglobins. Electrophoresis at pH 6.4, chromatography with *n*-butyl alcohol/acetic acid/water (3:1:1). The darkly and lightly shaded spots are those belonging to the peptide showing the difference.

(c) Chromatogram of fractions of hemoglobin A and S digests obtained by large-scale paper electrophoresis. (1) Slowest-moving, positively charged fractions; (2) neutral fractions [16]



two amino acids, the peptide "maps" will be very similar even if not identical (Fig. 23.1). In order to compare the peptides in the spots, the experimenter can elute them from the paper, hydrolyze further, and compare the amino acid content chromatographically.

Three varieties of normal hemoglobin are readily identifiable in humans. These are hemoglobin A (HbA),  $\alpha_1^A\beta_2^A$ , and hemoglobin A<sub>2</sub> (HbA<sub>2</sub>),  $\alpha_2^A\delta_2^A$ , in adults; fetal hemoglobin,  $\alpha_2^A\gamma_2^F$ , in infants. During the transition from fetal to extrauterine life, the hemoglobin  $\gamma$  chain is exchanged for a  $\beta$  or  $\delta$  chain. The sequences have been recorded in Fig. 22.5. In addition, there are a variety of abnormal hemoglobins. Examination and comparison with the normals show that, in each, there is a change in one amino acid.

Some substitutions that have been noted are:

68	$\alpha$	Asp	replaced by	Lys
6	$\beta$	Glu	replaced by	Lys or Val
58	$\alpha$	His	replaced by	Tyr
63	$\beta$	His	replaced by	Tyr
67	$\beta$	Val	replaced by	Glu

There is no forbidden substitution, and there may be a charge change. Interesting as such studies may be, they are beyond control experimentally. A large number of individuals are continually scanned by hospital laboratories, and the opportunity to discover abnormal hemoglobins is therefore great. The inherent disadvantage is that mutations cannot be produced at will. Although the  $\alpha$  and  $\beta$  chains are the gene products of unlinked genes, these genes cannot be located or mapped by experiment.

This experimental deficiency has been circumvented by using a bacterial protein. Yanofsky chose for this purpose the tryptophan synthetase system from *E. coli*. The enzyme, noted in Sec. 15, catalyzes three reactions and, in *E. coli*, consists of two proteins (A and B). The two proteins are chromatographically separable and inactive when separated.

It is possible to unite the genetic material of two bacterial mutants in the same cell to produce a diploid cell. When this is done with various mutants unable to synthesize tryptophan, two types of effects may be seen. (1) There still may be no synthetase action because both mutations are close and relate to the A, or the B, protein. (2) Synthetase action may be restored, since one mutation relates to the A protein and the other relates to the B protein. It is said that restoration of activity is due to *complementation*. Both the A protein and the B protein have been isolated and purified. Studies on A protein from mutants have shown that only one amino acid residue has been changed per mutational event. This result not only reinforces the hypothesis that mutation causes a specific and identifiable chemical event, it also heightens the conviction that there is no heterogeneity in polypeptide sequences.

Continued examination of the sequences in the A protein of mutants has shown that seven different amino acids can occupy the same position in the mutant chains. Two are not active enzymically, but others are. Further study of the strain that synthesizes the inactive chain showed that reversion can occur with a return of capacity to make an active A protein. The reversion can occur by a change at the original site, or by a second change in a residue situated 36 residues away. It can be concluded from these studies, and others, that mutation-linked changes can identify portions of a polypeptide chain that are functionally related. The two sites noted above are probably brought near one another in space by the folding pattern.

### isoenzymes

Possible variations in functional protein structure also have been noted in the study of enzymes by electrophoresis. For several years, workers using fixed-

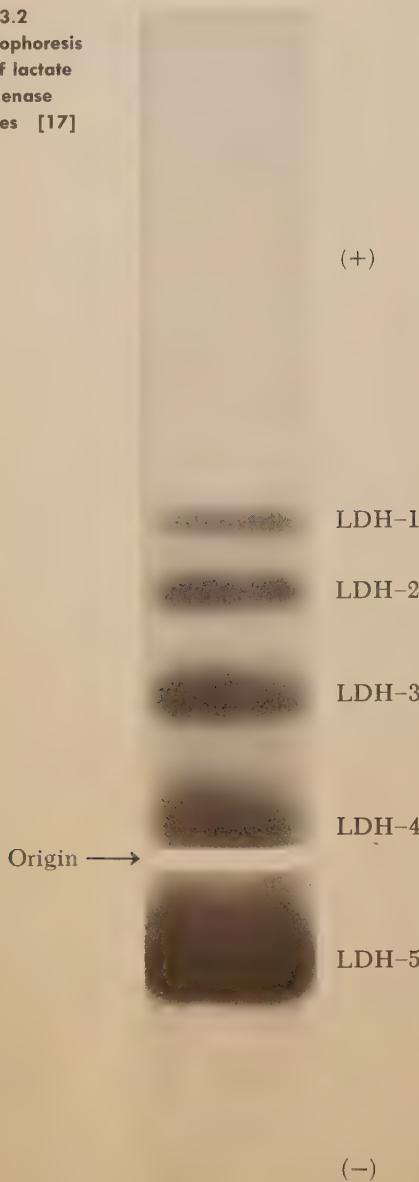
bed or gel electrophoresis noted several closely spaced bands where only one was expected. The question arose whether multiple bands of enzyme activity represented several types of the same enzyme or an experimental artifact. It was observed that the enzyme activity was in several protein bands and that the proteins of these electrophoretically produced bands were quite similar. They were called isozymes, or isoenzymes,\* and it was found that they had the same substrate specificity. The electrophoretic patterns obtained were tissue- and species-specific. Thus a new question arose: How should we refer to a protein with a certain enzyme activity, such as lactic dehydrogenase? Should we simply refer to lactic dehydrogenase? Obviously not, if there is more than one protein having this activity. It is not unusual to refer to the source in naming an enzyme, but in the case of isoenzymes we find *several* proteins in a single source, all having the same enzymic activity.

In the specific instance of lactic dehydrogenase, gel electrophoresis produces five bands, each containing an enzymically active protein (Fig. 23.2). It has been proposed that this enzyme (molecular weight 134,000) is an assembly of four subunits of two types and that the bands correspond to the various possible assortments  $A_4$ ,  $A_3B$ ,  $A_2B_2$ ,  $AB_3$ , and  $B_4$ , products of hybridization. Each type of chain has its own characteristic sequence and different properties; the chain syntheses are controlled by nonidentical genes. Apparently the chains are complementary enough in structure to associate in the five possible permutations. This variety is in contrast to the hemoglobin situation, where less freedom in modes of association is evident. Many enzymes have now been shown to exist as isoenzymes, and there is by no means total agreement concerning the explanations so far offered. What is certain is that enzymic activity can exist in a variety of similar proteins whose precise relations are as yet unknown.

The significance of such investigations, both for protein structure and for genetics, is obvious. It is probable that the genetic coding in the nucleic acids is transcribed into amino acid sequences of restricted length. If the molecular weight of mRNA is 150,000, then about 500 nucleotide residues are present. For a triplet code this length could provide a sequence coding for 170 amino acids, a polypeptide with a molecular weight of about 25,000. Very few polypeptide chains are reported to have a molecular weight above  $10^5$ , and a large number have weights in the range of 20,000 to 60,000. It is currently assumed that the secondary and tertiary structures dictated by the amino acid sequence appear at the polysome assembly point during synthesis. The structural sub-units formed associate in characteristic clusters, but it is not known whether they associate at the site of synthesis. In general, only such clusters have enzymic activity. That is, some single-chain enzymes do exist, but an enzyme that normally exists as an associated cluster of subunits does not retain activity when dissociated. It has also been shown that dissociation-induced losses of activity are often reversible, and this attests to the thermodynamic stability of enzymically active ensembles.

\* Preferable spelling according to a committee of the International Union of Biochemistry.

**FIGURE 23.2**  
gel electrophoresis  
pattern of lactate  
dehydrogenase  
isoenzymes [17]



Thus the term “one gene–one enzyme” is now not only incorrect but virtually misleading. It is an example of a working hypothesis that, although not quite correct, stimulated fruitful experimentation. In the years since the hypothesis was formulated it has been found necessary to add a modifier to the term *gene* and to view an enzyme not as a compound, but as an activity. In many cases, enzymic activity may be considered not an attribute of a poly-

peptide chain but more correctly a property of a special association of protein subunits.

The interplay between structural studies, the recording of protein electrophoresis patterns, and various kinds of genetics research has been quite fruitful and will probably continue to be for some time. Since the early ultracentrifugal investigations of Svedberg there has been evidence for the existence of subunits in proteins. As the knowledge of the DNA-RNA-polypeptide relation grew, it became increasingly evident that very long polypeptide chains require very large mRNA assemblies; hence, small subunits were suspected.

A good many tantalizing questions remain. Why is it that some enzymes exhibit a random assortment of subunits? Can some subunits serve as structural units in two quite different types of enzymes? When subunits of an enzyme derive from two different genetic loci, how do they "find each other"?

Many enzymes like the ribonucleases (2.7.7.17), or lysozymes (3.2.1.17), consist of single, small polypeptide chains. Others, such as aldolase (4.1.2.7) and lactate dehydrogenase (1.1.1.27), consist of small numbers of polypeptide chains of less than 30,000 molecular weight. Some enzymes contain only one type of structural subunit, and others may contain two or more subunits. It is quite common to find dimers, trimers, and tetramers of such subunits. At present, the dissociation behavior of each protein must be examined singularly until there are enough data for generalizations. In this book the numbering classification recommended by the Commission on Enzymes of the International Union of Biochemistry has been used. From the foregoing discussion it will be seen that this classification refers to *enzyme activities* and *not to enzyme proteins*. For example, the systematic name of 1.1.1.27 is L-lactate:NAD oxidoreductase. It is singular in activity but very plural indeed with regard to protein structure. The enzyme activity must be an attribute of certain sequences, but nothing forces us to conclude that all of this sequence must be in the same polypeptide chain.

Some proteins pose special problems. Bovine serum albumin has a molecular weight of 69,000 and no demonstrable structural subunits. If the foregoing reasoning is correct, the polypeptide chain seems very long for any mRNA. There is some evidence that it contains four repetitive sequences. Could it be that four subunits are connected by a special covalent bond after they leave the polysome? There are also several very large proteins such as glutamic acid dehydrogenase (1.4.1.3), urease (3.5.1.5), and  $\beta$ -galactosidase (3.2.1.23) with molecular weight of  $5 \times 10^5$  and greater. Each has been shown to consist of several subunits, and only the entire assembly is enzymically active. Experimentally, such proteins must be treated quite differently from small enzymes. We have much to learn before we will be able to build satisfactory models of such proteins.

At present there has been no attempt to segregate evidence concerning gene-protein relationships into one class obtained from haploid cells and one obtained from diploid cells. Certainly bacteria have many experimental advantages. Unfortunately for the experimentalist, many of the practical problems of life relate to diploid cells.

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## the cell nucleus—protein metabolism in nucleated cells

For many purposes, bacteria have provided useful experimental systems, and it is a working hypothesis that any event discoverable in a bacterial cell is likely to be demonstrable in other types of cells. The reverse is certainly not true. Most plants and animals familiar to us possess nucleated cells, and many nucleated cells produce substances not found in bacteria.

**nuclei** A variety of mammalian cells provide good experimental material for the study of the nucleus. Cells, such as those from liver, are easily broken by a mild shearing force in a Potter-Elvehjem homogenizer,<sup>\*</sup> or some similar device, and the liberated nuclei can be separated by centrifugation in a wide variety of media. Nuclei are relatively large subcellular structures and hence sediment readily. Care must be used to avoid swelling of the nuclei and to avoid undue abrasion of the nuclear membrane, but it is possible to obtain good yields of undamaged nuclei reasonably free from debris.

Isolated nuclei contain DNA, RNA, protein, and lipid. For any given species the amount of DNA is constant in the somatic (body) cells of various tissues. This value is about twice that found in the haploid spermatozoa. RNA/DNA weight ratios range from 0.13 to 1.6 in rat liver nuclei (for example), depending on the isolation media. The lipid/DNA ratio is in the range 0.12 to 0.60, and the protein/DNA ratio is 2.4 to 22. The range of values signifies that the nuclear membrane is probably quite permeable to large molecules.

There is no test of activity quite equivalent to that of oxidative phosphorylation for mitochondria. Strictly biological techniques have made possible the insertion of an isolated nucleus into an enucleated cell; thus, retention of biological activity can be observed directly. This transplantation has been done by using amoebas, for example. In liver cells this type of manipulation has not been developed. Rather, one finds many data recorded for the enzymic complement of the nucleus. The glycolytic sequence enzymes are present. Exclusive to the nucleus is the reaction (Fig. 15.3)



Amino acids are incorporated into peptide linkage in the nucleus, and nucleoside triphosphates are incorporated into RNA.

<sup>\*</sup>Just after World War II there was a spate of experimentation on cell-free preparations, or homogenates. Properly prepared, a homogenate is the sum of all the cell components obtained by rupturing the cell membrane. The homogenizer, credited to Potter and Elvehjem, of the University of Wisconsin, was simply a test tube with a glass pestle ground to fit. It was, and is, delightfully simple and often effective.

Studies on isolated thymus nuclei have revealed that ATP is synthesized inside the nucleus. Not only is the glycolytic sequence present but, probably, also NAD-dependent oxidative mechanisms coupled to ATP synthesis.

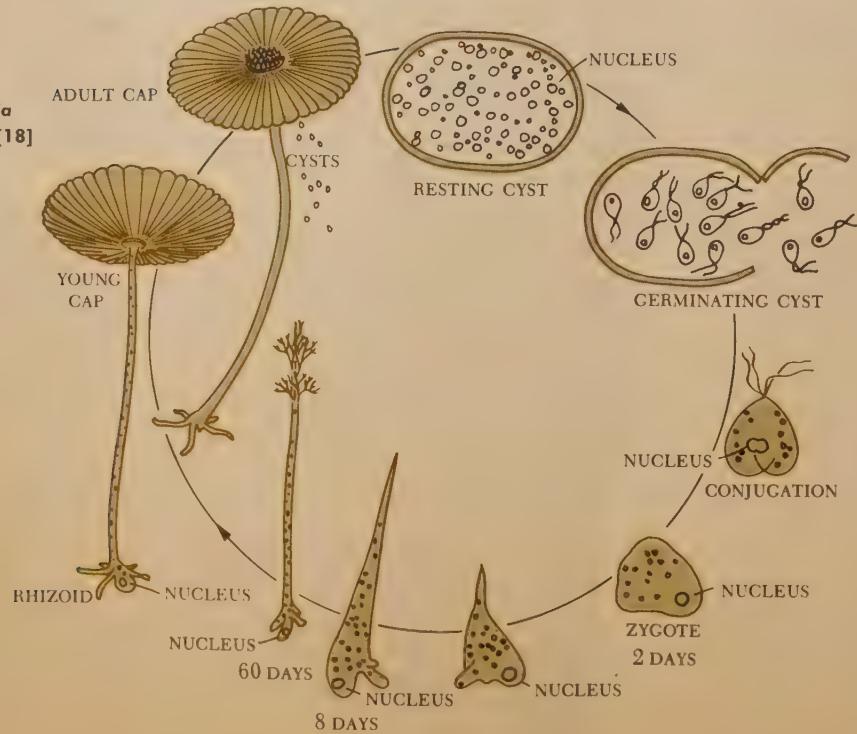
In the avian erythrocyte, hemoglobin has been demonstrated to be synthesized in the nucleus. The hemoglobin penetrates the nuclear membrane to appear in the cytoplasm. (The avian red cell differs from the mammalian in that the mature cell of the mammal lacks a nucleus.)

It has been possible to isolate ribosomes (Sec. 16) from thymus gland nuclei, to demonstrate amino acid activating enzymes, and to obtain evidence for the synthesis of mRNA, and its function, in the isolated nucleus. Altogether, there is clear-cut evidence for DNA-dependent protein synthesis. This evidence, in turn, implies substantial metabolic activity.

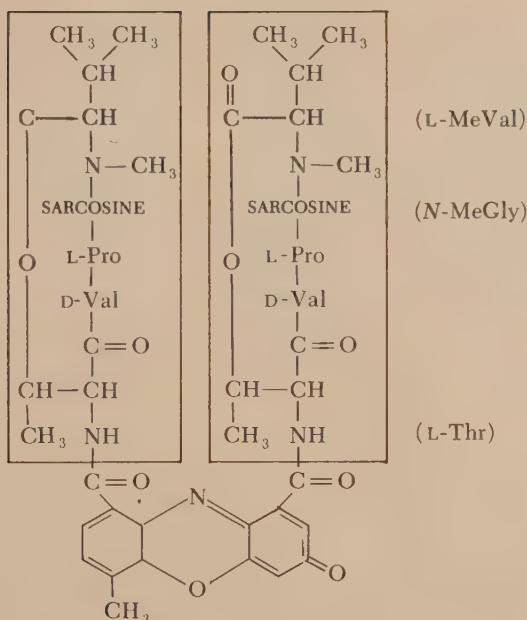
In man and higher animals there are cells, nerve cells, that do not multiply in number after birth, there are somatic cells that multiply during the period of rapid growth and then remain relatively quiescent, there are cells such as those of the liver that can be stimulated to multiply rapidly. Finally, there are mature erythrocytes lacking a nucleus and with a relatively short life. It is not surprising, then, to find that the nucleus is requisite only for cell division and not for metabolic activity.

The unicellular alga *Acetabularia* has been a convenient experimental subject for investigating nuclear activity. It consists of a rhizoid containing a nucleus, a stalk, and a "cap" at maturation (Fig. 24.1). The organism may be

**FIGURE 24.1**  
life cycle  
of *Acetabularia*  
*mediterranea* [18]



**FIGURE 24.2**  
Structure of  
actinomycin D



enucleated easily by severing the distal end of the rhizoid. In the absence of the nucleus, photosynthesis continues undisturbed and even a severed cap may be replaced. The conclusion, from a variety of experiments, is that the nucleus continually furnishes substances to the cytoplasm. One of these substances appears to be a relatively long-lived mRNA which eventually is destroyed but allows protein synthesis to go on for some time in the absence of the nucleus. In contrast to *Acetabularia*, enucleate amoebas cannot maintain normal metabolic activities. There is a loss of ability to ingest food, and the cell cannot survive for more than a few days.

Several kinds of differentiated cells have been tested for the presence of mRNA with a lifetime of more than 24 hr. This exploration has been done by treating tissues with actinomycin D (Fig. 24.2) to inhibit RNA synthesis<sup>o</sup> and then testing for polyribosomes active in protein synthesis. Lens tissue, feather cells, and reticulocytes produce an mRNA which persists more than 16 hr. It may be significant that each of these tissues produces a characteristic protein. Tissues, such as liver, muscle, and brain, that produce a wider variety of protein synthesize an mRNA that has a much shorter span of function. In rat liver the average mRNA has a half-life of about five days.

An experiment that has provided direct evidence for the flow of substances from the nucleus to the cytoplasm has been described as follows. *Neurospora* hyphae were allowed to grow on a grid in an ultracentrifuge cell in such a way

<sup>o</sup> The antibiotic actinomycin D, at concentrations as low as  $10^{-6} M$ , binds to the helical form of DNA. It appears to bind preferentially to areas containing guanine, and it appears to bind less strongly when the DNA is "denatured," melted, or uncoiled. It is very effective in inhibiting the DNA-dependent synthesis of RNA. Several other compounds such as mitomycin C, proflavin, and chloroquine also combine with DNA. Chloroquine, like actinomycin, forms a stable complex with the helical form of DNA and a less-stable complex with the single-strand form.

that they acted as their own centrifuge tubes. When centrifuged, the particles in each septum were sedimented into an order, from top to bottom: vacuoles, supernatant, microsomes, mitochondria, nuclei, large granules. The cells were then exposed to tritiated leucine and tritiated uridine and centrifuged again at appropriate intervals. Autoradiography after centrifugation showed that the labeled uridine appeared first in the nucleus and then in the microsomes. This strengthens the hypothesis that RNA is synthesized in the nucleus and moves to the cytoplasm. The labeled leucine, in contrast, appeared first in the microsomal fraction and moved to other parts of the cell.

The evidence certainly suggests that in cells that contain a nucleus there is a constant movement of substrates into the nucleus, a constant synthesis of macromolecules, and transport of these products out through the nuclear membrane.

#### protein turnover

Another apparent (not real) difference between bacteria and differentiated cells is the phenomenon of protein turnover (cf. Sec. 11). Early experimentation seemed to show that bacteria do not exhibit concomitant synthesis and breakdown of protein. It was eventually recognized that the experimentation had been confined to bacteria during active growth only. When observations were made on resting cells, cells that were metabolizing but not dividing, a substantial turnover was found.

The concept of turnover was an outcome of the (then) revolutionary work of Schoenheimer and Rittenberg (cf. Sec. 8) that definitely changed the patterns of thought in biochemistry. A few experiments have been selected to illustrate pertinent points.

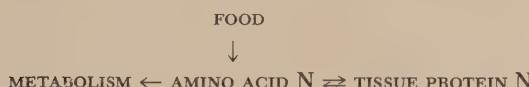
- A mobile equilibrium exists between tissue proteins and dietary amino acid nitrogen.

Adult rats of constant weight, in nitrogen equilibrium, were maintained on a 16% casein diet, a diet quite adequate in nitrogen.  $^{15}\text{N}$ -labeled glycine was fed to furnish 25 mg of nitrogen per day for three days. The percentages

TABLE 24.1  
distribution  
of  $^{15}\text{N}$  in  
tissues

tissue	atom percent
	$^{15}\text{N}$ content of protein N
serum	1.7
intestinal wall	1.5
kidney	1.4
liver, heart, testes	0.9
muscle, hemoglobin	0.3
skin	0.2
percent recovery	
muscle	66
internal organs	33

of  $^{15}\text{N}$  recovered were protein N, 44; tissue nonprotein N, 11; urine, 41; feces, 3. Such results led to the picture:



But it is not at all clear what is occurring here. Is it only the nitrogen that is in flux? Are the peptide bonds continually breaking and reforming? Are protein molecules continually disintegrating and being replaced? Can cells be disintegrating and multiplying at a rate sufficient to account for the data? Some of the questions generated have not been answered even today.

- Tissues differ with respect to the speed of exchange and the total quantity of nitrogen exchanged.

An experiment quite similar to the preceding one was done except that  $^{15}\text{N}$ -labeled leucine was used and the various tissues were examined. See Table 24.1 for the results. Several amino acids were found to yield similar results.

- $^{15}\text{N}$  is distributed among the various amino acids.

Again, the same experiment was made with leucine but now the liver amino acids were analyzed. Consult Table 24.2. As noted in Sec. 14, the nitrogen in lysine is not in rapid equilibrium with other amino acids.

- Amino acid nitrogen is transferred as  $\text{NH}_4^+$ .

The experimental conditions were changed only by adding  $^{15}\text{N}$ -labeled ammonium citrate to the diet at a level of 25 mg/day for nine days. Animals sacrificed at the end of this period were found to have  $^{15}\text{N}$  in their liver protein. Later experiments showed that the rate of  $\text{NH}_4^+$  utilization varied with the protein level of the diet. At low protein levels,  $\text{NH}_4^+$  was utilized almost as well as glycine. *Immature* rats fed on cornstarch, lard, and  $^{15}\text{NH}_4^+$  citrate also incorporated  $^{15}\text{N}$  into body protein despite the absence of real growth (because no essential amino acids were present).

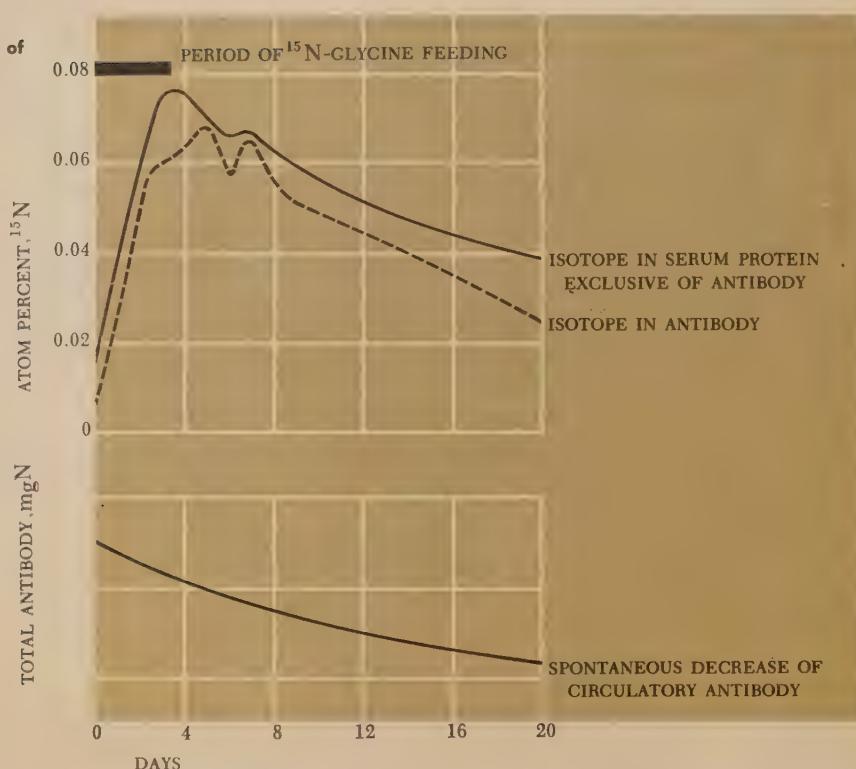
A rabbit was immunized with pneumococcus (type III) until the serum had a high antibody titer. It was then fed  $^{15}\text{N}$ -glycine for three days. Daily serum samples were taken, nitrogen-free type III specific polysaccharide

**TABLE 24.2**  
 $^{15}\text{N}$  in amino  
acids isolated  
from liver

amino acid	atom percent $^{15}\text{N}$ content
Leu	8
Glu	1.9
Asp	1.2
Arg	0.9
Gly	0.75
Tyr	0.5
Lys	0.06

By permission of Harvard University Press. From R. Schoenheimer, *The Dynamic State of Body Constituents*, 1942, Table 8, chap. 2.

**FIGURE 24.3**  
time variation of  
 $^{15}\text{N}$  content in  
antibody [19]



was added, and the precipitate of the antigen-antibody complex was analyzed for  $^{15}\text{N}$  (Fig. 24.3). The results of this experiment strongly suggested that  $^{15}\text{N}$  was being incorporated into new antibody even when the total antibody titer was declining. Hence a decrease in the total amount of a protein could be visualized as the resultant of two different rates: a rate of disposal greater than the rate of synthesis. The result mirrored a change in a *biochemical equilibrium*.

In other experiments various serum proteins were examined, and in each case  $^{15}\text{N}$  was found to be incorporated readily, indicating a continual synthesis. The mobility of plasma\* proteins had long ago been shown by Whipple, who used the technique of plasmapheresis: animals were bled and the cells were centrifuged down, resuspended in buffer, and reinjected in the animal. Plasmapheresis is a device for continually removing plasma protein; the ability of the animals to replace plasma protein is a measure of synthesis rates. Definite evidence of an interchange between plasma and tissue protein was obtained. Other workers have reported experiments in which liver slices were incubated with  $^{14}\text{C}$ -labeled plasma albumin. The protein appeared to go through the cell membrane and to be converted to  $\text{CO}_2$ , glucose, and glycogen, but not to amino acids.

\* Plasma proteins are those in the extracellular portion of the blood. Serum proteins are those in the serum squeezed out of the blood clot. Virtually the only difference is the absence of fibrinogen from serum.

Again, Whipple and Cannon had reported that dogs could be kept in good health for months on a low protein diet if blood protein were injected. Thus it appears that the plasma protein system, consisting largely of albumins, globulins, and fibrinogen, is a quite labile system. There is no reason to believe that the protein turnover exhibited by the blood proteins is extraordinary, although specific proteins exhibit a wide spectrum of rates. It has been shown that protein turnover occurs in tissue-culture cells as well. The rate is about  $1\text{ hr}^{-1}$  in mammalian cells. Under optimal conditions, cells in tissue culture double in about a day and hence there is an increase of about  $3\text{ hr}^{-1}$ . Thus, even during active growth, 25 to 30% of the protein is being degraded.

In plants also there is evidence for protein turnover. During seed germination, there is a period of rapid disintegration of seed globulins<sup>o</sup> and synthesis of other types of protein. During germination, asparagine and glutamine tend to accumulate and to serve as reservoirs of nitrogen for protein synthesis in the leaf. In adult plants there is also evidence for the continuing uptake of nitrogen, not only for new net synthesis, but for replacement of previously formed protein.

The study of protein synthesis in rapidly dividing bacteria is a restricted study. There is no reason to believe that the chemical and physical mechanisms of protein synthesis as such are different in bacteria than they are in liver cells. What seems certain is that the whole milieu in which protein synthesis takes place is different in the cells of differentiated tissues. The nucleus is capable of protein synthesis and, therefore, synthesis must be viewed as a process not confined to a particular subcellular particle. On the other hand, there is evidence that extranuclear protein synthesis is mediated by mRNA, a nuclear product.

Protein synthesis is by no means restricted to dividing cells, or to growing cells, and can be demonstrated in cells or tissues that are "resting" or that are in a metabolic steady state. Obviously, some process of protein removal must exist in order to maintain a steady state. In the animal as a whole there are mechanisms for the transfer of protein out of one tissue and into another, so that the steady state could embrace internal protein usage. However, studies of isolated tissues indicate this to be less than the whole explanation. There seems to be a protein "catabolism," a special degradative process aside from secretory loss of protein and cell death, and we know nothing of its nature. What factors determine the half-life of a particular protein type? The average life of protein in the rat has been estimated at 17 days, in the human at 80 days. Moreover, the estimated rate of protein synthesis in the rat has been found to be  $1\text{ g}/(\text{day})$  (kilo body wt.), whereas the figure for man is 0.2. Since the types of protein in the rat resemble those in man, it seems that the rate of breakdown is coupled to the rate of synthesis. It seems likely that all proteins are reasonably stable thermodynamically, and hence the analogy that

<sup>o</sup>Euglobulins and pseudoglobulins are globular proteins found in a wide variety of biological materials. Eu-(true) and pseudoglobulins are differentiated on the basis of solubility behavior. A pseudoglobulin is soluble at very low ionic strength, whereas euglobulins remain sparingly soluble until the ionic strength is raised. Euglobulins remain soluble at the isoelectric point, whereas pseudoglobulins do not.

proteins disintegrate because of "wear and tear" is outworn and inappropriate. Examination of preserved blood samples up to 20 years old showed that hemoglobin in solution had remained stable and unmodified. In the non-dividing cell there must exist degradative processes coupled to the synthetic processes.

Recent studies on subcellular particles have revealed that rat liver mitochondria have a half-life of about 10 days and that microsomal proteins have a half-life that is only 30 to 40 hr. The examination of specific enzymes has resulted in a wide range of half-lives. Specific but nonenzymic proteins display a similar range.

In the human, protein is distributed approximately as in Table 24.3. Albumin has been discussed. Hemoglobin has a half-life of about 60 days in man, but in other species this value may vary from 10 days to 11 months. The main proteins of skeletal muscle are about as labile as hemoglobin, whereas the protein of skeleton (mostly collagen) is very stable indeed.

An organism as complicated as the mammal presents to the experimenter an ensemble of rates so varied that it is difficult to make a precise statement about turnover in the whole animal. In general, however, we do sense turnover for the whole animal. When degradative processes achieve rates equal to those of synthesis, the animal stops growing.

TABLE 24.3  
protein distribution in man

protein	weight, g
total protein ( $N \times 6.25$ )	10,006
striated muscle	4,680
skeleton	1,864
skin	924
adipose tissue	361
hemoglobin	750
albumin	250

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# 25

## growth

It has been emphasized that protein synthesis has been studied most vigorously in organisms capable of rapid growth. The biochemist may be tempted to equate protein synthesis and growth but, as you have seen, protein synthesis can, and does, occur in cells that are not growing.

“Growth” has an everyday meaning difficult to dissociate from the word. It is the understanding that the growing subject is getting bigger. Yet the biological usage contains the notions of division, of differentiation and, in some circumstances, enlargement merely by an imbibition of water. Because so many processes are implied, the word “growth” should be used circumspectly in a scientific context.

In the life processes of bacteria there is nothing to inhibit cell division as long as the environment is favorable. Given an adequate nutrient supply and room for physical dispersion, there is no inherent mechanism to limit multiplication. The same can be said for many plants. Some trees will continue to grow indefinitely in a favorable environment and in the absence of noxious infestation. Such is not true of animals, whose very forms are evidence of intercellular, intertissue controls that affect cell division. Does it follow, then, that the DNA replication process in the nucleus differs from that in *E. coli*? The work of Taylor and his coworkers bears on this question. *Vicia faba* (broad bean) seedlings were grown for one root cell division in tritiated thymidine. This substrate was used because it has been found to be incorporated by living cells specifically into DNA. The substrate was removed and growth was allowed to continue in colchicine, which blocked cell division without inhibiting chromosome replication. At intervals, samples were taken for autoradiography. Since tritium radiation is very low energy  $\beta$  radiation easily stopped by the silver granules of the film, definition of the source of radiation is rather precise.

The working hypothesis was that DNA has no turnover and hence thymidine can be incorporated only during replication. Immediately after exposure to thymidine, all the chromosomes were found to be labeled, but after one further cell division in colchicine (in the absence of labeled thymidine) only half the chromosomes were found to be labeled, except where crossing over had occurred. The number of chromosome sets (i.e., the ploidy) was an index to the number of replications under these circumstances. Such evidence points to a semiconservative replication process in a nucleated cell. What has not been proved is that chromosomal DNA is the *only* substance bearing a genetic code and involved in the transmission of cell character.

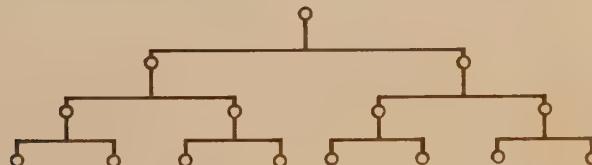
At the onset of mitosis the nucleolus and other granules inside the nucleus are present. When the chromosomes form, the nucleolus, as well as the nuclear membrane, disappears. The replication of the chromosomes can be

demonstrated with the light microscope, and nearly everyone who reads this book will have seen the patterns formed as the daughter chromosomes (chromatids) move apart. What happens when a mutation occurs in a cell of higher ploidy? This process has been studied in a diploid yeast (Fig. 25.1). In these diagrams the vertical distances are proportional to division times. After irradiation, many "lethals" were produced and the time of cell division was noted to be quite variable, but eventually some normal cells were produced. The explanation of such a recovery is not a simple one. The course of events may be determined by the rate of chromosome crossing over. This phenomenon is illustrated in Fig. 25.2, and this simple diagram serves to visualize several questions. It will be noted that only two strands are represented as crossing over. Occasionally all four are involved; why not always? The crossing over is represented as occurring physically in such a way that the two resultant strands are exactly the original length. In this way it can be seen that repair of an irradiation-damaged strand can be achieved. What secures the exact matching in length?

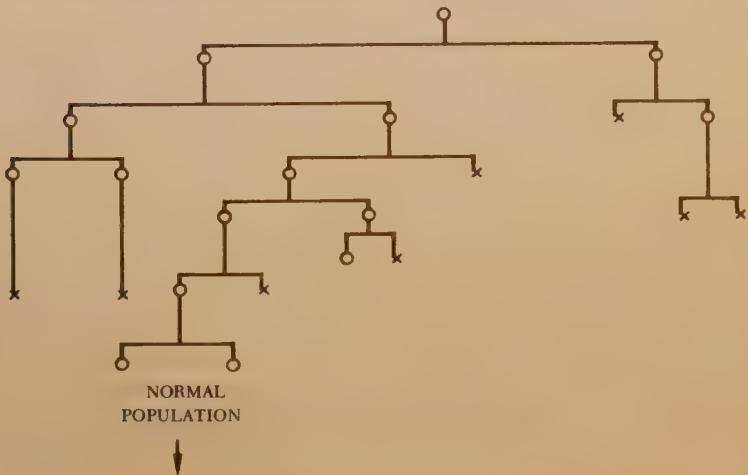
The chromosomes of such cells (unlike the purely DNA "chromosome" of bacteria) are composed of both DNA and protein. How does the protein come away from the DNA and return in good order during this process? From a biochemical viewpoint the present models are quite unsatisfactory. It has been shown that the rate of repair of chromosomes broken by X irradiation

**FIGURE 25.1**  
cell division in  
yeast (diploid)

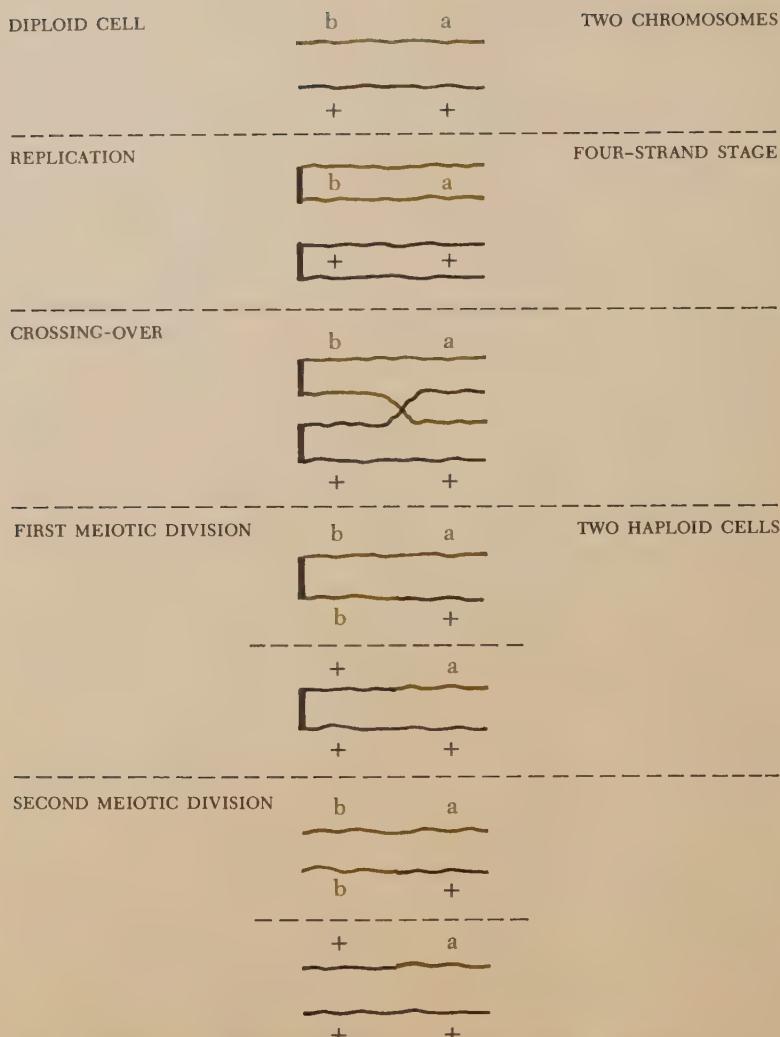
NORMAL DIVISION PROCESS



DIVISION IN AN IRRADIATED DIPLOID YEAST



**FIGURE 25.2**  
diagrammatic  
illustration of  
crossing-over



tion of hamster cells can be substantially increased by administration of DNA. Breaks become visible at metaphase in cells growing in culture. Most breaks are repaired by some unknown mechanism facilitated by the presence of DNA. It is an open question whether this repair process is similar to that in which DNA polymerase has been observed to participate.

### multiplication of sub-cellular particles

One aspect of cellular division and growth, aside from the replication of the nucleus, is the duplication of subcellular particles. It is now recognized that mitochondria in daughter cells arise from mitochondria in the parent and that the same is true for plastids. In the marine alga *Micromonas* there exist one nucleus, one mitochondrion, and one plastid per cell. During cell division, all three organelles divide synchronously.

Much more extensive documentation has been forthcoming from experi-

ments on the plastids of *Euglena gracilis* (a phytoflagellate) and the mitochondria of yeast. These have proved to be excellent experimental subjects: when *Euglena* is grown in the dark, proplastids divide but do not differentiate into mature plastids; in yeast, O<sub>2</sub> is the trigger to the differentiation of mitochondria. It now seems that each organelle contains some DNA, enough to code the production of a few hundred enzymes. These enzymes could be considered constitutive. In addition, some enzymes, inducible enzymes, can be synthesized in the organelle but only at a signal from the nucleus. Thus, the organelle does not divide in the absence of a signal from the nucleus, but differentiation and function are dependent on environmental variables. Mutations have been induced in the organelles without effecting any change in the nuclear material. Currently there is speculation that it may be possible to induce transformations in yeast and other cells that will result in enzymes for specific purposes.

Aside from the latter speculation, there does seem to be substantial evidence that extranuclear genes can mutate and that very high mutation rates can be produced. This situation points to the development of a new branch of biochemistry, organelle biochemistry, that can be closely coordinated with genetic experimentation.

### **differentiation**

In bacteria there is DNA synthesis during a major fraction of cell life, but in most other kinds of cells DNA is replicated only during a limited part of the intermitotic period. For example, in kidney cell preparations there is an initial stage of 12 hr when precursors enter nuclear RNA, followed by a sharp increase in this fraction during the period 12 to 22 hr. The new high level is maintained during the period 22 to 32 hr, and, at 32 hr, DNA synthesis begins, continuing for 50 to 60 hr. At present the coordination of these biochemical events is imperfectly understood.

Finally, there remains before us the enigma of cell differentiation. All somatic cells in a complex organism have the same genetic complement, and yet it is obvious that there is only partial gene expression in each type of cell. It seems certain that the control of gene expression is in the nucleus. For example, in the frog egg, injection of chromosomal protein causes differentiation to halt at the blastula stage. If this nucleus is now substituted for the nucleus in a normal frog egg, again differentiation halts at the blastula stage. It has been said that "genetic silence" exists up to the blastula stage, silence that may be due to the presence of a stable mRNA.

Many investigators feel that their evidence implicates the chromosomal protein as the controller of gene expression. The chief protein in the chromosome is termed a histone. Unfortunately, it is very difficult to characterize chromosomal proteins precisely, because of their perplexing heterogeneity. In a sense, this supports the idea that they are a versatile group of compounds that can "cover" a certain portion of DNA and prevent it from coding certain types of mRNA. But there has been no unequivocal demonstration of the control of gene expression by either histones or chromosomal nonhistone protein.

**nucleolus**

Much speculation has been stirred by the observation that a marked enlargement of the nucleolus is almost invariably seen in cancer cells. Since these are cells whose division rate is out of control, and in which differentiative processes have been suppressed, it is of interest to clarify the nucleolus function. To repeat, the nucleolus is a structure that disappears at the onset of mitosis and reappears after division. It is present in most, but not all, cell nuclei, and it may be multiple; the maximum number per cell is an index of the ploidy. The nucleolus appears to have a high protein content and to contain RNA, lipids, and carbohydrate, but no DNA is demonstrable.

During periods of intense protein synthesis, the nucleolus may be observed to become single and large. In contrast, cells which do not synthesize protein rapidly (muscle cells, leucocytes) exhibit very small nucleoli. Yet, even in nucleated cells the nucleolus is not omnipresent and is clearly not indispensable for protein synthesis.

There is evidence for the hypothesis that sRNA is synthesized in the nucleolus, that ribosomes are present, and that protein synthesis occurs within this structure. It is possible, but not proved, that the ribosomes of the cytoplasm are assembled in the nucleolus. Many studies have been facilitated by success in making preparations of isolated nucleoli by isolating nuclei, homogenizing, and centrifuging in the proper medium.

Possibly it is presumptuous to include a section on growth in a book of biochemistry. Certainly no topic is more clearly biological. Within the same thought, however, is the conviction that the moving force in growth is metabolic, and what is metabolic may be considered biochemistry. Growth processes involve the time scale of the epigenetic system; they are processes that are rather deliberate as cell events go. Some of the control systems will be discussed in the last section. Such processes are not easily explored by the classical biochemistry techniques, and there is no doubt that new experimental methods are badly needed.

Differentiation represents not only a different time scale but a discontinuity. Much of interest has arisen from studies of insect physiology and experimental embryology. Here biochemistry has served only as a tool and has contributed little in the way of concepts. Despite this, it is well for anyone who is interested in biological problems to attempt to phrase the problem in the setting of his own discipline.

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# 26

## metabolism of differentiated cells

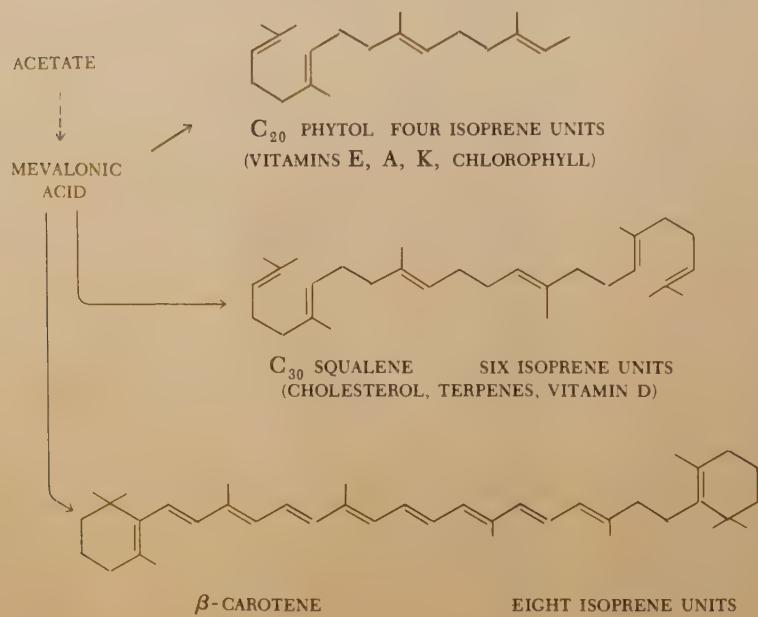
The topics treated thus far have been germane to the study of an undifferentiated cell; they are those expected to be pertinent for all cells. They include the chemical interrelations involved in furnishing energy for synthesis and for motion, the several metabolic functions of the subcellular particles, the mechanism of enzyme catalysis, the syntheses and structural features of biopolymers, and the chemistry of the replication process. It was stated at the beginning of this book that biochemistry, similarly to other sciences, deals with models. The enzyme-catalyzed reaction, the isolated mitochondrion, and the nucleus—these are but models, useful though they are. In the sections to follow we shall attend to the biochemical matters that accompany differentiation and that may, in some instances, be a consequence of cell association.

### cholesterol

One class of substances emphasizing such interaction is that of the sterols. Sterols are absent from cells without well-defined nuclei, such as bacteria and some algae, but are not restricted to multicellular animals or plants. For many years cholesterol was thought to be a universal constituent of animal cells, but this ubiquity is now known to be unlikely. Cholesterol seems to be absent from higher plants but has been isolated from some types of algae.

There is an extraordinary variety of sterols in biological material, and the

**FIGURE 26.1**  
**acetate-derived**  
**isoprenoids**



ability to synthesize some type of sterol is very widespread. One outstanding exception is the insect. For this biological class, sterols have a vitamin function and must be present in the diet.

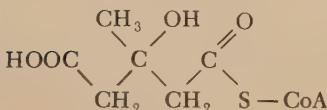
Acetate is the carbon source for the syntheses of steroids and related compounds. Some relations are outlined in Fig. 26.1. The site of cholesterol synthesis may vary from tissue to tissue. In liver, the microsomes and supernatant are involved. Dietary cholesterol inhibits synthesis, but the effective inhibitor has recently been suggested to be bile salts.

In 1942, Bloch and Rittenberg found that deuterio acetate fed to mice led to the formation of deuterio cholesterol. Deuterium was found to be associated with the carbon of the ring structures as well as the side chain. It was eventually demonstrated that 15 of the sterol carbons derived from the methyl group of acetate and 12 derived from the carboxyl group of the acetate. Evidence has been adduced for the following reaction sequences:

As noted previously:



In 1954 it was suggested that acetyl CoA and acetoacetyl CoA could interact in liver extracts to form  $\beta$ -hydroxy  $\beta$ -methyl glutaryl CoA:

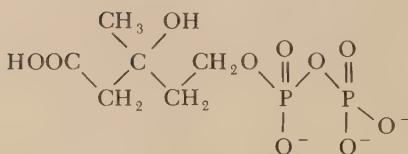
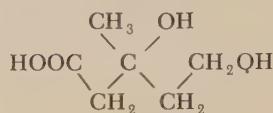


A reduction product, mevalonic acid, has been found to be a very efficient precursor for sterols. The reduction of  $\beta$ -hydroxy  $\beta$ -methyl glutaryl CoA to

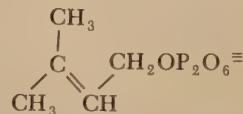
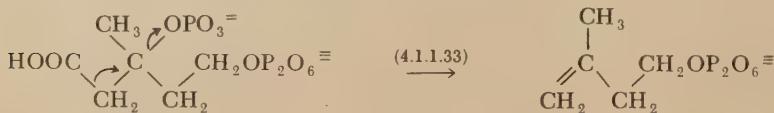
mevalonic acid



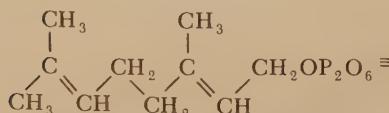
mevalonate is an NADP-coupled enzymic reaction (1.1.1.34). The mevalonate is further phosphorylated in a two-step reaction to mevalonic-5-pyrophosphate.



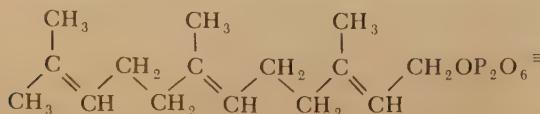
Another ATP-coupled phosphorylation yields a derivative that loses both  $\text{CO}_2$  and phosphate to form isopentenyl pyrophosphate. This compound is an intermediate in the formation of several others.



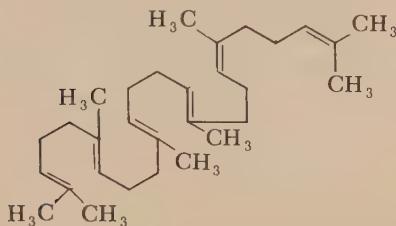
Isopentenyl pyrophosphate and the isomeric  $\beta\beta$ -dimethyl allyl pyrophosphate (with which it is in equilibrium) react to form geranyl pyrophosphate (2.5.1.1).



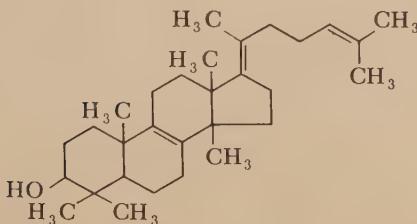
In another condensation, geranyl pyrophosphate and isopentenyl pyrophosphate react (2.5.1.1) to yield farnesyl pyrophosphate.



All the above steps plus the following reactions have been demonstrated in yeast and in liver. The "head-to-head" condensation of two molecules of farnesyl pyrophosphate yields squalene.

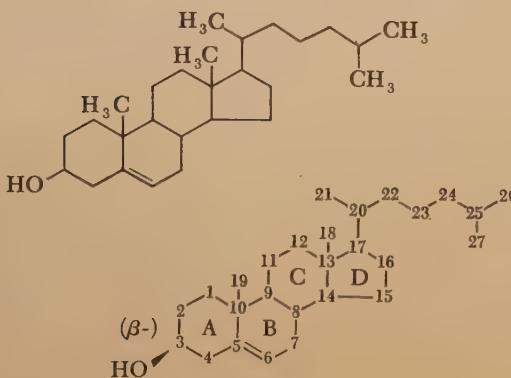


Squalene is more efficient than acetate, by an order of magnitude, as a precursor of cholesterol. The first sterol that arises from squalene is the C<sub>30</sub> compound lanosterol. It is thought that SQUALENE HYDROXYLASE (OXIDOCYCLASE) (1.14.1.3) catalyzes simultaneously all the ring closures and an oxidation involving molecular oxygen.

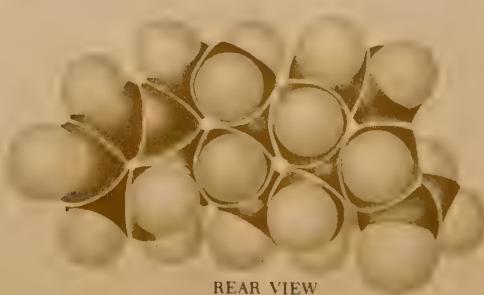
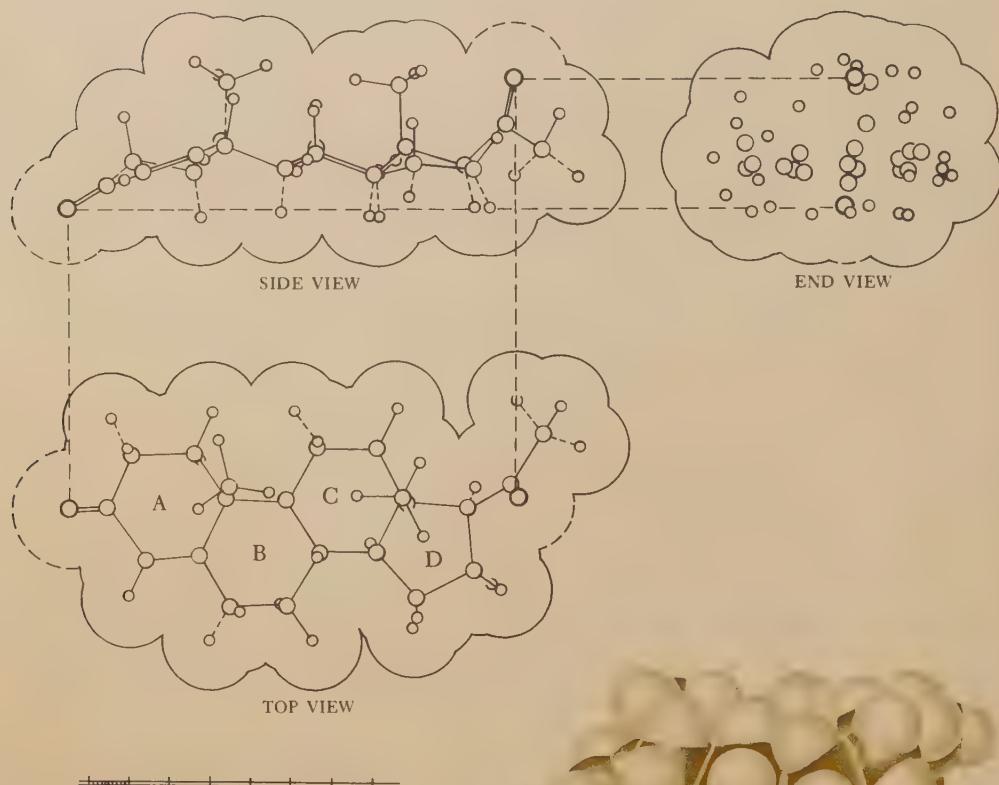
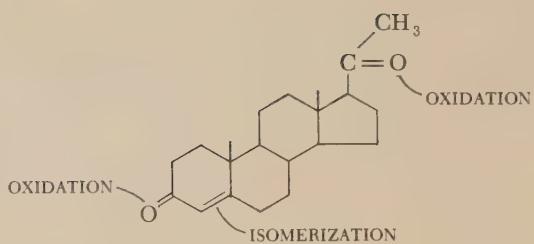


The derivation of cholesterol from this precursor involves the loss of three carbons (Fig. 26.2). Cholesterol is found in tissues as such and also as an aliphatic acid ester. In blood it is found as a component of lipoprotein. Its presence occasionally becomes a nuisance in the form of gallstones, and its occurrence in arteriosclerotic plaques has often been referred to. Of prime concern here is the position of cholesterol as a precursor of hormonal steroids and of bile acids.

**FIGURE 26.2**  
cholesterol and its  
numbering convention



**FIGURE 26.3**  
formulas of  
progesterone [20]

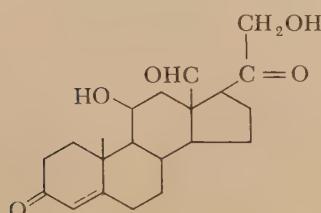


**adrenal  
steroids**

The adrenal gland has the ability to synthesize a variety of steroids. As its name indicates, this endocrine\* gland is found near the kidney. In some animals the adrenal medulla and the adrenal cortex are separate glands, but in mammals the gland is macroscopically singular. The dual function remains, however. The product of the medulla is the neurostimulatory compound adrenalin referred to in Sec. 15. The cortex tissue is essential for the proper balance of functions in the organism: the cortex is often said to be necessary "for life." Its loss is followed by a variety of symptoms: among them are water and salt disturbances, extreme susceptibility to stress, and lowering of the blood pressure.

A probing of cortical tissue has revealed 30 steroids, only a fraction of which appear in adrenal venous blood. Since steroids are not stored in the gland, presumably some of the compounds that have been described are intermediates. Some steroids (mineralocorticoids) function in the regulation of water and monovalent ion exchange, some (glucocorticoids) participate in the regulation of carbohydrate metabolism, and others (sex hormones) have an effect on accessory sex functions.

There is little doubt that cholesterol is an important intermediate in the biosynthesis of all these substances. The formation of progesterone, for example, requires two oxidations and an isomerization (Fig. 26.3). Progesterone is also secreted by ovarian tissue and is involved in the implantation of the fertilized ovum. It has no known action in the male. In the adrenal cortex it is further modified and mineralocorticoids, the most effective of which is aldosterone, are produced.

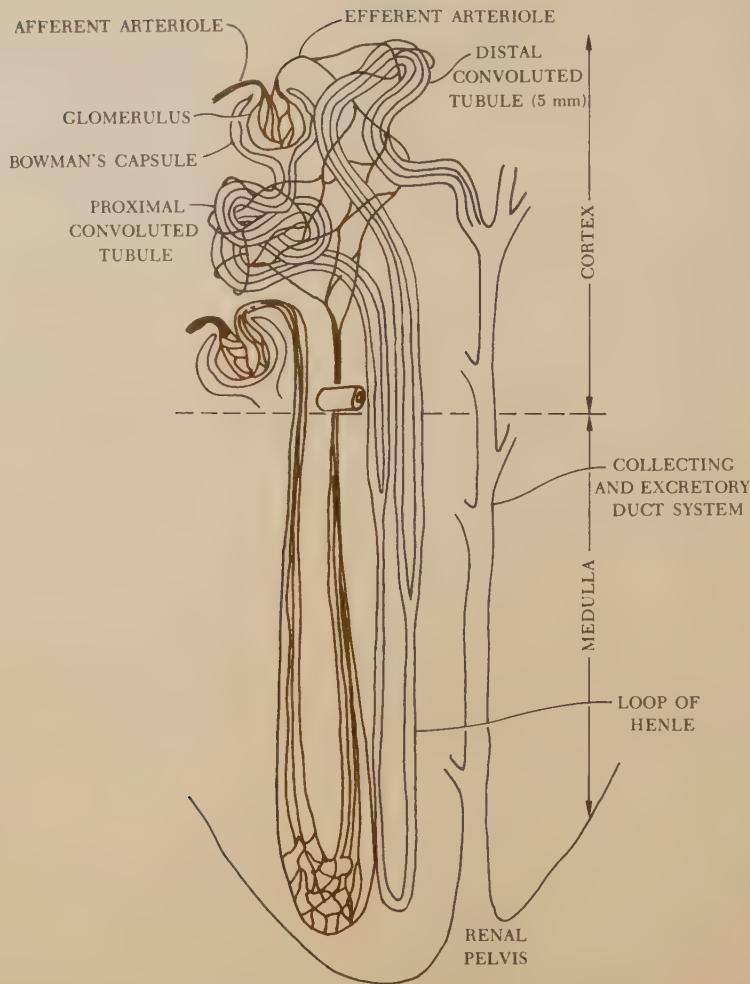
**kidney  
functions**

Aldosterone and other compounds similar in activity probably affect the processes that occur in the cells of the kidney tubule. The tubule, the chief functional unit of the kidney, contains several kinds of cells and exhibits a varying function from portion to portion. Figure 26.4 is a diagram of the anatomy of the nephron in a human kidney. In Fig. 26.5 are sketched the movements of monovalent ions and of water through various parts of the nephron.<sup>†</sup> The mineralocorticoids seem to shift the equilibria in favor of

\*The endocrine glands in the human are the adrenal, hypophysis (pituitary), thyroid and parathyroid glands, the islet tissue of the pancreas, and portions of the gonads. These glands have no excretory ducts, but the gland products enter the general circulation nonetheless. Exocrine glands are of several types, but each has some type of excretory duct.

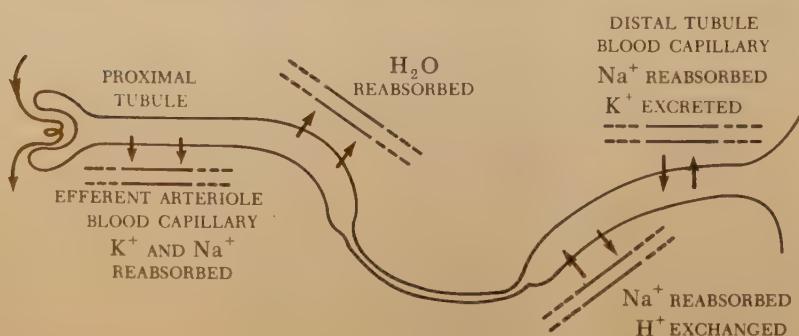
<sup>†</sup>It has been pointed out in a recent paper that the selection of solutes for reabsorption in the kidney tubule is responsible for the low thermodynamic efficiency of the kidney. Calculations based on osmotic work indicate an efficiency less than 1% (REF. 7).

**FIGURE 26.4**  
**a nephron, the functional unit of the kidney [21]**



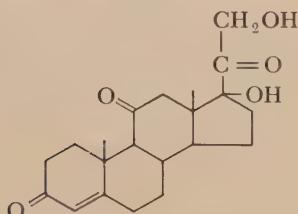
$\text{Na}^+$  retention and water reabsorption. As long as  $\text{Na}^+$  is being reabsorbed,  $\text{K}^+$  can be excreted. In the absence of corticosteroids, then,  $\text{Na}^+$  is not reabsorbed and hence is overexcreted, water is not reabsorbed,  $\text{K}^+$  cannot be

**FIGURE 26.5**  
**the flows of monovalent ions and of water through the nephron walls**



excreted and hence accumulates. From the discussion in this book, so far, there is no basis for understanding the results of ion imbalance. No generalities will serve, since so little is known of the fundamental aspects of monovalent ion interaction.\*

In another series of reactions are produced the glucocorticoids, the most active of which bears the trivial name of cortisone (17-hydroxy 11-dehydro corticosterone):

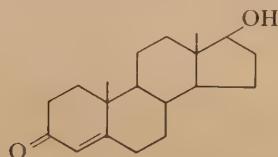


These compounds shift the equilibrium in favor of *non*reabsorption of glucose in the kidney tubule, so that glucose will tend to appear in the urine if the blood glucose rises. Normally, as the blood flows through the capsule (Bowman's) at the distal end of the tubule, it is ultrafiltered so that all of the blood components, except the cells and macromolecules, appear in the glomerular urine. Most of the glucose and much of the water is reabsorbed during the formation of bladder urine at the distal end of the tubule.

The glucocorticoids also favor glycogen accumulation in the liver and inhibit protein synthesis by stimulating the formation of glucose from amino acids (gluconeogenesis). The effect on glucose utilization in the extrahepatic tissue is inhibitive and is therefore the opposite of insulin action. To describe adequately the many effects of adrenalectomy, or the effect of an oversupply of hormone due to adrenal tumor, is not consonant with the balance of this book. Endocrine control involves several tissues, and the removal of any one affects the activity of the others. The disturbance of such a poised system (usually a push-pull system) often yields results difficult to interpret.

#### sex hormones

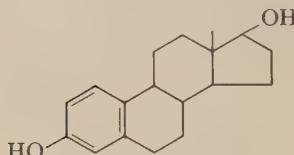
In the testes, cholesterol is converted, with progesterone as an intermediate, to one of the male sex hormones (androgens), testosterone:



This substance, also produced in small quantity in the adrenal cortex, is involved in the development of accessory sex functions in the male.

\* Monovalent ions are also involved in the swelling phenomena of mitochondria. Several substances, as diverse as  $\text{Ca}^{++}$ ,  $\text{P}_i$ , and thyroid hormone, induce swelling provided active oxidation is taking place. If oxidation is blocked by an inhibitor, for example,  $\text{CN}^-$ , swelling does not occur. During the swelling process,  $\text{K}^+$ , as well as water, accumulates inside the mitochondrion. There is a substantial literature on this subject but no knowledge of causation.

In the ovary, cholesterol is converted into progesterone, already noted, and to estrogens such as estradiol:

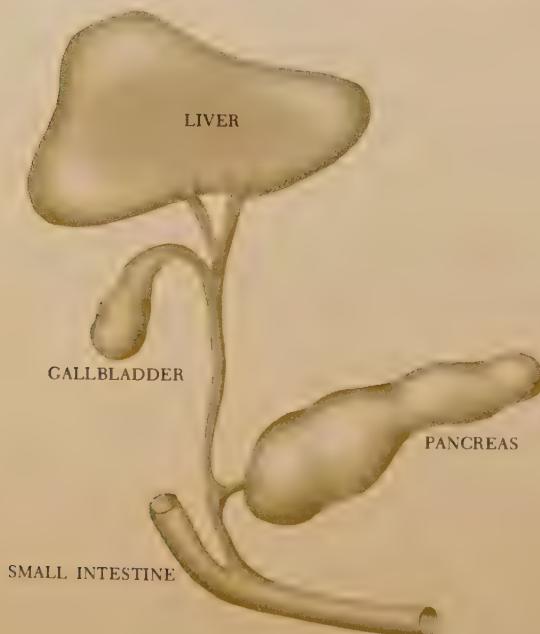


Here again, the variety of actions which progesterone and the estrogens may have upon various tissues in different physiological states defies simplification. Students should consult works on endocrinology for further elucidation. It is an interesting but unevaluated observation that estrone causes a reduction in blood cholesterol in the intact animal and also inhibits the synthesis of cholesterol in rat liver preparations. The inhibition is believed to be due to prevention of mevalonate decarboxylation. Limited success has attended efforts to demonstrate the direct effect of such steroids on enzymes, and the usual causative relations are unclear. It seems likely that in some instances such hormones react with the mRNA that codes for the synthesis of specific enzymes. In a few cases, hormones have been noted to affect protein conformation, but the biological significance has not been adequately evaluated.

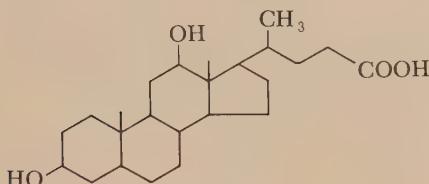
### bile acids

Yet another family of compounds deriving from cholesterol is elaborated in liver tissue. These products have been termed bile acids because they accumulate in gallbladder bile, a concentrated liver secretion (Fig. 26.6). Because of some, as yet obscure, relation, bile acids appear at an evolutionary

**FIGURE 26.6**  
anatomy of bile flow



level where calcified skeletons appear. A typical bile acid is deoxycholic acid:



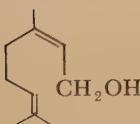
The bile acids are excreted into the small intestine and are involved in fat absorption. Since the bile salts are reabsorbed, in part, a cyclic process develops; in it a certain quantity of bile acids is continually synthesized and balanced by a steady excretion. The result is a steady-state concentration of bile acids.

Bile acids have unique properties deriving from their reactions with *both* hydrophobic and hydrophilic compounds. They are, thus, surface-active agents, and when they are added to a mixture of fats and aqueous solutions, emulsions are formed. Biological structures, such as the membranes of red cells and of other cells, are disordered by the bile salts, and this has been attributed to disturbance of the lipid layer arrangement. However, bile salts also cause the dissociation of several protein complexes, possibly by disturbing hydrophobic bonding. The student may well wonder how the liver escapes damage by such potent products. Actually, in the bile these compounds exist in amide linkage with glycine and with taurine, but it may be presumed that a complex polysaccharide film insulates the tissues from the harmful effects of the bile compounds.

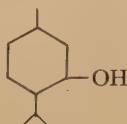
### **terpenes**

It has been noted that cholesterol does not occur generally in plants, but there is no reason to think that the syntheses of plant sterols are dissimilar to the processes described in animals. The synthesis of ergosterol from squalene has been demonstrated in yeast.

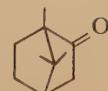
As a group, plants exhibit the ability to synthesize a bewildering array of substances from acetate via mevalonate. Geranyl pyrophosphate is the parent compound of the *terpenes*, which form part of the essential oils of plants. Some familiar compounds are



CERANIOL



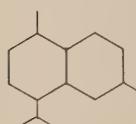
MENTHOL



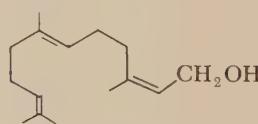
CAMPHOR

From farnesyl pyrophosphate (the precursor of squalene) derive the 15C *sesquiterpenes*. For the most part these are neither familiar nor biologically

interesting. Simple examples are

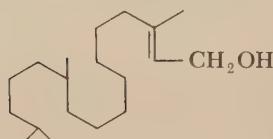


CADINENE

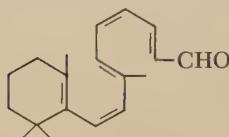


FARNESOL

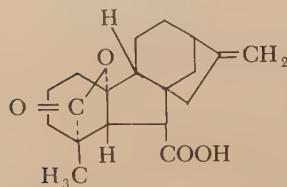
The *diterpenes*, containing 20 carbons, derive from the fusion of two molecules of geranyl pyrophosphate. Among the more interesting compounds to be found are phytol, a constituent of chlorophyll,



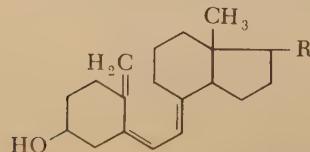
and vitamin A:



In flowering plants the gibberellins are growth regulators which have been demonstrated to derive from geranyl pyrophosphate:



The *triterpenes* (30C) arise from the reactions of farnesyl pyrophosphate via squalene. They include the sterols already considered and the vitamins D:



The above formula represents vitamin D<sub>2</sub> (calciferol), which also arises from the ultraviolet irradiation of ergosterol. Other triterpenes include digitonin, other cardiac glycosides, and alkaloids.\*

\* Most alkaloids, compounds containing nitrogen, usually in a heterocyclic ring, arise from other precursors. These precursors include tyrosine, tryptophan, phenylalanine, ornithine, and amino pentoses.

The *tetraterpenes* (40C) are found in many biological sources as carotenoids.  $^{14}\text{C}$ -Mevalonate has been shown to be incorporated into  $\beta$ -carotene in fungi and in carrots.

*Polyterpenes*, the natural rubbers, are also known to be formed from mevalonic acid.

It is precisely this area that has become the center of interest for the many organic chemists who concern themselves with "natural products." The terpenoids, alkaloids, carotenoids, and sterols provide a magnificent, or overwhelming, variety of compounds. The mechanisms of biosynthesis cannot fail to interest the organic chemist, and the mastery of the organic reactions cannot fail to interest the biochemist.

This section might have been titled "the nonsaponifiable lipids." Most of the substances discussed do derive metabolically from acetate- and fat-related metabolism. As you have seen, the variety is great, both in chemistry and in biological action. Another lipid-associated group of compounds will be considered next.

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# 27

## phospholipids and the light-sensitive pigments

As a class of chemical compounds the sterols have received wide and constant attention. It is known that the biological requirements vary with the species, and several sterols have been identified with specific processes. In general, it is not possible to write a chemical description of the way in which sterols react with other cell components to produce the unique effects observed. The phospholipids share some of the characteristics of the sterols. They are ubiquitous, being synthesized in nearly every tissue, but their precise functions are not yet formulated. Several segments of the metabolic apparatus are involved in their formation and several types of phospholipids are formed. These several varieties have rather similar, but not identical, solubilities.

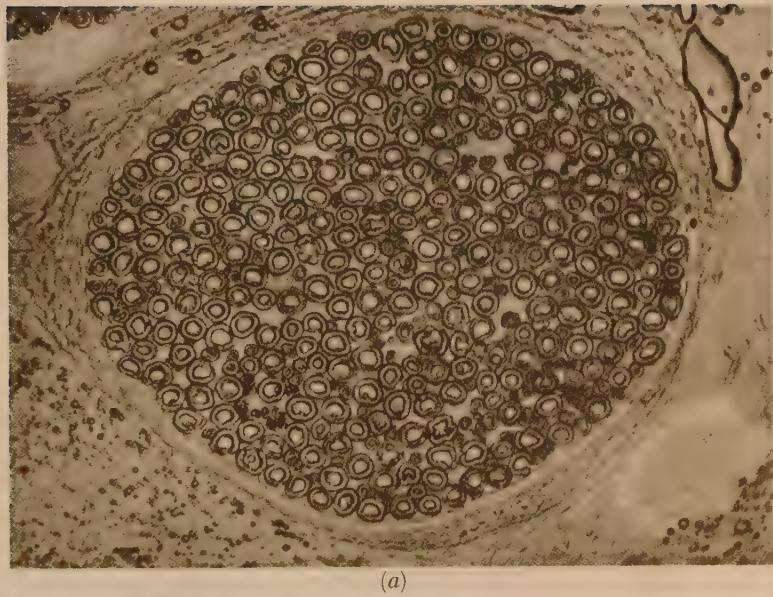
### membranes

In contrast to the uncertainty surrounding the metabolism of the phospholipids is their patent involvement in biological membrane structure. It was noted in Sec. 3 that lipids are important for the structure and functioning of mitochondria.\* Some specialized tissues, such as nerve, contain phospholipid as a major constituent. Since phospholipids contain long hydrocarbon chains as well as polar groups capable of water solvation, investigators have long postulated their special function in the formation of membranes. In mammalian cell surfaces phospholipid is demonstrable, and there is little doubt that water-insoluble lipoprotein structures form part of the cell surface.

How the lipid is bound to protein is conjectural. One possibility is that the relation resembles that in protein-detergent complexes. Detergents such as sodium dodecyl sulfate (SDS), even at very low concentrations, are bound to many proteins, and in many cases this binding is accompanied by subunit dissociation. This, in turn, indicates that the apolar residues from both detergent and protein are probably in close contact. However, there is also evidence that hydrogen bonding is present. Some proteolipids, which are extractable by methanol-chloroform mixtures, are precipitated by solvents more effective in interfering with hydrogen bonding. Apparently, methanol and chloroform associate in such a manner that an amphiphilic solvent system results.

Still other evidence has revealed some ionic binding between the protein and lipid moieties. The characterization of mixed lipid layers is quite diffi-

\* If lipid is removed from mitochondria, the enzymic functions disappear but the electron micrographs of such mitochondria strongly resemble those of the intact organelle. The enzymic activity can be restored if the proper phospholipid fraction is added to mitochondria freed of lipid. Currently it is thought that cytochromes *c* and *a* are in the phospholipid layer but bonded to the structural protein. Coenzyme Q and some cytochrome *c* are postulated to be mobile in the liquid layer.

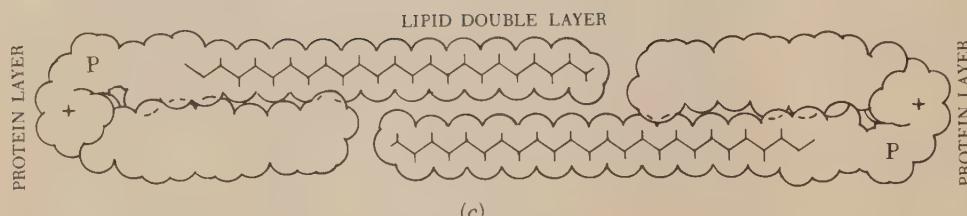
**FIGURE 27.1****myelin structures:**(a) transverse section  
of peripheral nerve  
showing rings of  
myelin around  
individual axons

(a)

**FIGURE 27.1(b)****single myelinated  
axon showing  
concentric layering  
of the myelin membrane**

(b)

**FIGURE 27.1(c)**  
**cholesterol-phospho-**  
**lipid or -sphingomyelin**  
**complexes [22]**



(c)

cult. Fatty acids, phospholipids, cholesterol, and cholesterol esters can exist in a single lipid layer packed into a continuous structure. Polar lipids like soap and phospholipids swell when wetted and at the same time can incorporate cholesterol, which is quite hydrophobic. Such structures can be quite variable in composition and yet show relatively small changes in form.

The myelin membrane has been much studied. It is a sheathlike structure surrounding the nerve axon, as shown in Fig. 27.1b and has a fine structure, shown in the same figure, that resembles a membranous wrapping. There is experimental support for the view that the center of the membrane is hydrophobic and nonpolar and consists of the hydrocarbon portion of phospholipids, cerebrosides, and sterols. The periphery contains polar groups that can interact with protein as well as water. Such a model would, two-dimensionally, be a bimolecular lipid layer bounded by two protein monolayers. Several such models have been proposed, but none can accommodate all the phenomena. It is not clear, for instance, why the lipid components of myelin show such a minimum turnover rate. This question urgently needs answering, since it is central to an understanding of demyelinating diseases.

It may turn out that the problem of understanding membrane structure is related to that of understanding the three-dimensional structure of protein. In the case of protein we have seen that the primary and secondary structures are varied but repetitive and that the tertiary structure is usually a less regular arrangement dictated by the requirement for stability. Most proteins are associations of such structural subunits, and the entire assembly is dependent upon a variety of interactions. When a nonprotein molecule is present in the vicinity of such a complex, it is conceivable that a more stable new complex can and does form. The new complex of protein and, in this case, lipid will be asymmetric and probably could achieve symmetry only by the kind of structural configuration mentioned in the preceding paragraph. This may lead to a variety of membranes each having a similar macroscopic form but quite different composition.

Erythrocyte membranes have provided readily available material for examination. Red cells burst when exposed to solutions of low osmotic pressure, and the hemoglobin-free membranes can be collected by centrifugation. The red cell is circular when viewed in one orientation, but when it is seen

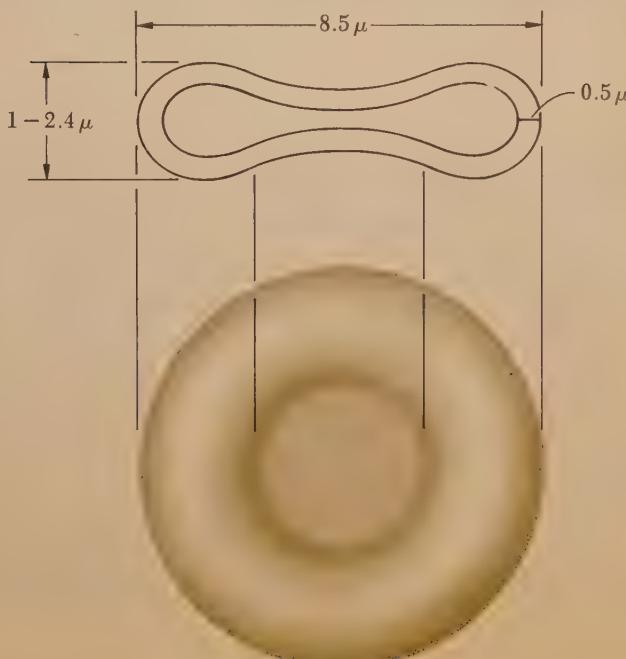
in another orientation, it may appear as in Fig. 27.2. It has been found that the hemoglobin and lipoprotein contents have a reciprocal relationship in different regions of the cell interior. That is, there is very little lipoprotein except near the cell surface. The red cell does not consist of a membrane surrounding a structureless fluid; rather, the hemoglobin solution is almost a gel. The membrane contains a protein (stromatin) and cholesterol as well as phospholipid. It is probable that not all of the phospholipid is linked to protein and that, in any event, little is bonded covalently.

The membranes (not the cell wall or the capsule) of bacteria contain substances and structures similar to those in mitochondrial membranes and show a variable lipid pattern.\* The cell walls of plants contain complex oligo- and polysaccharides and protein. Phospholipids may thus have a specialized function in membranes and one that may be restricted to certain animal cells. In contrast, the function (presumably different) of phospholipids in mitochondria and other subcellular structures may be more widely shared.

Phospholipids have also been considered to be involved in the mechanism by which dietary triglyceride is absorbed from the intestine of animals (Fig. 8.5). Much of the absorption depends on the formation of chylomicrons secreted from intestinal mucosa cells into the lymphatic system. Chylomicrons are small droplets of triglyceride and other lipids, including phospholipids and cholesterol, surrounded by a protein film.

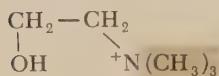
\* Sterols are absent from most bacterial membranes, as is the glycerol phosphoryl choline type of lipid found to predominate in the endoplasmic reticulum. There are glycerol phosphoryl ethanolamine lipids, polyglycerol phosphates, and frequently uneven-numbered fatty acid derivatives.

**FIGURE 27.2**  
shape and dimension  
of erythrocyte



## synthesis

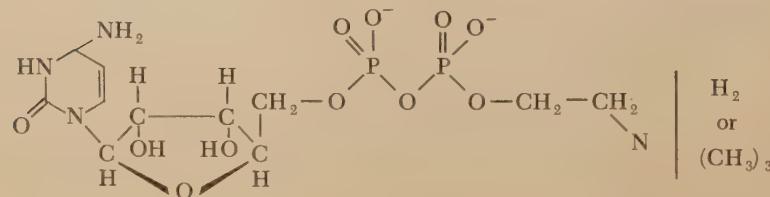
Phospholipid synthesis involves both the microsomes, or endoplasmic reticulum, and the supernatant fraction. To recapitulate: phosphatidyl ethanolamine and serine react to form phosphatidyl serine and ethanolamine (Fig. 11.5). In the animal this occurs in most tissues. The conversion of ethanolamine to choline,



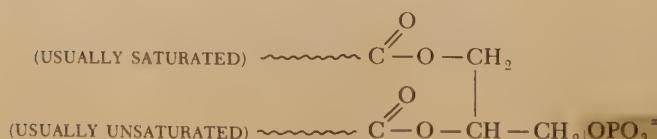
by contrast, is restricted to the liver. A variety of methyl donors may be involved (REF. 8). Both ethanolamine and choline have a steady-state concentration which, when exceeded, potentiates phosphorylation by ATP in the presence of CHOLINE KINASE (PHOSPHOKINASE) (2.7.1.32) to yield the following derivatives:



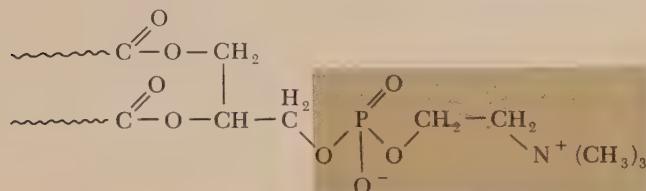
In the presence of CTP and CYTIDYL TRANSFERASES (2.7.7.14 and 2.7.7.15) cytidine diphosphate choline and cytidine diphosphate ethanolamine are formed. These enzymes are in the cell supernatant.



Coincident with these syntheses are others having their origin in carbohydrate metabolism.  $\alpha$ -Glycerophosphate, arising from dihydroxy acetone phosphate, is produced by an NADH-coupled dehydrogenase (1.1.1.8) in the supernatant. The glycerophosphate reacts (2.3.1.15) with two moles of fatty acyl CoA to form a phosphatidic acid.

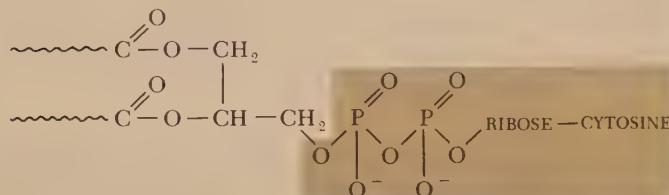


This can be further hydrolyzed (3.1.3.4) to diglycerides. The diglycerides in turn react with cytidine diphosphate choline in the presence of a TRANSFERASE (2.7.8.2) in microsomes, and this results in the production of a lecithin, a diacylglycerophosphatide.



An analogous reaction (2.7.8.1) yields phosphatidyl ethanolamines that were termed cephalins in the older literature.

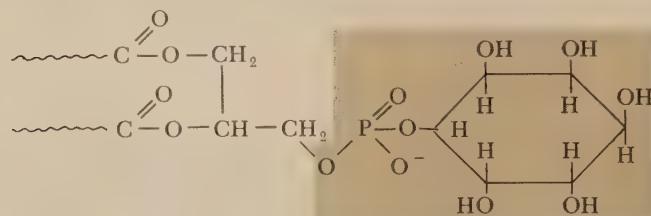
Diglycerides may also interact with CTP to form a cytidine diphosphate diglyceride.



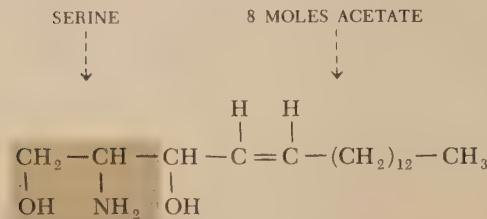
a lecithin with short fatty acid chains



Compounds of this class (variable acyl groups) react with inositol in the presence of a transferase to yield phosphatidyl inositol and CMP.



Another group of phospholipids, termed sphingomyelins, arises in the following manner:



The above compound, sphingosine, is acylated to the *N*-acyl derivatives which are the ceramides. The ceramides, in turn, are substrates for a TRANSFERASE (2.7.8.3) that catalyzes reaction with cytidine diphosphate choline. The resultant group of compounds, in which phosphoryl choline is attached to the terminal oxygen of the ceramides, is the sphingomyelin group. In nerve tissue there also exist cerebrosides, in which galactose forms a glucosidic link with the same terminal group of the ceramides.

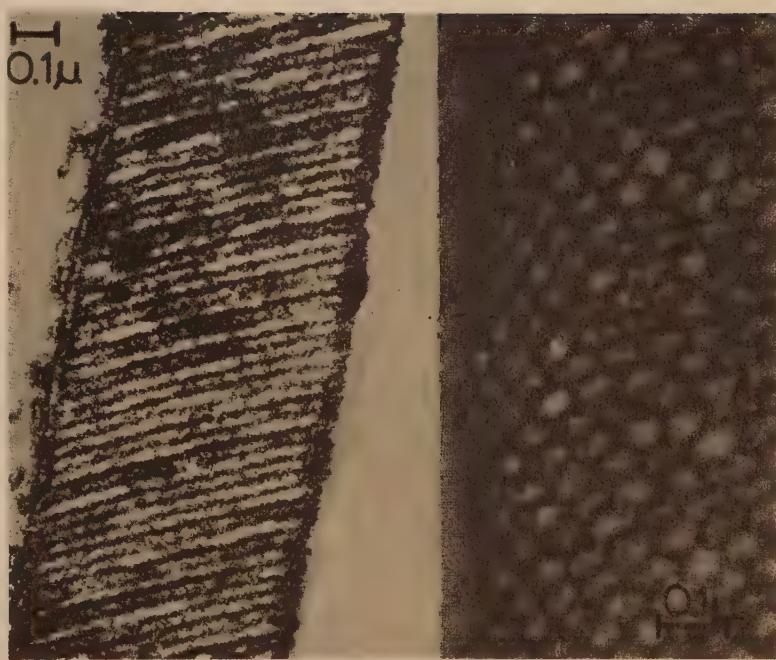
### **light-sensitive pigments**

Associated with the phospholipid-containing membranes in some tissues are the light-sensitive pigments. Light perception ranges from a primitive sensing of light by the starfish to the highly complex eye of the vertebrate. In the invertebrate eye the photoreceptors are assemblies of tubules; in the vertebrate eye they are assemblies of plates (Fig. 27.3). Both types of structure are formed from layers of lipid and protein. The precise disposition of the visual pigments within the structures has not been defined, but it is presumed to be in layers.

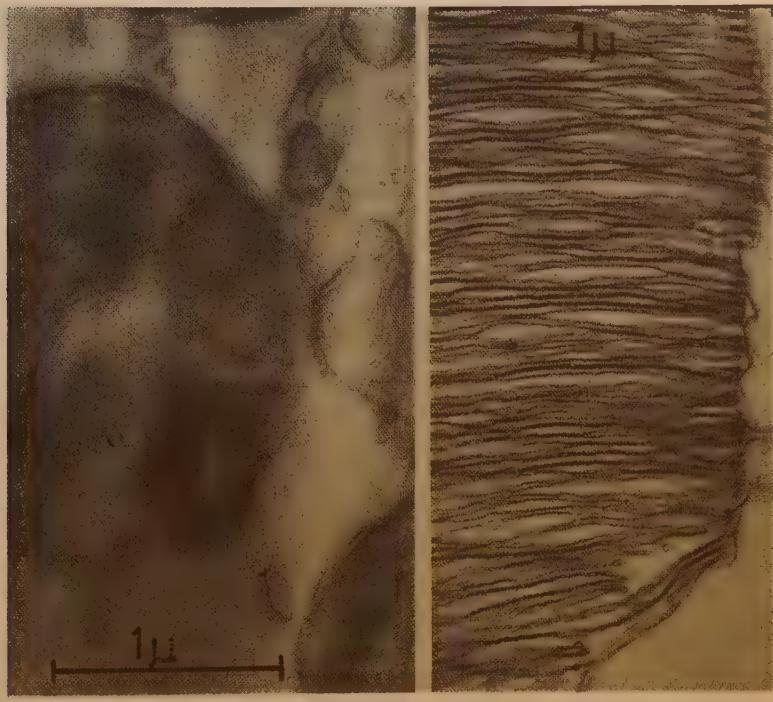
This is not the proper occasion to discuss the physiology of sight, but it is instructive to consider the light-sensitive compounds and their metabolic derivations.

In a general discussion of light-sensitive compounds none merits attention more than chlorophyll. There are yet other pigments in the chloroplast—carotenoids and xanthophylls—but their function seems accessory rather than

**FIGURE 27.3**  
vertebrate and  
invertebrate photo-  
receptor structures:  
(a) oblique longi-  
tudinal section through  
a rhabdomere of the  
housefly retinula  
showing lamellar  
type of internal  
structure;  
(b) transverse section  
through basal region  
of outer segment of a  
retinal rod of the  
guinea pig showing  
characteristic tubular  
structures and  
vesicular  
formations [23]



(a)



(b)

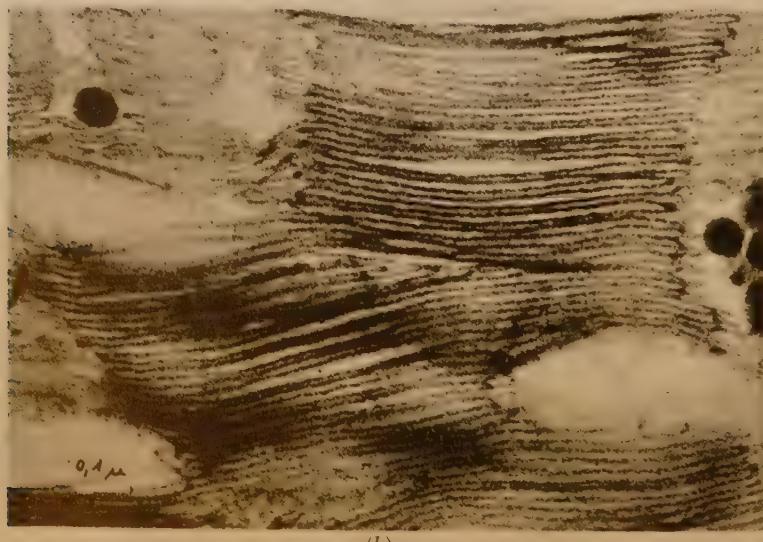
primary. The characteristic lamellae in the chloroplast (Fig. 27.4) do not develop until chlorophyll appears, and thus there is reason to believe that the synthesis of this pigment is coupled to the synthesis of phospholipid and protein. *Euglena* has been used for studies of this phenomenon. As noted in Sec. 25, chloroplasts develop when this organism is grown in the dark on glucose, but they do not fully differentiate unless exposed to light.

The initial step in chlorophyll biosynthesis is the formation of  $\delta$ -amino levulinic acid. A SYNTHETASE catalyzes the formation of a complex involving glycine, pyridoxal phosphate, and succinyl CoA.

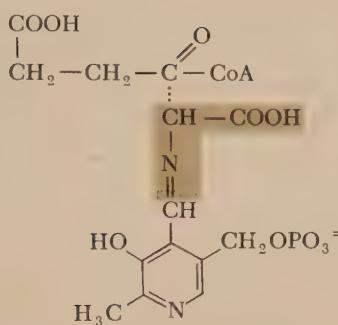
FIGURE 27.4  
lamellar structure of  
chloroplast [24]



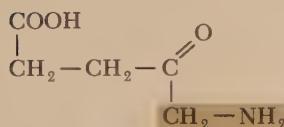
(a)



(b)



This complex rearranges with the formation of  $\text{CO}_2$ , CoA, pyridoxal phosphate, and  $\delta$ -amino levulinic acid.



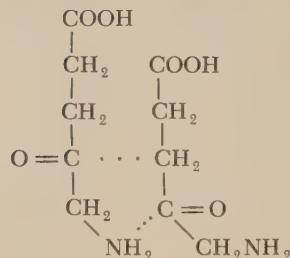
The step is common both to chlorophyll synthesis and to heme synthesis. This being so, it might be expected to occur in a wide variety of tissues. It can be demonstrated in immature erythrocytes by washing away the soluble enzymes for which  $\delta$ -amino levulinic acid is a substrate. It has also been demonstrated in the photosynthetic bacterium *Rhodopseudomonas sphaeroides*. The synthetase is not completely specific, since it also catalyzes a reaction between glycine and acetyl CoA.

Once formed,  $\delta$ -amino levulinic acid may be deaminated to  $\alpha$ -keto glutaraldehyde (which may be incorporated into the pool of citrate cycle inter-

#### $\delta$ -amino levulinic acid

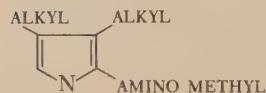


mediates) or it may undergo a condensation reaction (4.2.1.24) forming



porphobilinogen. The *synthase* involved catalyzes a reaction between two identical substrate molecules but exhibits simple Michaelis kinetics. The enzyme is widely distributed among plants, animals, and bacteria.

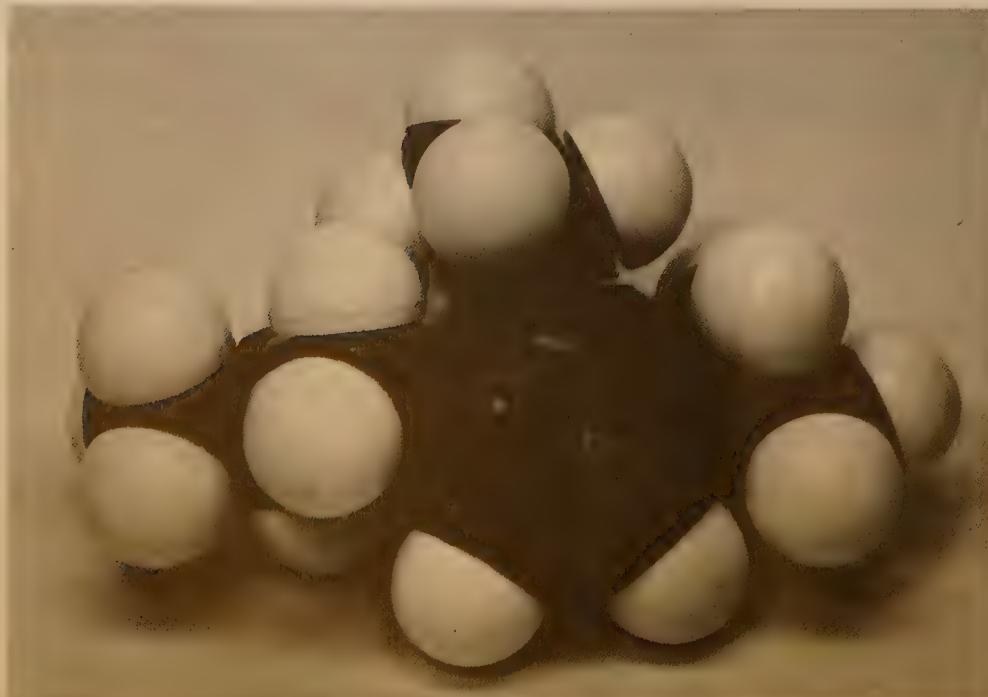
Porphobilinogen is a  $\beta, \beta'$  dialkyl  $\alpha$ -aminomethyl pyrrole. Pyrroles of this



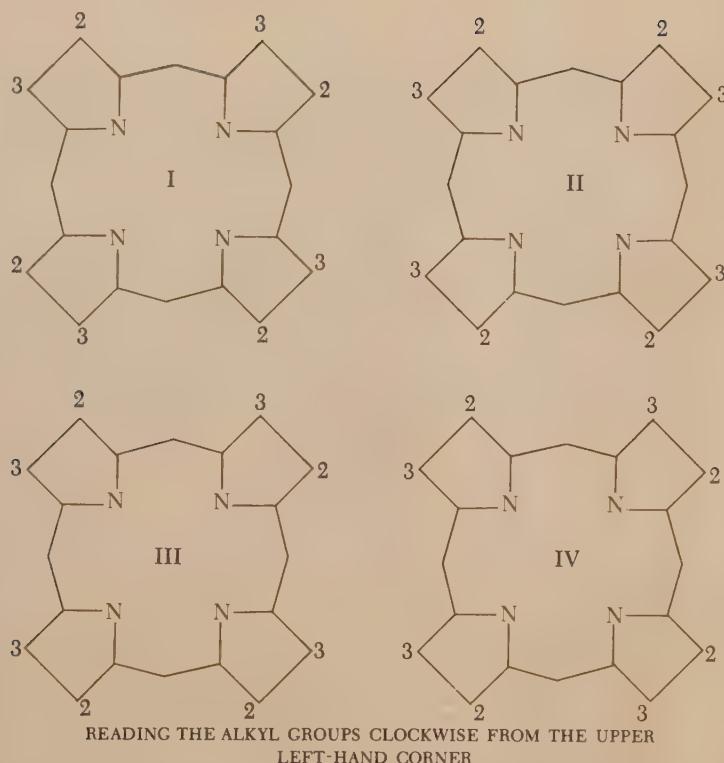
type readily self-condense,<sup>°</sup> as does  $\delta$ -amino levulinic acid at pH 8. Four isomeric uroporphyrins are possible, as shown in Fig. 27.5. Only III is seen to be unsymmetrical, and this is the naturally occurring form. Uroporphyrin III

<sup>°</sup> A mechanism of tetramerization has been proposed and is briefly discussed in reference 12.

### porphobilinogen



**FIGURE 27.5**  
the isomeric  
*uroporphyrins*



I    3,2    3,2    3,2    3,2

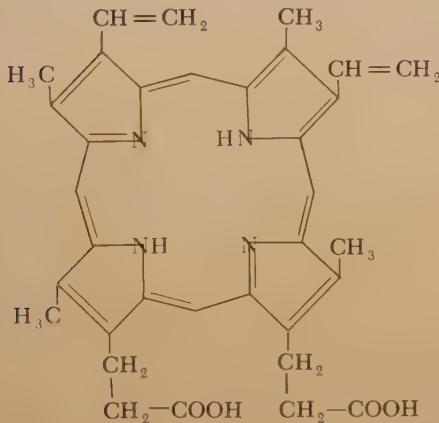
II    3,2    **2,3**    3,2    **2,3**

III    3,2    3,2    3,2    **2,3**

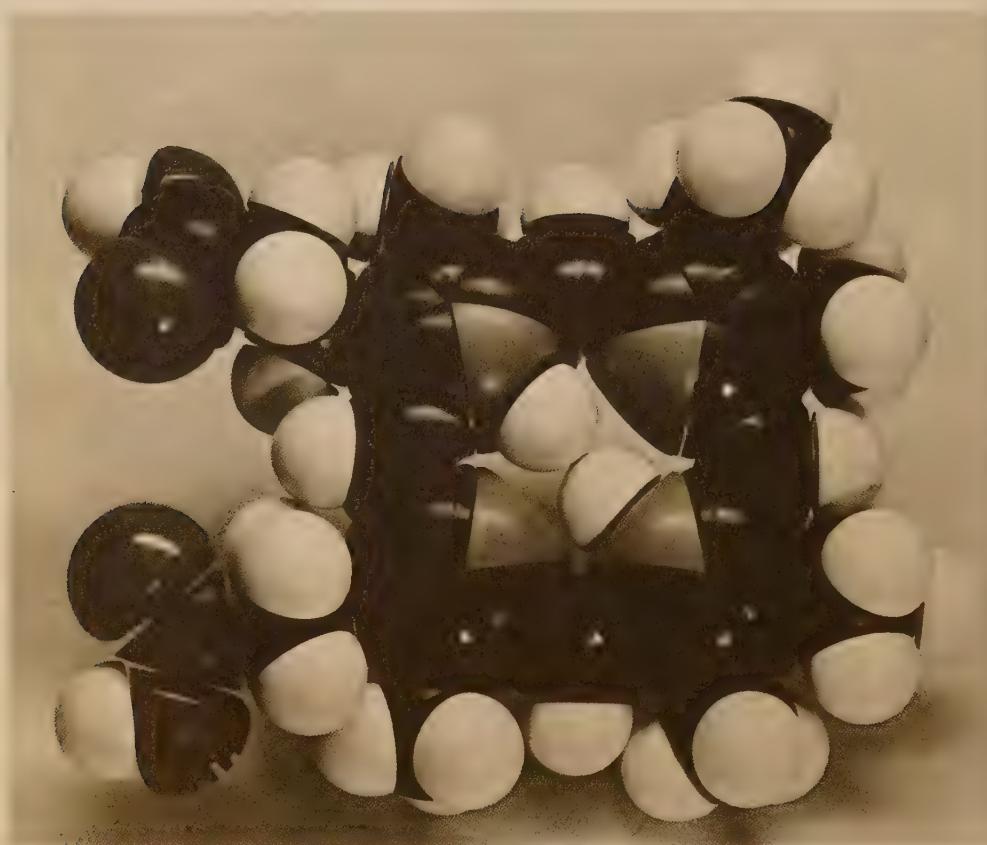
IV    3,2    3,2    **2,3**    2,3

is converted in several steps into protoporphyrin IX in the presence of particulate enzymes (Fig. 27.6). Free protoporphyrin can be isolated from immature erythrocytes, and it also occurs in plants in traces.

**FIGURE 27.6**  
protoporphyrin IX

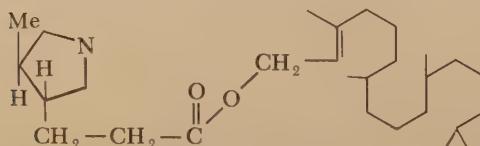


## protoporphyrin IX



### chlorophyll synthesis

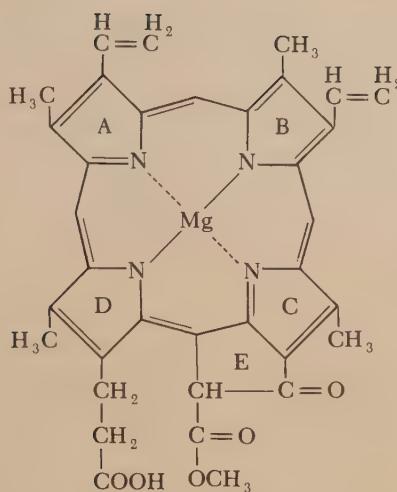
Magnesium has been found to be incorporated in a compound called magnesium vinyl phaeoporphyrin a<sub>5</sub> (MgVP), or proto chlorophyllide (Fig. 27.7). This pale green compound accumulates in etiolated barley leaves supplied with δ-amino levulinic acid. The conversion of MgVP to chlorophyll involves a modification of ring D, which, in chlorophyll *a*, has the following structure:



The hydrogens added to ring D are trans, and the diterpene added to the acyl side chain is the C<sub>20</sub> alcohol, phytol. *Chlorella* and some other plants can reduce ring D in the dark, but for most plants this is a one-quantum light reaction. It is likely that several steps and several components are required for the entire synthesis. There is some evidence that MgVP is attached to a protein, as is chlorophyll.

Some chlorophyll *b* is also present in higher plants. In this molecule the methyl group on ring B is supplanted by an aldehyde group. Bacteriochloro-

**FIGURE 27.7**  
proto chlorophyllide

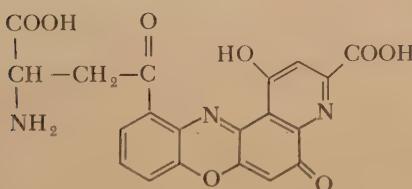


phyll is derived from chlorophyll *a*. Ring B is reduced, and the vinyl group on ring A is modified to —CO—CH<sub>3</sub>.

The process of the transduction of light energy into chemical energy, and the exact mechanism by which chlorophyll is incorporated in this process, remains a challenge. The problem is not simply a chemical one, or a physical one. A particular type of organelle structure seems to be required, as well as molecules, such as chlorophyll, with very special properties. Light sensing is much less restrictive in requirements, and there is a rather interesting assortment of molecules that participates in this process.

### visual pigments

The small creatures *Chlamydomonas*, *Volvox*, and *Euglena* have light-sensitive eyespots, usually colored. Both  $\beta$ -carotene and astaxanthin (3,3'-dioxy 4,4'-diketo  $\beta$ -carotene) have been proposed as the photopigment. In the arthropods and cephalopods there exist pigments called ommochromes. These have a very different origin; they arise from tryptophan via 3-hydroxy kynurenine. One such eye pigment called xanthommatin is found in the blowfly, *Calliphora erythrocephala*.



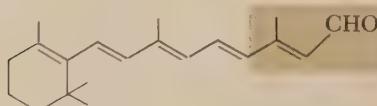
This compound has been found to derive from two molecules of 3-hydroxy kynurenine. The *Drosophila* geneticists have studied eye pigment changes for some time, and a substantial literature correlates genetic and biochemical data.

The most common visual pigment in the eyes of all species is the aldehyde of vitamin A<sub>1</sub> termed retinene<sub>1</sub>. It forms the prosthetic group of the protein

complex, rhodopsin (visual purple). The following reaction is initiated by light.

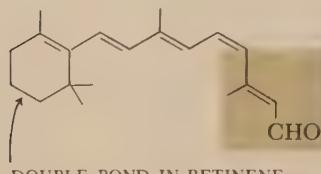


+



ALL-TRANS RETINENE

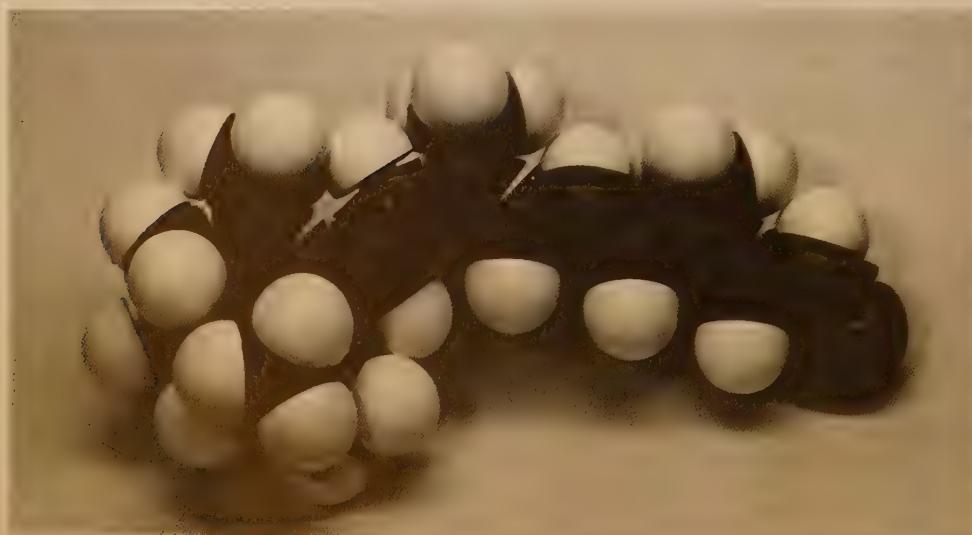
This reaction is not reversible, and, in order to reform rhodopsin, opsin must be united with neo-*b* retinene<sub>1</sub>.



DOUBLE BOND IN RETINENE,  
AND VITAMIN A<sub>2</sub>

Two mechanisms are involved in this conversion. There is the action of a RETINENE ISOMERASE (5.2.1.3), and there is a reduction process. In the latter, the all-trans retinene is reduced by an NADH-coupled ALCOHOL DEHYDROGENASE (1.1.1.1) system present in the retina. The vitamin A resulting from this reaction is in equilibrium with that of the circulating blood. In the presence of light, vitamin A isomerizes so that a mixture of all-trans and neo-*b* forms are constantly being produced. It is the latter form of vitamin A that

#### **transretinene**



reacts with opsin. The protein-pigment complex can be oxidized by an NAD-coupled system with the formation of rhodopsin.

The light-catalyzed dissociation of rhodopsin is thus seen to be accompanied by the release of an isomer that cannot bind opsin. It is believed that the action of light on rhodopsin is to cause the cis-trans isomerization that induces dissociation and, by an unknown mechanism, initiates a nerve impulse. The sensitivity of the process may be gauged by the fact that a human can detect a flash of light containing only 100 quanta, much of which may suffer absorption before it reaches the visual pigment. It has been suggested that the retinal rods consist of a stack of alternating disks of protein and pigment-containing lipoprotein.

The above process is the only one in which carotenoid function is understandable at this time. Carotenoids occur widely and have been implicated in a number of processes, such as the light-sensitivity of the seedlings of higher plants. An oat or wheat coleoptile, for example, bends toward the light, and the light receptor has been postulated to be a carotenoid.

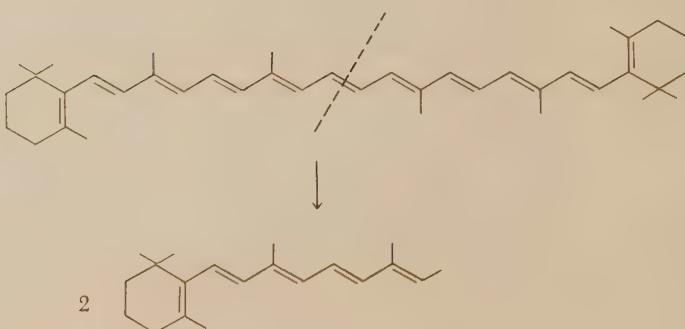
Again, carotenoids are almost invariably associated with chlorophyll, and it seems almost certain that they are involved somehow in photosynthesis. The presence of an extensive conjugated double-bond system has elicited the idea that carotenoids form charge transfer complexes and act to transfer energy from light quanta to chlorophyll *a*.

One of the puzzles in photobiology derives from the fact that four quanta of visible light appear to be required for the reduction of one mole of CO<sub>2</sub>. It is difficult to formulate a process that accumulates four quanta, one after the other, and then discharges the total into a chemical form. Some investigators have speculated that other pigments might be energy-coupled to chlorophyll and might help to funnel energy into it. Carotenoids may act in this way, but there is no compelling evidence. Nevertheless, spectrophotometric evidence

neo-*b* retinene



**FIGURE 27.8**  
biosynthesis of  
vitamin A



lends credence to the view that there is a  $\pi$  complex between chlorophyll and carotenoids and that the electronic systems of the two molecules can be closely coupled. Stanier has offered the alternative suggestion that the function is to protect the cell from photodynamic destruction by chlorophyll. He has isolated a *Rhodopseudomonas* mutant that has lost the ability to synthesize carotenoids and has found that it can exist in the light only if oxygen is excluded.

The function of carotenoids in animals is as provocative and ill-defined as in plants. The color of the salmon is due to an extraordinary concentration of carotenoids, and this is also true of the Garibaldi fish. Hens kept on carotenoid-free diets lay eggs with yolks that are nearly white. Desert locusts can be grown on diets that will eliminate the normal yellow color. Animal health does not suffer, provided vitamin A is present, even when carotenoids are rigorously excluded. Even in man, excessive consumption of carrots or peppers may cause accumulation of yellow pigment in the skin, but this has little correlation with health.

The synthesis of vitamin A<sub>1</sub> from  $\beta$ -carotene (Fig. 27.8) occurs in the intestinal wall. For some time there was doubt that each molecule of carotene yielded two molecules of vitamin A, but recent results tend to support such a mechanism. The mechanism details are uncertain, but it is known that molecular oxygen is required.

The function of vitamin A in the chemical aspect of vision is now well documented, but the function as a growth factor for mammals, birds, and perhaps other animals remains obscure. Lack of vitamin A results in skin and mucous membrane pathology.

The synthesis of  $\beta$ -carotene has been studied in *Phycomyces blakesleeanus*, and mevalonate has been found to be a precursor. It has also been shown that leucine is efficient in stimulating carotene synthesis. This reaction was demonstrated to result from the conversion of leucine to hydroxymethyl glutarate CoA and thence to mevalonate.

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# 28

## more on metalloporphyrins

Protoporphyrin IX, discussed in the preceding section, may complex with magnesium, as seen, and it may also complex with iron. An enzyme controls the rate of combination of  $\text{Fe}^{++}$  and protoporphyrin, although, *in vitro*, the reaction proceeds readily without a catalyst. The incorporation of  $^{59}\text{Fe}$  into heme has been found to be a mitochondrial function in several tissues.

### iron metabolism

Inorganic iron ions react readily, but they are not the form in which  $\text{Fe}^{++}$  is made available to the developing erythrocyte. Rather, the element is transported in the animal as the metal-protein complex, transferrin. This compound is a glycoprotein, molecular weight 83,000, found in the circulating blood, that can bind two atoms of iron ionically. (The transferrins have proved to be intriguing for genetic reasons: about fifteen electrophoretic species have been described in human blood.) In the animal most of the iron that is transferred from the plasma to tissue cells enters the bone marrow, the site of erythrocyte maturation. The iron transferrin complex appears to be absorbed to the developing red cells, and after the iron equilibrium has been established, the transferrin is released again to the circulating blood.

In several kinds of cells, but particularly liver parenchyma cells, there are to be found two remarkable iron complexes, ferritin and hemosiderin, often referred to as "storage" forms. The kinetic relation of iron in these forms to the form bound to transferrin shows great variation in different pathological states. Ferritin is an unusual complex. The subunits of the apoferritin (the protein moiety) have a molecular weight of about 25,000, and 20 of these structural units are associated in such a manner that they stabilize a complex with micelles of ferric hydroxide-phosphate. The inorganic content may vary somewhat but is of the order of 20%. One current hypothesis is that the apoferritin "denatures" after the iron content reaches a certain level and this change is accompanied by a loss of water solubility. The apoferritin disappears; the iron-containing micelles aggregate; and the result is the hemosiderin granule described by cytologists.

In Table 28.1 the data clearly show that a large fraction of the iron (in the

TABLE 28.1  
distribution of iron  
in the human  
(70-kg male)

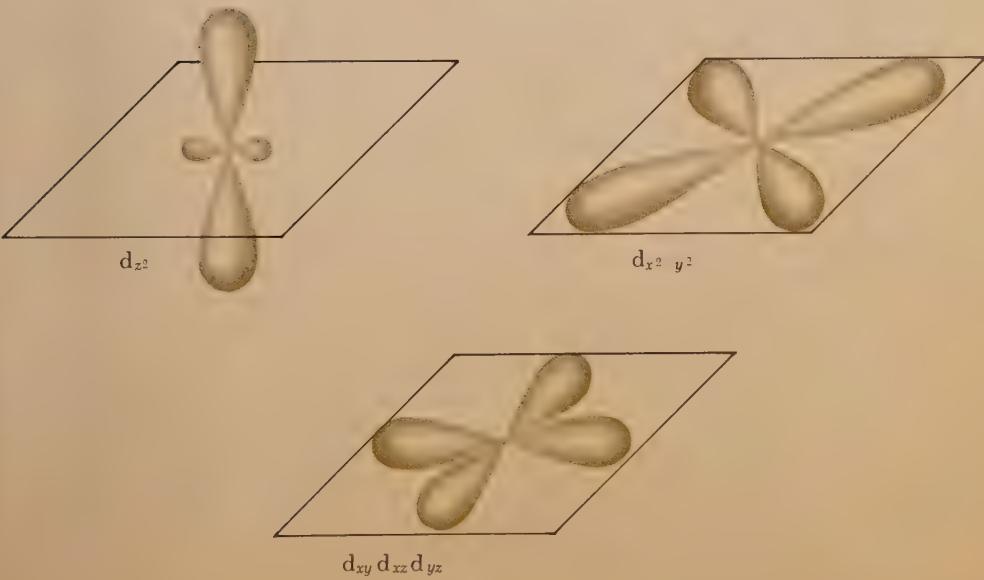
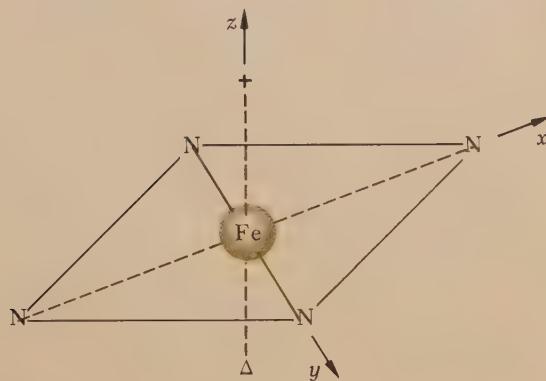
fraction	iron, mg
hemoglobin	2,700
transferrin	3
ferritin and hemosiderin	500–1,500
myoglobin	120
cytochromes, catalase, siderophilin	10
nonheme iron in muscle	500

animal) is present as a heme complex. Some nonheme iron-containing flavoproteins were referred to in Sec. 3, but other forms probably remain to be described.

**heme compounds** All hemes are ferrous protoporphyrin IX with side-chain modifications. Iron forms complexes by virtue of its empty 3d orbitals (Fig. 28.1). For ferrous iron the following may be noted.

			orbitals
<i>unpaired electrons</i>	<i>Bohr magnetons</i>		$d_{yz}$ $d_{xz}$ $d_{xy}$ $d_{x^2-y^2}$ $d_{z^2}$
4	4.90		↑ ↑ ↑ ↑ ↑
2	2.83		↓ ↓ ↑ ↑
0	0		↓ ↓ ↓

**FIGURE 28.1**  
structural relation of  
ligands in heme  
compounds and  
directions of d orbitals



Since substances containing unpaired electrons are paramagnetic (they will be drawn into a magnetic field), the measurement of the magnetic properties of hemes, and heme derivatives, yields clues to structure.

In the past many chemists have been involved in determining how the iron fits into heme structure. Valence-bond theory was insufficient, but more recently, ligand-field theory has contributed to the formulation of a more accurate description. The structure of the porphyrin makes probable a square-planar structure exemplified in Fig. 28.1.  $O_2$ ,  $H_2O$ , CO,  $NH_3$ ,  $H_2O_2$ ,  $CN^-$ , pyridine, imidazole, and several other substances may occupy the ligand positions above and below the plane. In hemoglobin one of the ligands is a histidine imidazole of the globin polypeptide chain. The other site combines reversibly with  $O_2$  (only in the presence of globin). When  $O_2$  is absent, the site is probably occupied by  $H_2O$ .

As has been implied, iron as an inorganic ion has some catalytic properties, heme without protein has some catalytic properties, but heme attached to protein has very special properties. These can be compared in myoglobin and hemoglobin.

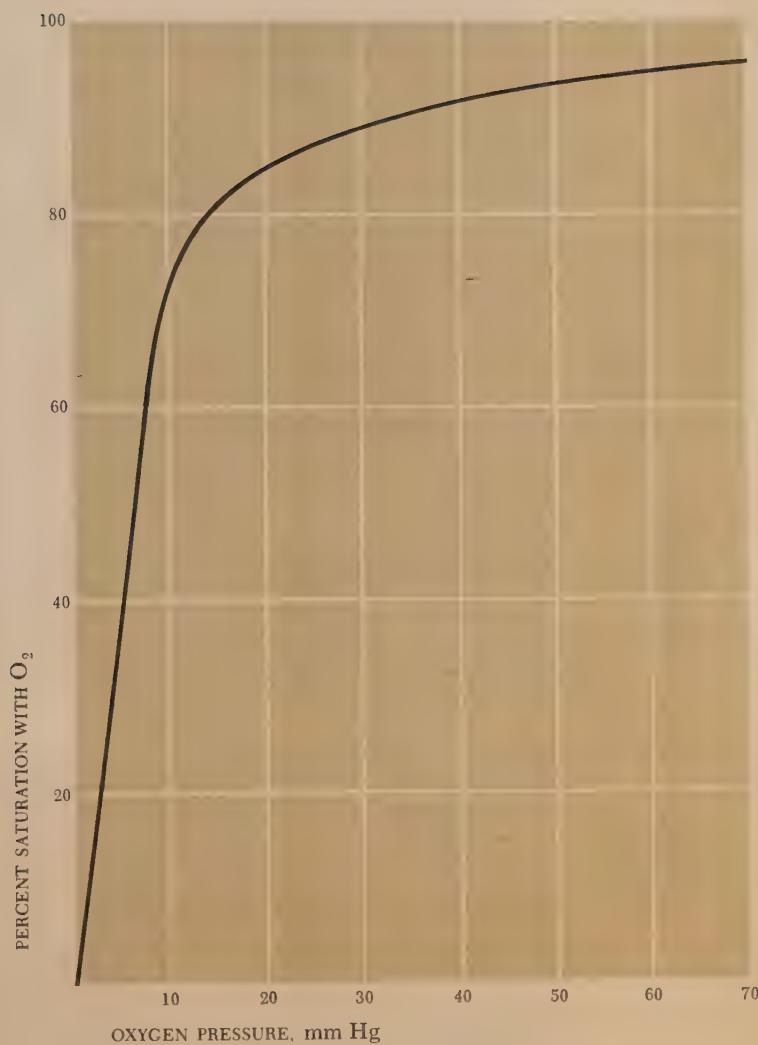
**heme proteins** Myoglobin contains one polypeptide chain and one heme. It combines reversibly with  $O_2$ , and the oxygen-saturation curve (Fig. 28.2) is similar to the plot of a reaction exhibiting first-order kinetics. Myoglobin remains oxygenated in muscles and is deoxygenated only at low oxygen tension (partial pressure). Significantly, the muscle tissue of diving mammals contains unusually high concentrations of myoglobin.

The heme group is probably involved in stabilizing the structure of myoglobin, as suggested by the sketch in Fig. 28.3. Reference to Fig. 22.2 will reveal the position of the heme group with respect to the entire folded myoglobin chain.

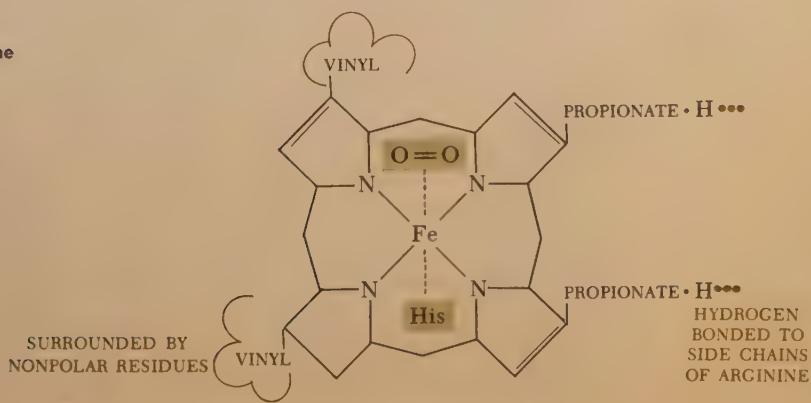
Hemoglobin contains four units of rather similar conformation. The units associate in such an arrangement that the heme groups are near the surface, each in its own cul-de-sac and surrounded by nonpolar residues (Fig. 22.4). The shortest distance between any two iron ions is 25 Å, and hence there can be no direct interaction between them.

**oxygen transport** Yet it is known that interaction between the hemes of hemoglobin exists, since the oxygen-saturation curve is that of Fig. 28.4. Long ago the physiologists recognized the advantages of such an oxygen-dissociation curve for animals. Relatively small changes in oxygen tension can cause hemoglobin to yield its oxygen, and this permits the animal to respire and to maintain an internal environment that exhibits no large changes in oxygen tension. Oxygen tension in arterial blood is about 80 mm Hg and in venous blood about 35 mm Hg. How the iron ions are coupled in such a way that an S-shaped dissociation curve results remains unexplained. It is perhaps suggestive that oxygenation is accompanied by a structural change that alters the relations of the subunits in space. It is also suggestive, as seen in Sec. 31, that some enzyme-catalyzed reactions exhibit sigmoidal kinetic curves. The inference is strong that this

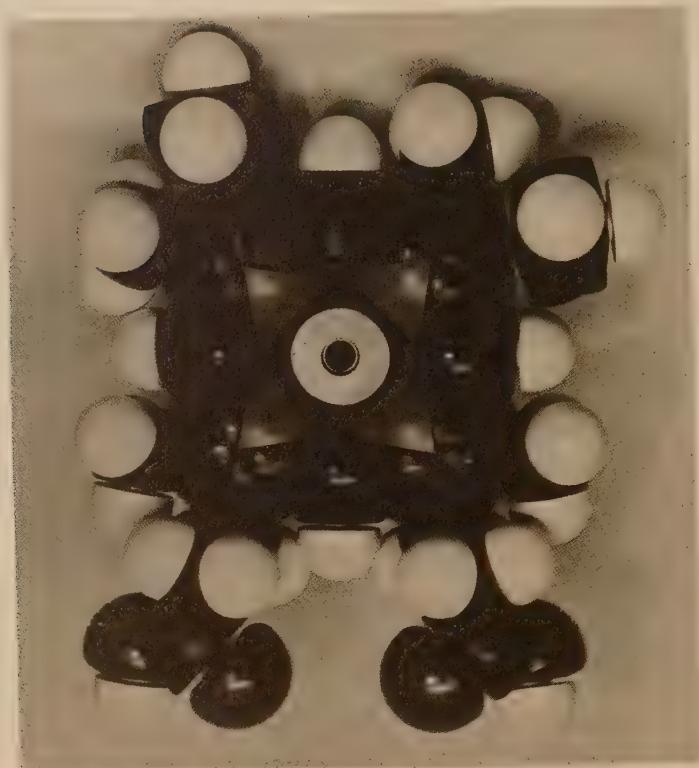
**FIGURE 28.2**  
oxygen-saturation  
curve for myoglobin



**FIGURE 28.3**  
the bonding of heme  
to myoglobin



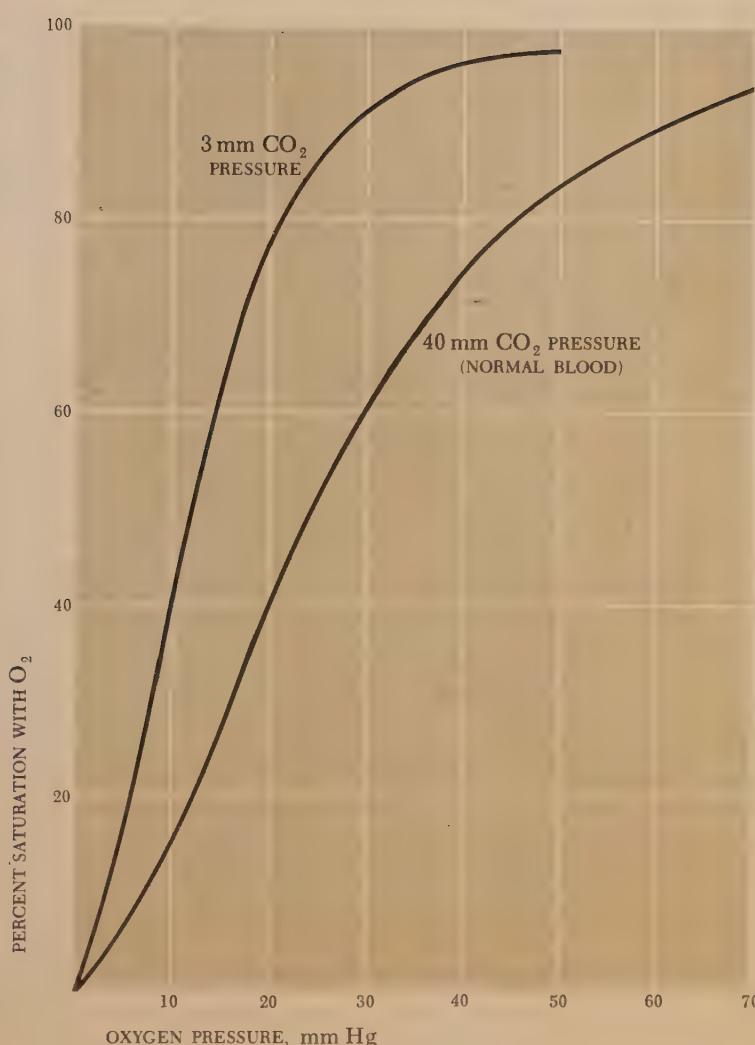
heme



heme with O<sub>2</sub> in place



**FIGURE 28.4**  
oxygenation-saturation  
curves for hemoglobin



kinetic behavior is concomitant with an allosteric effect. It should be remembered that hemoglobin and oxygenated and deoxygenated myoglobin contain ferrous ion. Derivatives containing ferric ion are easily produced—metmyoglobin and methemoglobin—by oxidizing agents other than  $O_2$ , but these derivatives do not combine reversibly with  $O_2$ .

In the homeostatic mechanisms of respiration, oxygen tension,  $CO_2$  tension, and hydrogen-ion concentration are interdependent. Hemoglobin is a center of this interaction; it reacts not only with  $O_2$  but also with  $CO_2$ . The sites of reaction are not the same. When  $O_2$  leaves the molecule, a heme-linked acid group also disappears and simultaneously either  $HCO_3^-$  or  $Cl^-$  is attached to hemoglobin. Subsequent oxygenation causes a discharge of  $CO_2$ . Probably the acid group involved is that of a histidine which becomes exposed

during the structural changes occurring during oxygenation. It has also been observed that hemoglobin becomes saturated with O<sub>2</sub> faster at a high pH than at a low pH. This pH dependence is often referred to as the "Bohr effect." No pH dependence of this type is exhibited by myoglobin.

It has been remarked that hemoglobin is contained entirely within erythrocytes in mammals. There are lower forms in which hemoglobin is not so restricted. We may suppose that confinement of this protein to cells reduces the viscosity and hence the amount of work that must be performed by the heart.

Hemoglobin combines with O<sub>2</sub> easily even in dilute solution, and this reversible reaction may be observed spectrophotometrically. Oxyhemoglobin is not paramagnetic and presumably contains no unpaired electrons. In contrast, deoxyhemoglobin is paramagnetic.

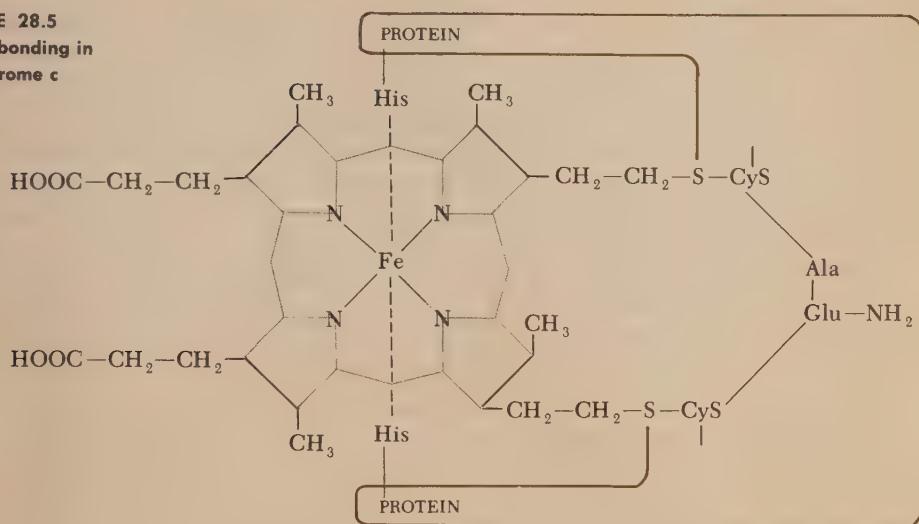
In a certain sense hemoglobin and myoglobin are part of the environment. They are involved in the presentation to the cell of diatomic oxygen—oxygen in the same state of chemical activity, or inactivity, in which it exists in the atmosphere. Perhaps one can think of these substances as providing an enriched atmosphere from which the even more unreactive element, nitrogen, has been removed. Of course, O<sub>2</sub> has a measurable solubility, and is present as dissolved oxygen in tissues, but at this level it would be quite inadequate for metabolic needs in multicellular systems. Oxygen exchange by the hemoglobin system is not a catalyzed reaction, but facilitated diffusion (REF. 5) has recently been discussed. Facilitation is inversely proportional to the size of the oxygen-binding protein involved and therefore is more notable for myoglobin than hemoglobin.

### cytochromes

When oxygen enters into the metabolic network as an electron acceptor, undergoing reduction to OH<sup>-</sup>, a heme complex, a cytochrome, is involved. Many different cytochromes have been described on the basis of the absorption spectra and characteristic oxidation-reduction potentials. In heart muscle mitochondria cytochromes a, a<sub>3</sub>, b, c, c<sub>1</sub> are found; in bacteria there are a<sub>2</sub>, b<sub>1</sub>, c<sub>1</sub>, c<sub>4</sub>, c<sub>5</sub>; in chloroplasts are b<sub>6</sub> and f; in plant microsomes is b<sub>3</sub>; and so on. Presumably each has its characteristic protein and binding sites.

Cytochrome c has a molecular weight of 13,000, and the heme group is bound as shown in Fig. 28.5. Transfer of an electron to Fe<sup>3+</sup> in the cytochrome, to produce Fe<sup>2+</sup>, may involve the coordinated histidine. In contrast to cytochrome c, the heme of neither cytochrome b nor cytochrome a is attached to the polypeptide chain by sulfur bonds. Cytochrome a (cytochrome oxidase) has a different iron-heme group as shown in Fig. 28.6, as well as one atom of copper. The concentration of cytochrome a in most tissues is about 10<sup>-6</sup> M. As yet no model compound that agrees entirely in its spectrum with this cytochrome has been found. It is assumed that the heme is bound to an imidazole group (histidine in the polypeptide chain) and that it is surrounded by a polypeptide matrix such as described for myoglobin. Investigators have had little success in studying this protein because it is insoluble.

**FIGURE 28.5**  
heme bonding in  
cytochrome c

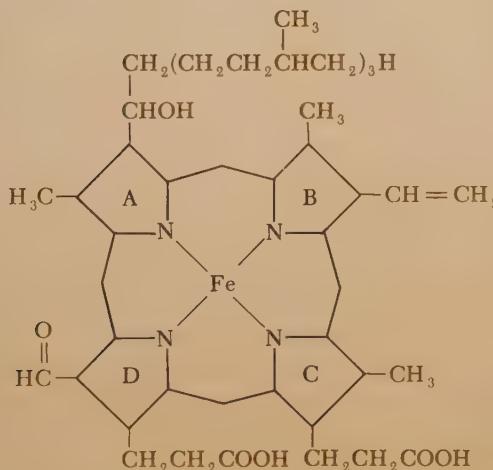


Cytochrome b, found in yeast and in animals, has been purified and found to contain ferroprotoporphyrin IX, which is not attached to the protein by covalent bonds. When solubilized, this protein has a molecular weight of 28,000. In the mitochondrion it probably forms a very large complex with cytochrome c<sub>1</sub>, possibly in a protein matrix.

Cytochrome b<sub>1</sub> has been prepared from *E. coli* in crystalline form after liberation from the organism by sonic treatment. Again the prosthetic group has been shown to be ferroprotoporphyrin IX. The molecular weight of the protein is about 60,000, but an aggregated form exists in solution.

Despite the commonality of components in the cytochromes, hemoglobin, and myoglobin, there are marked contrasts in function. To some extent this difference can be referred to varying orbital states of the iron atom. Cyto-

**FIGURE 28.6**  
heme group of  
cytochrome a



chrome c has a magnetic moment corresponding to one unpaired electron for the oxidized form; the reduced form is diamagnetic. Supposedly the loss and gain of one electron can occur without appreciable effects elsewhere in the molecule. In contrast, hemoglobin has a magnetic moment corresponding to four unpaired electrons; oxyhemoglobin is diamagnetic. The iron complex is more tentative, weaker than that in cytochromes, and the gain and loss of oxygen leads to substantial structural changes. Our knowledge of the structures of the iron complex in these compounds is by no means firm. As an example, evidence that has been presented recently would justify seven ligands, three of them to imidazoles from histidines 58, 87, and 89 of the polypeptide ( $\alpha$ ) chain.

**comment**

The comparative biochemistry of the metalloporphyrins, together with the related chemistry and physiology, is an intellectual edifice of vast bulk. Almost every theme is present in variations. Virtually all animals contain hemoglobin, although a few exceptions exist. More exceptionally still, hemoglobin is found in the nitrogen-fixing *Rhizobium* in the root nodules of legumes; there the function is unknown. More than one metal is involved; a variety of porphyrins have been described; and a large variety of proteins are associated with these complexes.

Electron transfer and oxygen transfer are the important, but not the only, functions. There are enzymes such as CATALASE (1.11.1.6) and PEROXIDASE (1.11.1.7) that also contain iron porphyrin complexes. Long ago it was found that hydrogen peroxide is a substrate for these enzymes. Since  $H_2O_2$  is a product of some reactions known to occur in cells, it has been assumed that these enzymes "protect" the cell by preventing accumulation. It is less than clear why  $H_2O_2$  is not used in some coupled reaction, and hence the biological function of these nearly ubiquitous enzymes remains a subject for speculation.

From an evolutionary point of view the metalloporphyrins may be clues to past events. As noted,  $Mg^{++}$  is sequestered as chlorophyll in the chloroplast. Presumably the analogous reaction in the animal cell is the complexing of  $Fe^{++}$  in cytochrome by the mitochondrion, although this also occurs in plant cells. Thus, the analogous reaction may be the formation of hemoglobin, but here we encounter an area of relatively little information about hemoglobin formation in cells other than the highly specialized erythrocyte. It will be appreciated from this and earlier sections that our recently augmented knowledge of hemoglobin and myoglobin has permitted advances in the understanding of function-structure relationships. Probably it should be emphasized that these molecules are rarely found in bacteria and that care should be taken in extrapolation across phyla.

In the following section is considered a related series of compounds deriving from the same metabolic sources but embracing yet another metal, cobalt.

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# 29

## biosynthesis of other cofactors

### the corrin group

Another type of metal-heme compound that combines reversibly with O<sub>2</sub> and may be involved in oxidation processes has been discovered. The metal complexed is cobalt, and it is found in a series of related compounds referred to as the vitamin B<sub>12</sub> family. These compounds contain the corrin nucleus, compared with the porphyrin nucleus in Fig. 29.1. When cobalt and the appropriate side chains are added to the corrin nucleus, the compound of Fig. 29.2 results. In this figure the numerals 1, 2, and 3 symbolize the methyl, acetate, and propionate groups, respectively. In the related compound, cobinic acid, the propionate side chain of ring D is in amide linkage with 1-amino-2-propanol. The name cobinamide has been given to the derivative in which the acetate and propionate groups have been converted to amides and the amino propanol group is present as in cobinic acid. If the hydroxyl group of the amino propanol group is substituted with phosphate, the total compound is termed cobamic acid. In yet another derivative the phosphate is joined, in turn, to a ribose molecule, and the resulting complex is a cobamide.

The formula for the vitamin B<sub>12</sub> series is shown in Fig. 29.3. In addition

FIGURE 29.1  
comparison of corrin  
and porphyrin rings

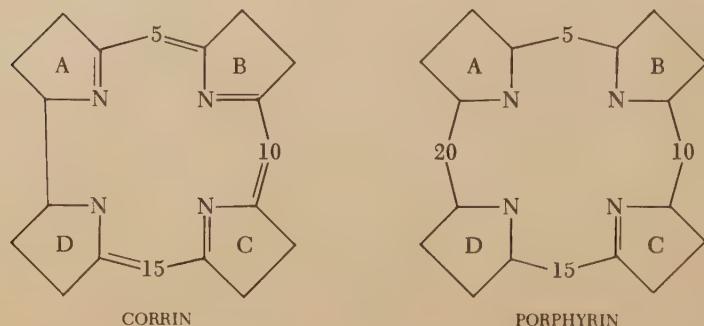
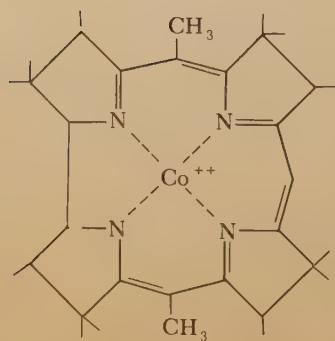
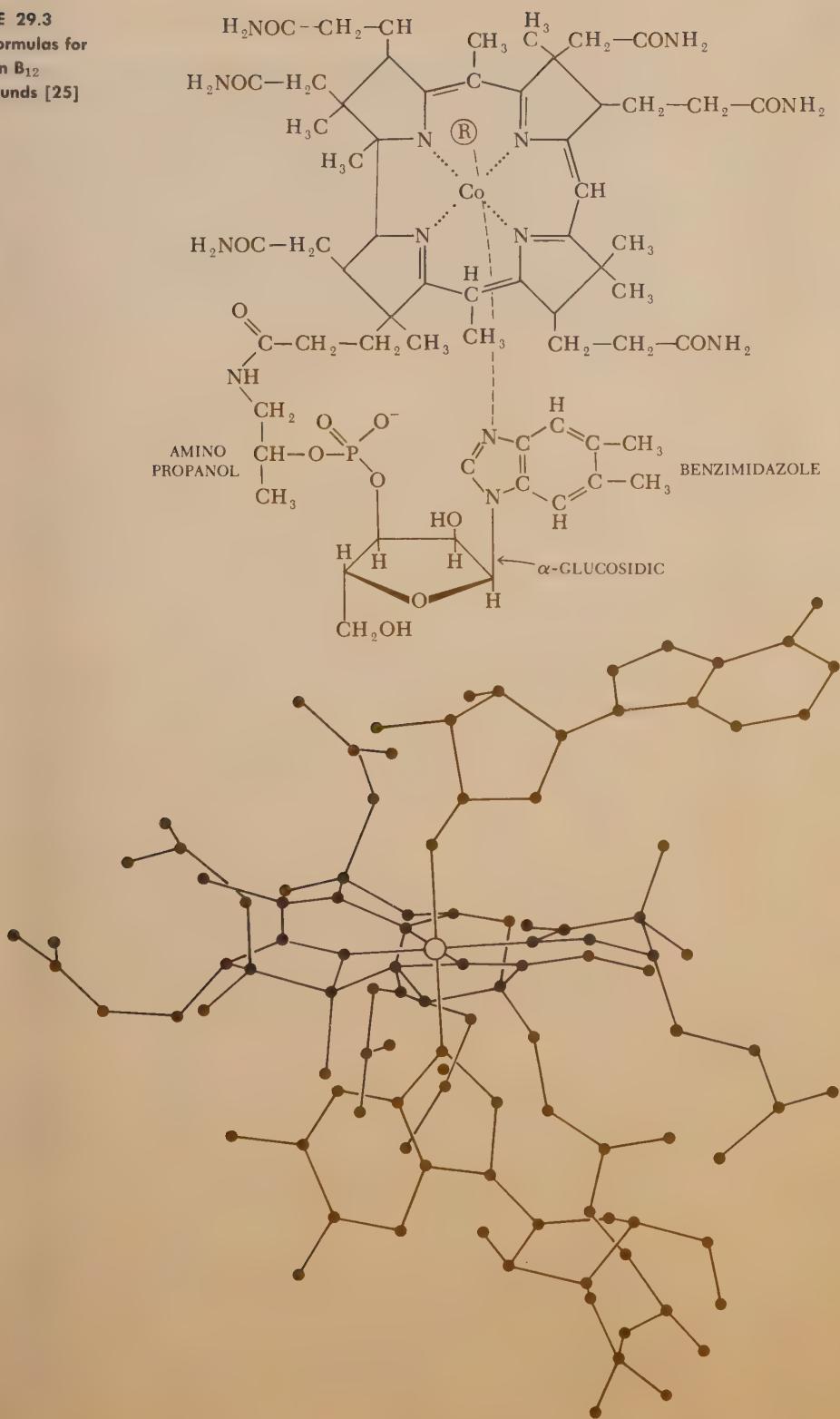


FIGURE 29.2  
cobyrinic acid

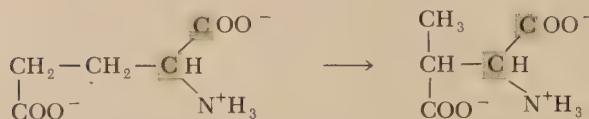


**FIGURE 29.3**  
 type formulas for  
 vitamin B<sub>12</sub>  
 compounds [25]

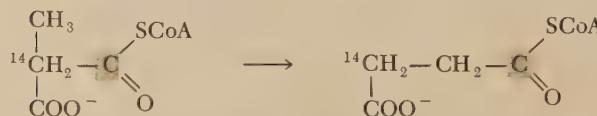


to the B<sub>12</sub> series, several cobamide coenzymes in which R is a 5'-deoxyadenosyl group are now known. The link to the cobalt atom is through the 5' position. All the cobamide coenzymes are very labile compounds readily inactivated by light, by acid treatment, or by complexing with cyanide ion.

The corrin nucleus arises from metabolic reactions similar to those leading to the porphyrin nucleus. In animals most of these cobamide compounds are found in liver, but in vanishingly small quantities. Animals, plants, and many bacteria cannot synthesize cobamide coenzymes; they were originally discovered during an investigation of the conversion of glutamate to  $\beta$ -methyl aspartate in *Clostridium tetanomorphum*.



Later another isomerization reaction was found to require a cobamide coenzyme. In this reaction, methyl malonyl CoA is converted to succinyl CoA.



Tracer studies have revealed that the carbon atoms indicated by the shading are those that migrate.

Unexpectedly, these coenzymes have also been implicated in the conversion of ribose to deoxyribose, the conversion of 1,2 diols to aldehydes, and the transfer of methyl groups (Sec. 12).

From some points of view the action of an analog that does not occur naturally is much more dramatic. Vitamin B<sub>12</sub> is usually isolated in the form known as cyanocobalamin, with the CN<sup>-</sup> anion as R in the formula (Fig. 29.3). The presence of CN<sup>-</sup> is actually an artifact due to the use of activated charcoal in the preparation of vitamin B<sub>12</sub>. Surprisingly, cyanocobalamin is biologically active in vivo. Its isolation resulted from efforts to obtain a factor known to be in liver and effective in ameliorating pernicious anemia. Castle in 1953 said (REF. 3)

*It can be stated with assurance that pernicious anemia is usually an example of a highly specific isolation of the affected person from his alimentary environment. This disease would not develop if the patient could effect daily transfer of a microgram of vitamin B<sub>12</sub> a fraction of a millimeter across the intestinal mucosa and into the blood stream.\**

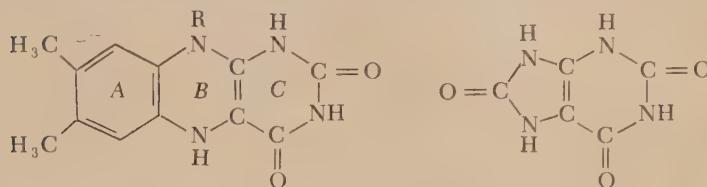
Like hemoglobin, vitamin B<sub>12</sub> forms many derivatives. R may be OH<sup>-</sup>, NO<sub>2</sub><sup>-</sup>, Cl<sup>-</sup>, SCN<sup>-</sup>, or (CN)<sub>2</sub><sup>-</sup>, as well as the nucleosides or the coenzyme forms. Moreover, there are series for each of the cobaltous and cobaltic states.

\*By permission of *The New England Journal of Medicine*. From W. B. Castle, *New Engl. J. Med.*, 249, 603 (1953).

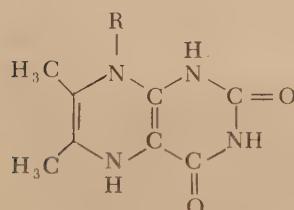
There is no doubt that the cobamide coenzymes are required by many organisms and, probably, for more than one type of reaction.

### the flavins

Any consideration of biologically important pigments must include a discussion of the flavins. These respiratory pigments are found in nearly all cells, although some bacteria, and all animals, lack the enzymic competence for their biosynthesis. As in the case of vitamin B<sub>12</sub>, experimentation with the *Clostridia* has been fruitful. These organisms synthesize riboflavin during fermentation, as do species of *Candida*, *Ashbya*, and *Eremothecium*. The relation of flavin structure to purine structure may be seen below.

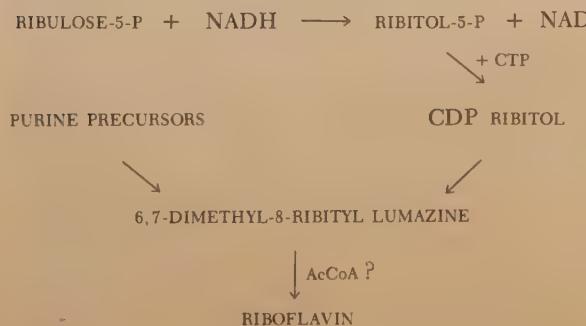


This comparison has led to speculation that rings *B* and *C* might originate from purine, and experiments with labeled acetate, formate, and glycine have revealed a labeling pattern very similar in each. Moreover, labeled purines, but not pyrimidines, were found to be flavin precursors. Ring *A* appears to derive from two-carbon fragments, perhaps from AcCoA. Several kinds of evidence indicate that the ribityl side chain at *R* is attached before ring *A* is completed and that the following intermediate, 6,7-dimethyl-8-ribityl lumazine, may be involved:



The ribityl side chain apparently is provided by the pentose phosphate reaction series (Fig. 29.4).

**FIGURE 29.4**  
metabolic sequences  
in riboflavin  
formation



The mechanism of formation of the coenzyme forms of riboflavin is more certain. In yeast, plants, and perhaps animal tissues a RIBOFLAVIN KINASE (2.7.1.26) catalyzes

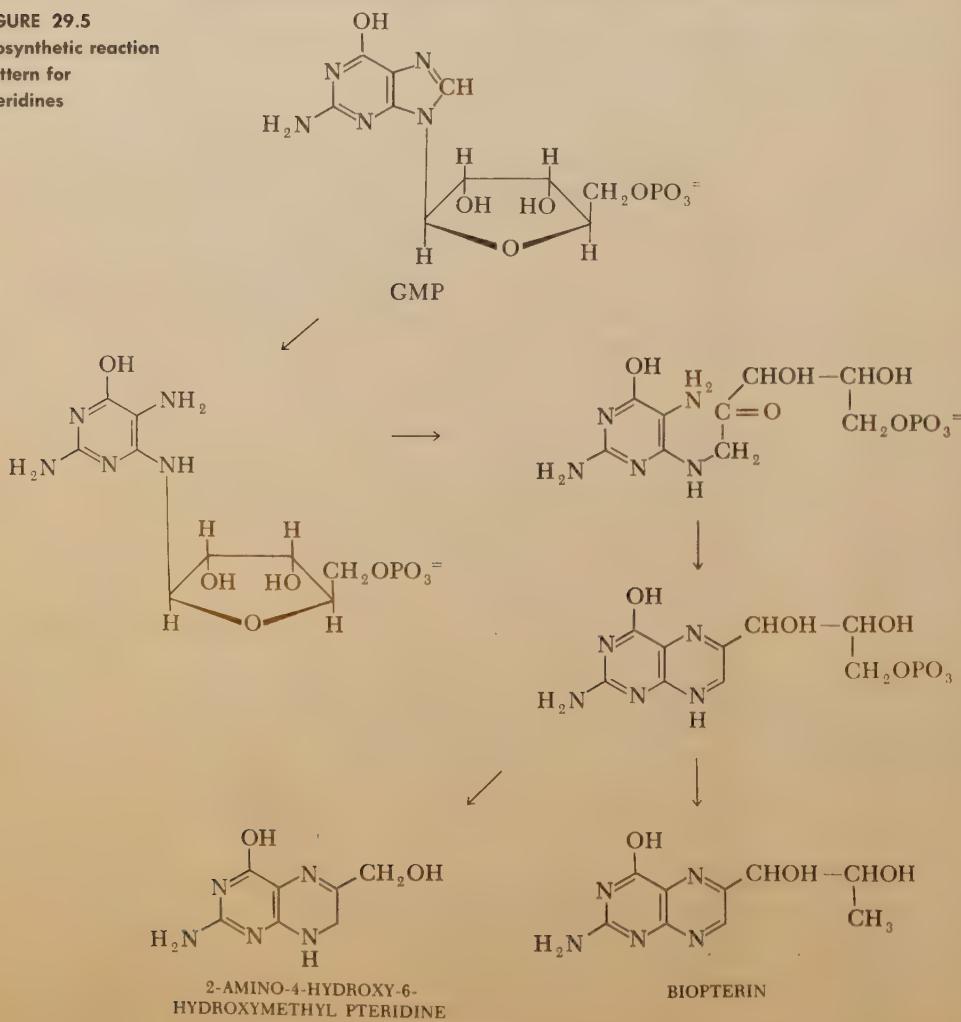


A subsequent reaction catalyzed by FAD PYROPHOSPHORYLASE (2.7.7.2) yields the dinucleotide.

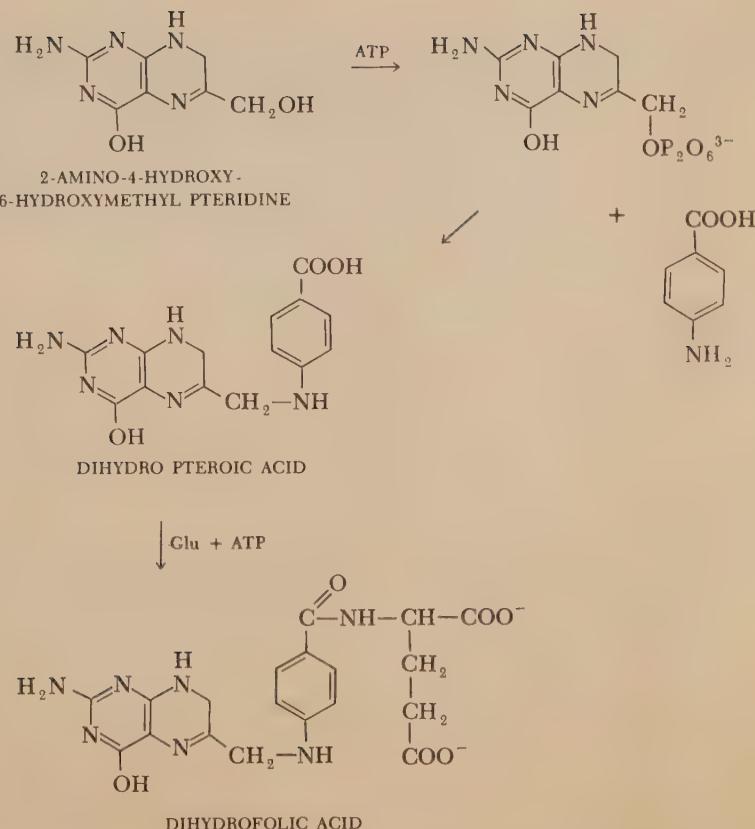


**the pteridines** The pteridines form another class of coenzymes ultimately derived from the purines. As the name suggests, these compounds are butterfly (*Pteridae*) pigments (quite nonfunctional as coenzymes in that case). The biosynthetic reactions have been suggested to be similar to those sketched in Fig. 29.5.

**FIGURE 29.5**  
biosynthetic reaction  
pattern for  
pteridines



**FIGURE 29.6**  
the biosynthesis of  
dihydrofolic acid



*N*<sup>5</sup>,*N*<sup>10</sup>-methenyl  
tetrahydrofolic acid



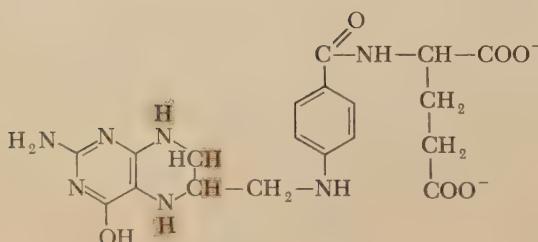
The biosynthesis of the derivative, dihydrofolic acid, is thought to consist of the reactions in Fig. 29.6. The coenzyme form is tetrahydrofolic acid, a compound that is readily oxidized. In Fig. 29.7 are the formulas of this compound and previously mentioned derivatives.

The processes in which tetrahydrofolic acid and its derivatives are known to participate should be recalled. They include the conversion of serine to glycine (Sec. 17), methionine synthesis (Sec. 12), histidine synthesis (Sec. 14), and thymine synthesis (Sec. 17).

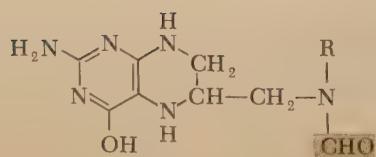
**FIGURE 29.7**  
tetrahydrofolic acid  
and derivatives

DIHYDROFOLIC  
ACID

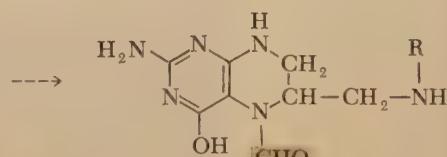
↓ (1.5.1.3)



↓ (3.5.1.10)  
↓ (6.3.4.3)



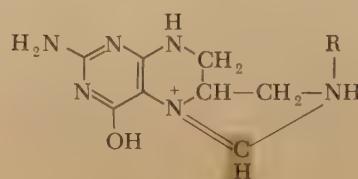
*N*<sup>10</sup>-FORMYL DERIVATIVE



*N*<sup>5</sup>-FORMYL DERIVATIVE

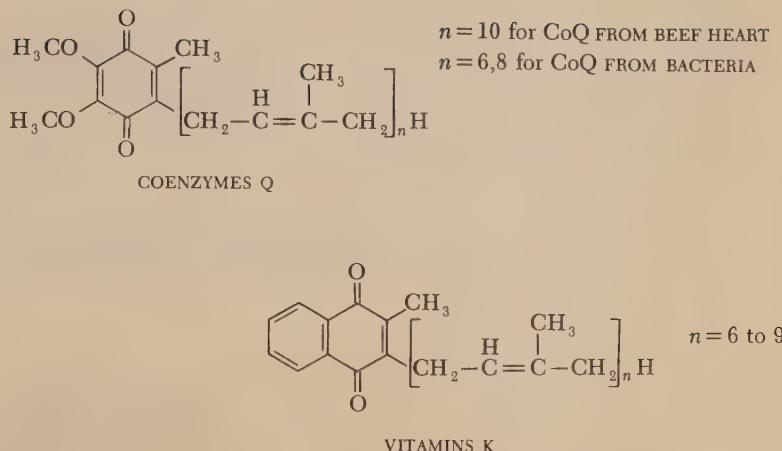
↔

ATP



*N*<sup>5</sup>, *N*<sup>10</sup>-METHENYL TETRAHYDROFOLIC ACID

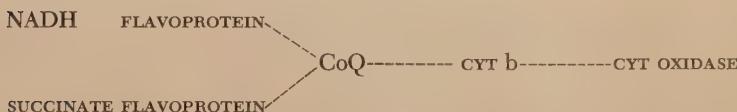
**FIGURE 29.8**  
biologically important  
quinones



### the quinones

The oxidation-reduction coenzymes being considered in this section include ubiquinone, or coenzyme Q (Fig. 29.8). The side chain is known to derive from mevalonate, but the mechanism of the benzoquinone biosynthesis remains to be discovered.

The complete function of CoQ is still being debated. Removal of this lipid-like molecule from mitochondria inhibits succinate oxidase activity. The sum of evidence indicates that in many tissues the linear progression of electron exchange includes CoQ at a branch point.

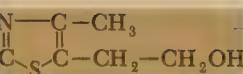
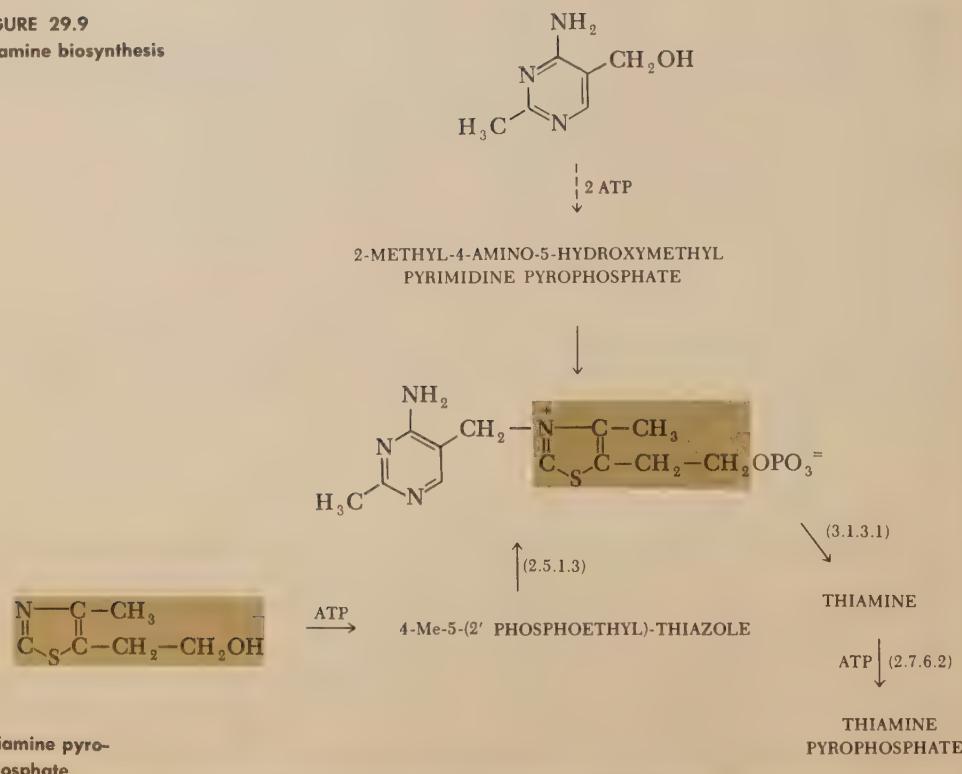


Another biologically important group of quinones, and one that has been studied for a longer period of time, comprises the vitamins K (Fig. 29.8). This "substance," eventually found to be a group of closely similar compounds, was first recognized as a blood clotting "factor." More recently, the vitamins K have been shown to function in the oxidation-reduction processes in plants. The significance of the polyisoprenoid side chain remains to be fathomed, but it may be surmised to "fit" a species-specified protein.

### thiamine

In this brief survey of small-molecule biosyntheses, it is appropriate to note the synthesis of thiamine, historically the oldest of the vitamins or "cofactors." That there is still an interesting cluster of problems relating to thiamine (or vitamin B<sub>1</sub>) attests to the complexity of this portion of metabolism. The several steps in thiamine biosynthesis are outlined in Fig. 29.9. It is by no means certain that hydroxymethyl pyrimidine is synthesized in the same manner as nucleic acid pyrimidines. Moreover, the biogenesis of the thiazole nucleus is obscure. Remarkably, the coenzyme form (the pyrophosphate) is derived from thiamine and *not* from thiamine phosphate.

**FIGURE 29.9**  
thiamine biosynthesis

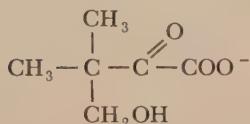


thiamine pyro-  
phosphate

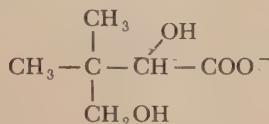


**coenzyme A**

The chemical definition of an important component of coenzyme A was initiated by R. J. Williams. A substance which he named pantothenic acid was found to have growth activity for yeast, and this factor, in turn, derived from a substance that had been called "bios." The biosynthesis was later shown in *E. coli* and other bacteria to include the sequence valine → α-keto isovalerate → α-keto pantoate → pantoic acid.

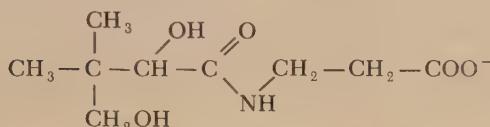


α-KETO PANTOATE



L-PANTOIC ACID

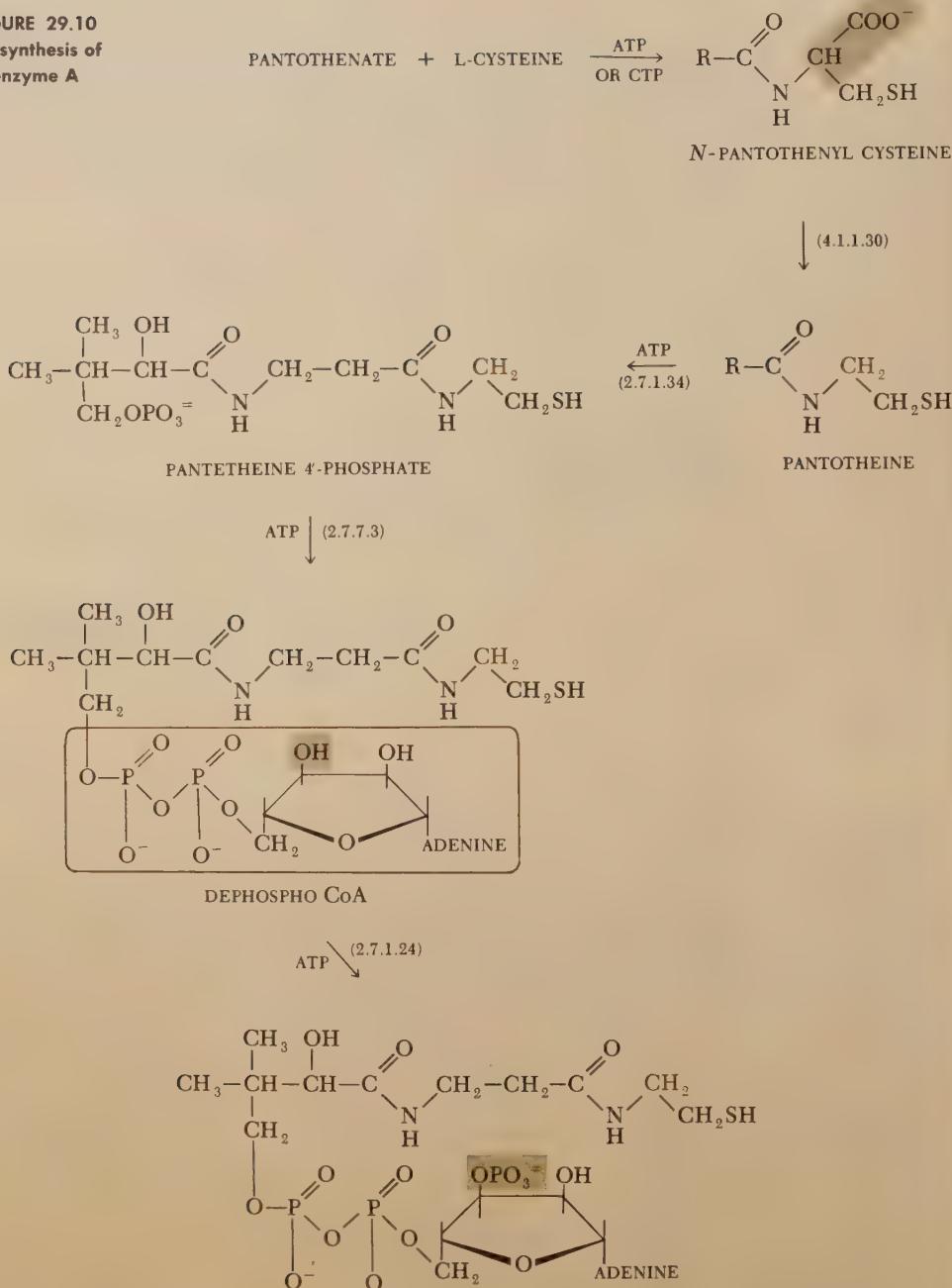
Pantoic acid forms a peptide bond with β-alanine (which derives from aspartate by decarboxylation in some organisms and by amination of propionate in others). In *E. coli* extracts there is an enzyme (6.3.2.1) that can be used to demonstrate the reaction of pantoic acid, β-alanine, and ATP to form pantothenic acid.



This reaction does not occur in mammalian tissues. However, in mammalian liver (and in many other cells) the subsequent syntheses do exist (Fig. 29.10).

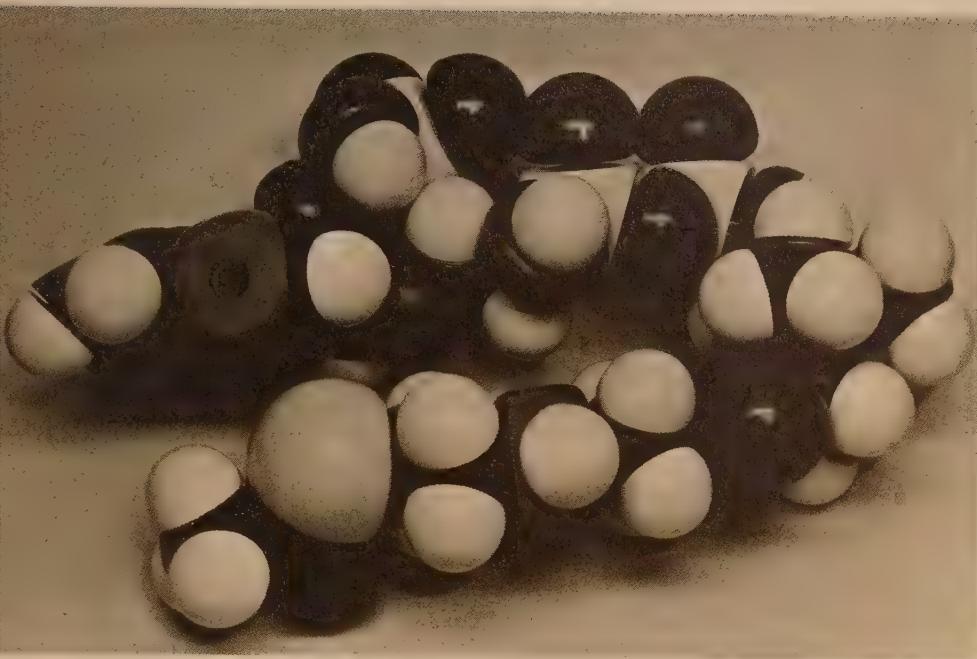
**pantothenic acid**

**FIGURE 29.10**  
biosynthesis of  
coenzyme A



**biotin** Another component of the historically interesting bios was identified and came to be known as biotin. Like many other cofactors, it is present in biological materials in very low concentration. Accounts of earlier investigations on such substances invariably stress the unprecedented amounts of material that had to be processed in order to obtain even a few milligrams of

acetyl CoA

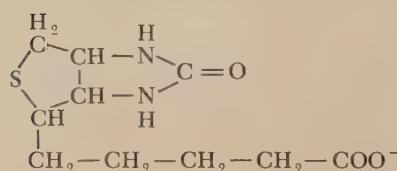


biotin

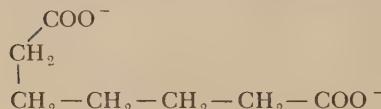


a compound. For some years there was a field, recognized as bio-organic chemistry, whose practitioners concerned themselves with just such problems. To some extent this concern has been assumed by "natural products" investigators.

The biosynthesis of biotin, like its fundamental mechanism of action, remains a subject for continued inquiry. This compound, together with an



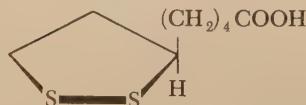
analog in which the sulfur atom is replaced with oxygen (oxybiotin) and one in which the sulfur atom is not present (desthiobiotin), is involved in carboxylation and decarboxylation reactions. Curiously, it is interchangeable with



pimelic acid as a growth factor for the diphtheria bacillus.

Lipoic (thiotic) acid, a compound that participates in enzymic acyl transfer.

### **lipoic acid**



### **lipoic acid**

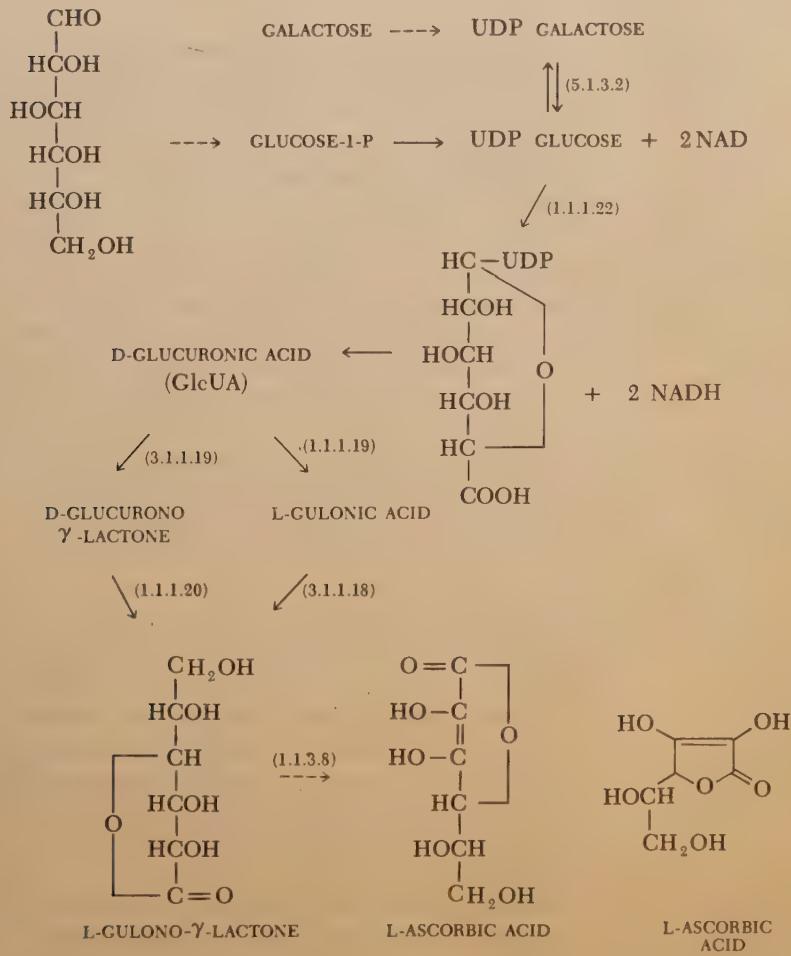


has the preceding structure. Its existence was recognized because it is a growth factor for bacteria (*L. casei*) and was later found to be a part of the pyruvate dehydrogenase system. Lipoic acid is synthesized in animals at a sufficient rate and is not a vitamin.

### ascorbic acid

Key reactions in the synthesis of ascorbic acid (vitamin C) are outlined in Fig. 29.11. Fischer projection formulas have been used in order that the D to L conversion might be easily visualized. Carbon atoms entering, either as glucose or galactose, can become incorporated in uridine diphospho glucose, the immediate precursor of uridine diphospho glucuronic acid. The latter is formed in the presence of a soluble liver enzyme, but the formation of the free glucuronic acid requires an enzyme restricted to the microsomes. The conversion of D-glucuronate to L-gulono- $\gamma$ -lactone can occur by the two mechanisms shown. Lactonization, followed by reduction, occurs in micro-

**FIGURE 29.11**  
biosynthesis of  
ascorbic acid



somes; the alternate, reduction followed by lactonization, occurs in the supernatant fraction. The final conversion to L-ascorbic acid (for which two alternate formulas are shown) depends on an incompletely characterized reaction requiring diatomic O<sub>2</sub>.

It is the absence of the microsomal enzymes (3.1.1.19 and 1.1.1.20) that prevents the successful synthesis of ascorbic acid in primates, guinea pigs, the Indian fruit bat, and the bubul. What the evolutionary significance may be is unknown. All other animals, and all higher plants, synthesize ascorbic acid. Microorganisms neither synthesize it nor require it for growth.

The participation of ascorbic acid in collagen synthesis has been remarked (Sec. 15), and this correlates with the significance of the acid for the metabolism of plants and animals as well as the absence from microorganisms. One of the most outstanding chemical properties of ascorbic acid is its powerful reducing action. Repeated attempts to demonstrate ascorbic acid participation in electron transport have implicated it in that process in some plant tissues.

**comment**

From the discussion it is seen that the molecules involved as acceptors or mediators in enzyme-catalyzed reactions, and present in catalytic quantities themselves, are derived from many kinds of metabolism. In the web of interrelated substances they are catalytic intermediates. In the animal many of them must be supplied in the diet as "vitamins." How shall we reason about this? Does it mean that this biosynthetic inability is due to an evolutionary loss, an accumulation of mutational damage? If this is so, can we also assume that the loss of this ability has some biological advantage for survival? If it does not have survival value, how explain the loss?

It has sometimes been stated that bacteria have certain biochemical activities because they are more primitive. This seems incorrect. The similarities between bacterial cells and mammalian cells are very numerous; the bacteria have continually evolved since evolution began in multicellular animals; and, in any case, many bacteria *do* require growth factors. Indeed, bacteria have been most useful in assaying for growth factors. Thus, vitamin or growth-factor requirements are not characteristic of any particular form of life and are not a consequence of a certain organization of cells.

Attempts to generalize have brought forth the observation that the water-soluble vitamins *tend* to be those required by all species, whereas the fat-soluble vitamins are found in many species-specific forms. Vitamins are undoubtedly part of the complex control apparatus necessary to all cells. In general, they are the end products of very long reaction sequences, often involving many precursors. Table 29.1 summarizes a portion of these data.

At first it may seem surprising that so many questions concerning the biosynthesis of several cofactors remain; but the quantities of cofactors produced are usually very small, and this makes for difficulty in experimentation. In some instances organisms have been recognized to produce unusually large quantities of such a metabolite, and in those cases the experimentation has produced far more certain results. Examples are the synthesis of riboflavin

by *Ashbya gossypii* and *Eremothecium ashbyii* (ascomycetes) or  $\beta$ -carotene by *Phycomyces blakesleeanus*. Needless to say, the metabolic flux through cofactor molecules in most organisms is small, however important. For perfect biochemical function there must be maintained a certain steady concentration of cofactors, each of which exhibits its characteristic turnover rate. Moreover, it may be expected that these levels and rates will vary from species to species.

**TABLE 29.1**  
metabolic  
origins of  
cofactors

cofactor	precursor molecules	
iron protoporphyrin IX	succinyl CoA	glycine
vitamin B <sub>12</sub>	transferrin	iron
riboflavin	succinyl CoA	glycine      Co <sup>++</sup>
coenzyme Q	adenine	benzimidazole
vitamin K	acetyl CoA	ribulose-5-P
vitamins A	purine	
tetrahydrofolic acid	acetyl CoA + ?	
thiamine	acetyl CoA + ?	
biotin	AcCoA or leucine	
coenzyme A	aromatic amino acid	glutamate
pyridoxal phosphate	purine      ribose	
nicotinamide	pyrimidine and thiazole precursors	
lipoic acid	pimelic acid and sulfur compound	
ascorbic acid	valine, aspartate, or propionate	
	cysteine      ATP	
	unknown	
	tryptophan	
	uncertain	
	glucose or galactose	

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# 30

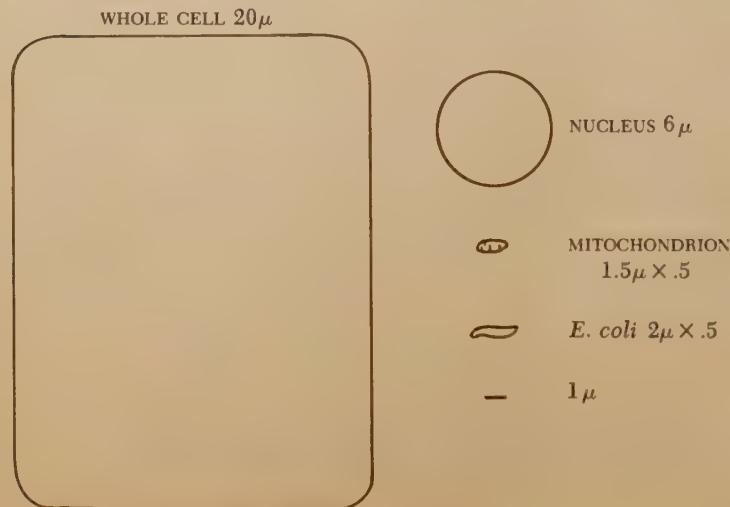
## cell membranes and cell walls

At some point in any course of study relating to biological subjects it is advisable to fix attention on dimensions. Any consideration of form and function must take into account the *size* of the form as well as its *shape*. It is well known among engineers and physical scientists that unforeseen difficulties arise in attempts to "scale up," because some functions are coupled to area, others to volume. Reasonable compromises based on one linear dimension may be grossly awry when that dimension is expanded by five or ten times, since the volume increases as the third power.

In a system as closely coupled and complex as a living cell it may be expected that some functions will reflect size changes very sensitively. A comparison of a typical plant or animal cell with a microorganism will show a relation similar to that in Fig. 30.1. Since the size of the *E. coli* cell is roughly that of a mitochondrion, it is to be expected that some striking differences will be discerned biochemically, as well as functionally, when cells of such disparate sizes are compared. It is undoubtedly clear by now that the general knowledge of metabolism is based on experimentation with the most varied kinds of biological tissues. Some reactions have been demonstrated in only a few tissues. That some tissues lend themselves to investigation more readily than others leads to the query whether there is indeed a unity in biochemical events in the cell.

In 1962 Stanier and van Niel set out the proposition that the structural differences among cellular forms are so great that there are really two distinct lines. See Table 30.1.

**FIGURE 30.1**  
some comparisons  
of size



**TABLE 30.1**  
biological  
cell  
comparisons

	<i>bacteria and blue-green algae</i>	<i>other cells</i>
	no nuclear membrane	nuclear membrane present
	no mitotic mechanism	mitotic mechanism present
	single circular chromosome	multiple chromosomes
	no membrane-surrounded substructures	mitochondria, chloroplasts
	respiratory center in protoplast membrane	separation of respiratory and photosynthetic organelles
	biosyntheses in contiguous cytoplasm	compartmentation
	if flagella, only one fibril	if flagella, multiple fibrils
	muramic acid in mucopeptides of cell wall	sialic acid in mucoprotein
	diamino pimelic acid in cell walls	

**cell  
membranes**

To illustrate the idea of a respiratory center in the bacterial cell membrane, Table 30.2 exhibits data based on analysis of *Staphylococcus aureus* protoplast membranes. A protoplast is an organism grown under conditions which prevent the formation of a cell wall external to the membrane. If the medium used to grow the organism has the correct osmotic pressure, the cell membrane is undisturbed. Cell rupture may be occasioned at will by lowering the osmotic pressure of the medium. The feasibility of preparing protoplasts was demonstrated by Weibull in 1953. It was shown that LYSOZYME (Sec. 22) treatment removed the cell wall of *B. megaterium* but that a living bacterium could still persist in the proper kind of medium. Other methods have been discovered in the interim. Examination of the chemical nature of these membranes reveals a high content of lipoprotein.

From the enzymic content of the plasma membrane it has been concluded that the membrane is the structure mainly concerned with the oxidative processes in bacteria. If so, then bacterial metabolism may be said to begin before an incoming molecule is fairly "inside." If metabolic products are to enter into the processes inside, then the reaction sequences in the membrane

**TABLE 30.2**  
enzymic  
complement of  
*S. aureus*  
protoplast  
membranes

<i>enzyme</i>	<i>percent activity</i>	
	<i>plasma membrane</i>	<i>soluble fraction</i>
cytochromes	>90	<10
succinic dehydrogenase	>90	<10
lactic dehydrogenase	80–95	5–20
malic dehydrogenase	>90	<10
glucose-6-P dehydrogenase	3	97

From P. Mitchell, in *Biological Structure and Function*, vol. 2, T. W. Goodwin and O. Lindberg (eds.), Academic Press Inc., New York, 1964, Table 1, p. 585. By permission of Academic Press Inc.

must be structured to ensure this. The term "active transport" is entirely appropriate in such a circumstance. There is also evidence that biosynthetic activities, as well as energy-yielding sections, occur in the membrane structure.

The metazoan cell membrane consists largely of lipoprotein also (Sec. 27). Details of structure are still controversial, but one widely held view is that each surface of the cell membrane is protein and that each sheet or web of protein has embedded in it the polar groups of lipids whose apolar chains, or groups, are apposed. The use of permanganate in staining sections for electron microscopy results in a preparation in which the cell membrane is seen as two dense lines. The cell membrane is eight or nine times thicker than that of the mitochondrion and does not contain respiratory enzymes. In other respects, the mitochondrial membranes are quite similar to the bacterial membranes. In animal cells also membranous structure is discernible in the cytoplasm; it is referred to as the endoplasmic reticulum (Sec. 16). These membranes, like those of the mitochondria, are very thin, seem to be lipoprotein, and often have ribosomes attached. It is not difficult to dissociate the ribosomes; and the membranes, <sup>o</sup> in isolation, are found to contain phospholipid, protein, and small amounts of RNA. In the animal and plant cell, then, there are a variety of membranes and a specialization of function.

### bacterial cell walls

The bacterial cell wall that ordinarily encloses the plasma membrane has no strict counterpart in the animal cell. Electron microscope examination of both isolated cell wall and intact bacteria reveals apparent homogeneity and occasionally a somewhat granular structure. Layering, if present, is not obvious.

Christian Gram, in the nineteenth century, devised a staining technique † that separated bacteria into groups and was very useful technically. Simple acid hydrolysis has revealed a chemical basis for this difference, and some chemical characteristics of each type of cell wall are noted in Table 30.3.

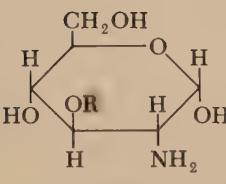
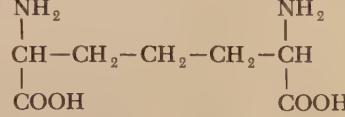
Solutions to the puzzles of cell wall structure are not simple, and many

<sup>o</sup>Much effort has been required to achieve a correspondence of objects, or organelles, or fragments obtained by differential centrifugation with structures seen by electron microscopy. A case in point is the lysosome, first obtained by centrifugation as a particle midway in density between mitochondria and microsomes. Electron microscopy revealed dense bodies surrounded by a single membrane. Several hydrolases with a pH optimum at 5—ACID PHOSPHATASE (3.1.3.2),  $\beta$ -glucuronidase (3.2.1.31), ribonuclease (2.7.7.16)—are found in these dense bodies, and it is suspected that these enzymes derive from the endoplasmic reticulum. The role of lysosomes in a number of biological phenomena is being explored. One of the most intriguing is that many carcinogens modify the lysosomal structure so that deoxy ribonuclease is released. The release of this enzyme would occasionally result in modification of DNA so that a "genetically new" cell lacking the controls of growth of the cells surrounding it would be produced.

Microsomal activities include the conversion of cytidine and uridine diphospho sugars into phosphatides, glucuronides, and ascorbic acid, participation in steroid synthesis, catalysis of reactions involving NADPH and O<sub>2</sub>, and the synthesis of glycerides.

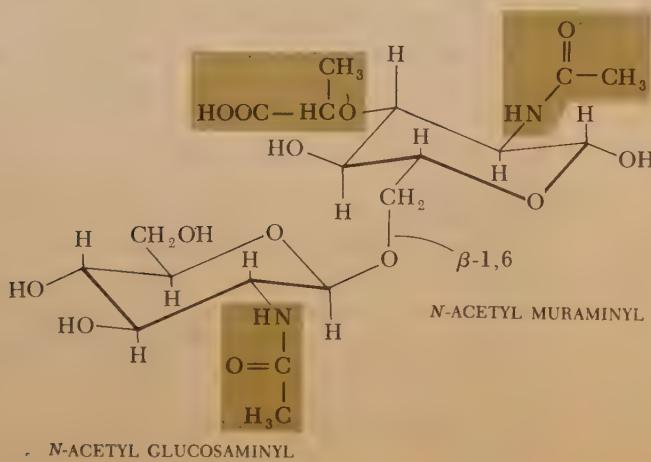
<sup>†</sup>The general nature of the Gram staining procedure may be gained from the following abridged directions: Stain smears one minute with ammonium oxalate crystal violet. Wash in tap water two seconds or less and immerse in iodine solution for one minute. Wash in tap water, blot dry, and counterstain for ten seconds in safranine solution. Wash in water and dry. Gram-positive organisms are stained blue and Gram-negative organisms are stained red.

**TABLE 30.3**  
comparison of  
gram-positive  
and -negative  
organisms

gram-positive organisms, e.g., <i>Streptococci</i> , <i>Lactobacilli</i>	gram-negative organisms, <i>e.g.</i> , <i>Bacillus</i> , <i>Corynebacterium</i> <i>Mycobacterium</i>
a restricted number of amino acids	all amino acids found in protein
amino sugar	2,6-diamino pimelic acid (cf. Sec. 14)
hexoses and pentoses	some amino sugar
	lipopolysaccharide, phospholipid
	20 to 30% lipid
	no teichoic acid
 <b>MURAMIC ACID</b> (3-O- $\alpha$ -CARBOXYETHYL D-GLUCOSAMINE)	$R = -\begin{matrix} CH \\   \\ CH_2-COOH \end{matrix}$  <b>2,6-DIAMINO PIMELIC ACID</b>

different courses of action have been taken by investigators. One straightforward move is to hydrolyze cell walls by various means and to seek repeating units.

Egg white lysozyme hydrolyzes the wall substances of the Gram-negative organisms *M. lysodeikticus*, *Sarcina lutea*, and *B. megaterium*. About 50% of the wall is reduced to soluble but nondialyzable compounds. The remainder of the product mixture is also soluble but of lower molecular weight. About 10% is the disaccharide, 6-O- $\beta$ -N-acetyl glucosaminyl-N-acetyl muramic acid.



Another 10% of the hydrolyzate is a tetrasaccharide:

*N*-Ac glucosaminyl  $\beta$ -1,6-

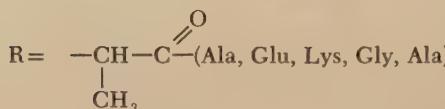
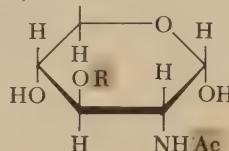
*N*-Ac muraminy  $\beta$ -1,4-

*N*-Ac glucosaminyl  $\beta$ -1,6-

*N*-Ac muramic acid

This yield indicates that considerable portions of the wall consist of an alternating structure of glucosamine (GlcN) and muramic acid units. It is probable that lysozyme potentiates the hydrolytic scission of the  $\beta$ -1,4 bond. Among the hydrolysis products are also mucopeptides that can be further simplified, hydrolytically, by an enzyme from *Streptococcus albus*, to compounds with the following structure:

*N*-Ac GLUCOSAMINYL  $\beta$ -1,6—CH



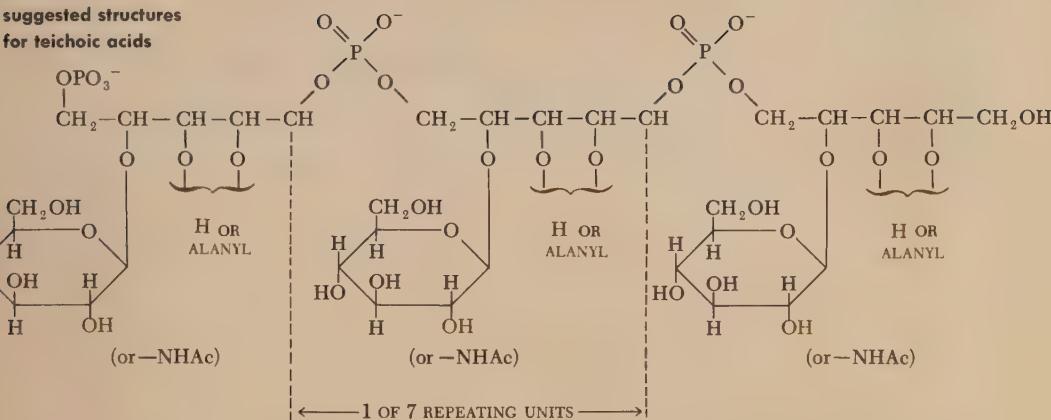
This chemical result points to a structure in which peptide chains are bonded to the saccharide matrix by the carboxyl group of muramic acid. Both Gram-negative and Gram-positive organisms contain this rigid polymer, but many Gram-negative organisms are rather resistant to lysozyme action, and it is believed that the lipopolysaccharide complement of the wall may be a protective agent.

### teichoic acids

When the cell walls of Gram-positive organisms, such as *Staph. aureus*, are extracted with 5% trichloroacetic acid in the cold, polymeric compounds called teichoic acids are obtained. They are composed of ribitol or glycerol, phosphate, alanine, and either hexose or hexosamine. Products with the probable structure shown in Fig. 30.2 have been isolated. Teichoic acids have been found to be polymers of glycerophosphate, or ribitol phosphate, linked by phosphodiester bridges. A particulate enzyme from the cell membrane of *B. licheniformis* or *B. subtilis* catalyzes the conversion of CDP glycerol to polyglycerol phosphate,<sup>\*</sup> a linear polymer about thirty units long. Another preparation from the membrane catalyzes the addition of glucosyl units from UDP glucose. An analogous polymer of ribitol phosphate shown in the above formula can be formed from CDP ribitol by a particulate enzyme from *L. plantarum*. In this case, the chain length is 7 to 9 units.

\* Compare with phospholipid syntheses, Sec. 27. CDP glycerol is formed from L- $\alpha$ -glycerophosphate and CTP.

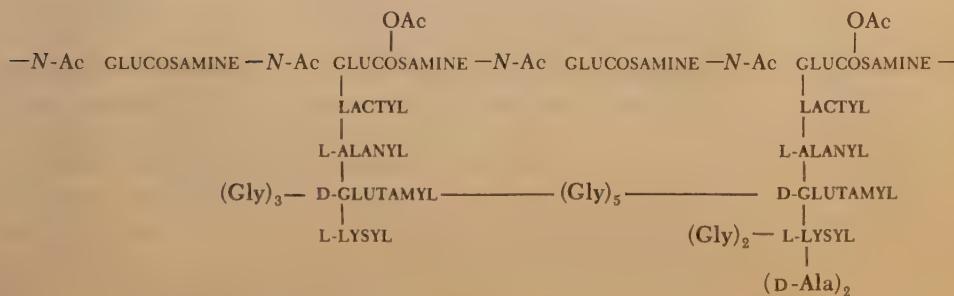
**FIGURE 30.2**  
suggested structures  
for teichoic acids



Enzymes from *Strep. albus* produce from *Staph. aureus* walls both teichoic acids and a glycopeptide thought to have the structure in Fig. 30.3.

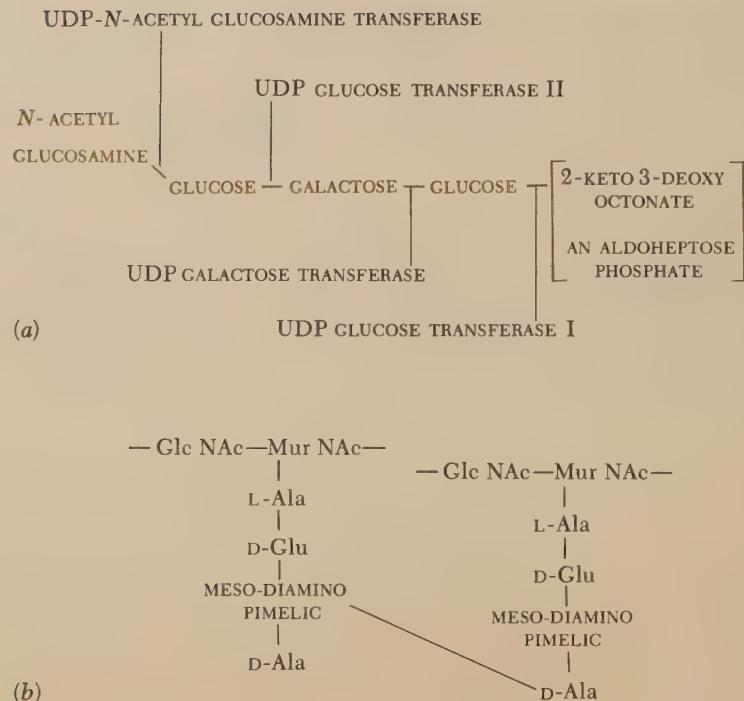
From the examples given, it can be seen that some of the wall polysaccharides have a resemblance to nucleic acids. In the nucleic acids there is a linear polymer of ribose, or deoxyribose phosphate, whereas in teichoic acids the polymer is formed from ribitol, or glycerol phosphate, units but, of course, the chains are also much shorter. There are also the polymers characterized by *alternating* units, and this raises the question of the mechanism of synthesis. Horecker and his associates have used the lipopolysaccharide of *Salmonella* as a model to study the mechanism of synthesis of such a complex polysaccharide. The lipid of this substance is of less interest to us here, but it is a special problem all by itself since it has an unusual composition. What is of central concern is that the model is a structurally specific heteropolysaccharide containing up to eight different monosaccharides. Antigenic specificity indicates a specific linear sequence. The use of mutants has allowed the observation that incomplete heteropolysaccharides may be formed. The synthesis of the core, or backbone, polysaccharide is believed to involve the mechanisms and components of Fig. 30.4. The incomplete sketch of the process, so far, implies that in such complex heteropolymers a backbone polymer is formed by serial enzyme action. It appears that side chains are added later to this acceptor polymer.

**FIGURE 30.3**  
glycopeptide  
(*S. aureus*) structure



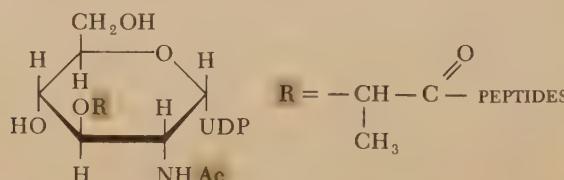
**FIGURE 30.4**

(a) suggested synthesis of *Salmonella* polysaccharide;  
 (b) glycopeptide cross-linking in *E. coli* walls



### uridine nucleotides

It was observed in 1949 that uridine nucleotides accumulated in the medium of *Staphylococcus aureus* cultures treated with penicillin. Later the structure of the nucleotides was found to be that shown here.



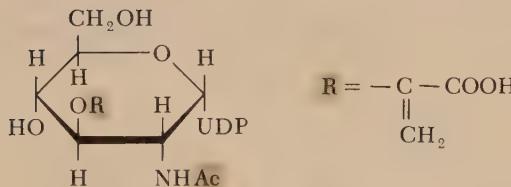
It was then discovered that lysine deprivation caused this organism to accumulate UDP-*N*-acetyl glucosamine lactyl L-Ala-D-Glu. Therefore, it began to appear that the starting point for such structures might be glucosamine or galactosamine and that such uridine derivatives might be intermediates or precursors in cell wall formation. Since unsubstituted amino sugars are not found in living cells, it is thought that glucose 6-phosphate is an acceptor for an amino group donated by glutamine. The glucosamine 6-phosphate so formed is further acetylated by acetyl CoA in the presence of an ACETYL TRANSFERASE (2.3.1.3). A mutase (2.7.5.2) catalyzes the formation of *N*-acetyl glucosamine 1-phosphate, which may then react (2.7.7.23) with UTP to yield UDP-*N*-acetyl glucosamine. Galactosamine (GalN) analogs derive from the

isomerization (5.1.3.7) of the hydroxyl of carbon 4 of UDP-N-acetyl glucosamine. The formation of muramic acid is sketched below.

UDP N-Ac GLUCOSAMINE

+

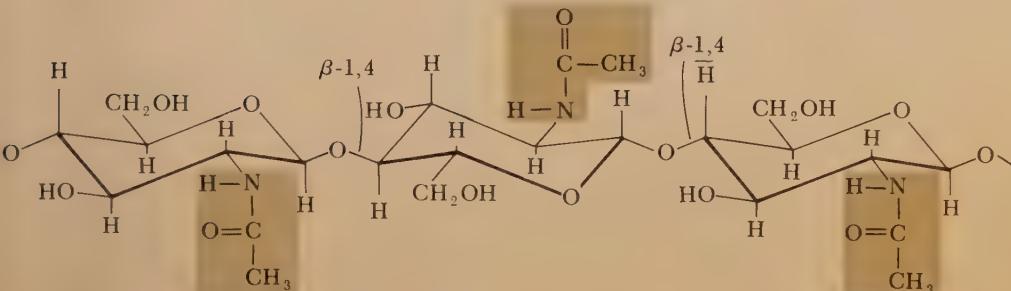
PHOSPHO ENOL PYRUVATE



The product, UDP N-acetyl 3-O- $\alpha$ -carboxyvinyl  $\alpha$ -D-glucosamine, is reduced to muramic acid, seen to be a derivative of lactic acid.

Recently a study of cell wall compounds of *E. coli* has suggested the mechanism inhibited by penicillin as evidenced by the cessation of growth (REF. 17). The enzymes involved in the formation of the peptide cross-link between diamino pimelic acid and D-alanine shown in Fig. 30.4(b) have been shown to be penicillin-sensitive. Thus, despite the relative insensitivity to penicillin of Gram-negative organisms, such as *E. coli*, it has been proposed that penicillin-sensitive reactions are involved in cell wall formation. Insensitivity is postulated to be due to impermeability of the membrane to penicillin.

In the cell wall of some microorganisms, and in the cuticle of insects, may be found chitin, a  $\beta$ -1,4-N-Ac glucosaminide. This polysaccharide has been demonstrated to form from UDP-N-acetyl glucosamine in *Neurospora* extracts, presumably by glucosyl transfer.

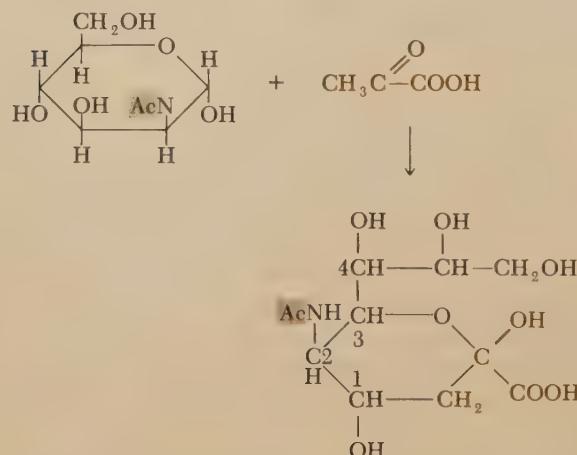


Cell walls also commonly contain unsubstituted hexose polymers, such as cellulose and glucans, galactans, and mannans. The latter are linear polymers in which the sugar residues are linked by  $\alpha$ -1,6 bonds.

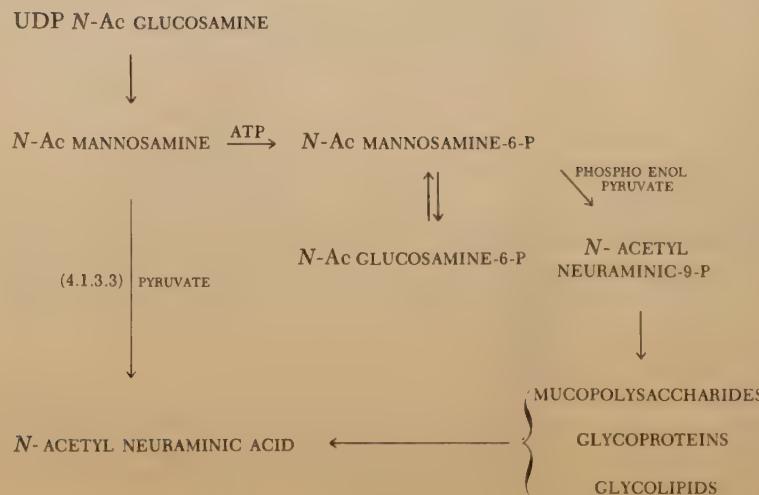
### sialic and neuraminic acids

The extracellular spaces in animals contain substances that resemble the cell wall materials of microorganisms. Some aspects of this type of tissue have been discussed in Sec. 21. As noted, collagen is the major protein of connective tissues such as skin, tendon, ligament, and cartilage. There is some tendency toward calcification of such tissues with hydroxyl apatite,  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ , which forms most of the mineral of bone. Besides the collagen there is a "ground substance" postulated to be composed of mucopolysaccharide complexes with noncollagenous proteins. There are also saccharide components in mucus, an imprecise term for a product of certain secretory cells and a component of the membranes lining the respiratory and gastrointestinal tracts in animals. Mucoproteins are also found in plasma, in egg white, and in the blood components responsible for the antigenic blood groups. One of the compounds characteristic of such substances is an amino

**FIGURE 30.5**  
sialic acid synthesis



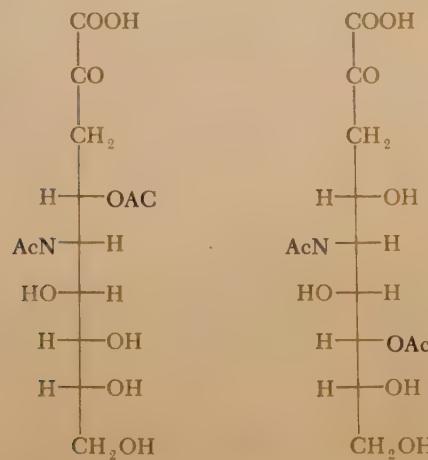
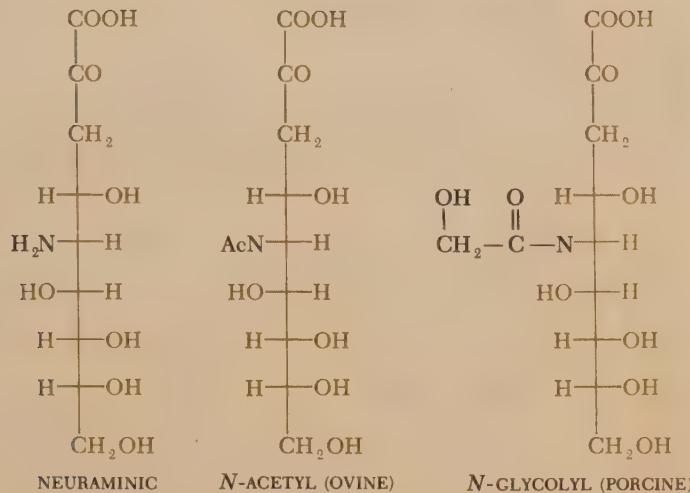
**FIGURE 30.6**  
metabolic relations of  
*N*-acetyl neuraminic  
acid



sugar derivative, sialic acid, whose synthesis is described here. UDP *N*-acetyl glucosamine is the precursor of mannosamine, the configuration of which is shown in Fig. 30.5. An unusual condensation with pyruvate occurs, and deacylation of the nitrogen in the product yields neuraminic acid. That is, *N*-acetyl neuraminic is sialic acid. Enzymes for each of the reactions in Fig. 30.6 have been demonstrated to be present in rat liver and in bovine submaxillary gland. Species specificity is exhibited in these compounds by variability in substituents (Fig. 30.7).

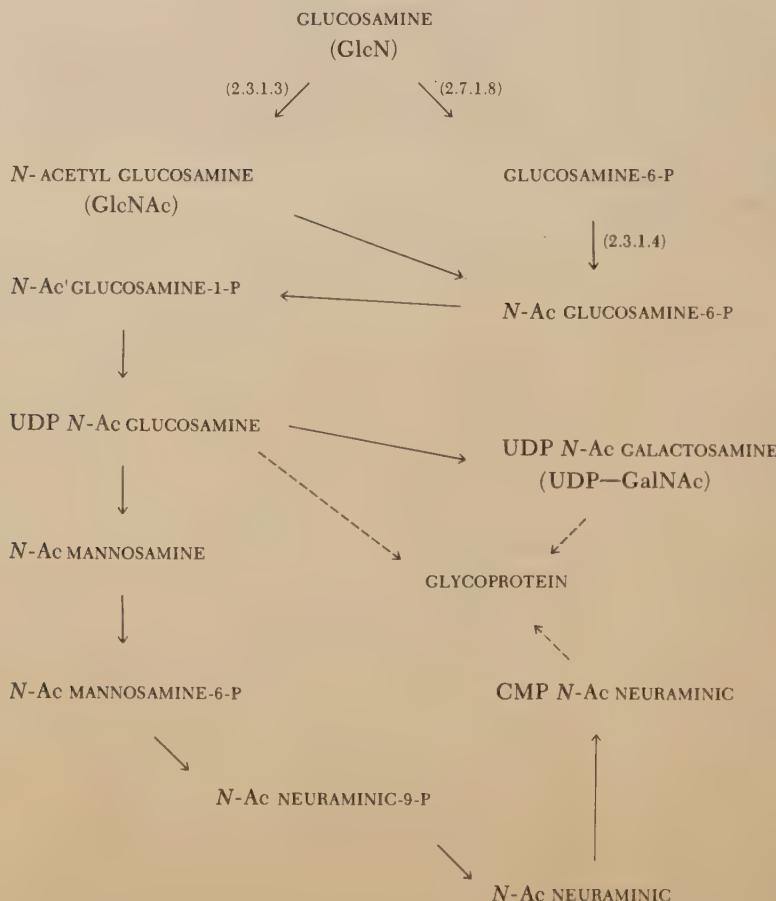
In the scheme just presented the term glycoprotein has been used without prior definition. There exist in many kinds of tissues proteins such as serum albumin and fetuin from umbilical cord, that have been shown to contain carbohydrate components. The carbohydrate moieties are quite variable in

**FIGURE 30.7**  
species differences in  
neuraminic acids



saccharide types and in chain length. Many of them contain glucosamine or galactosamine. The scheme in Fig. 30.8 is a more detailed sketch of glucosamine metabolism and depicts glycoprotein formation as presently known. In fact, it is not yet known whether the carbohydrate moieties are added after the protein is synthesized or whether carbohydrates are incorporated in a fashion similar to that of amino acids.

**FIGURE 30.8**  
glycoprotein synthesis



**animal  
extracellular  
poly-  
saccharides**

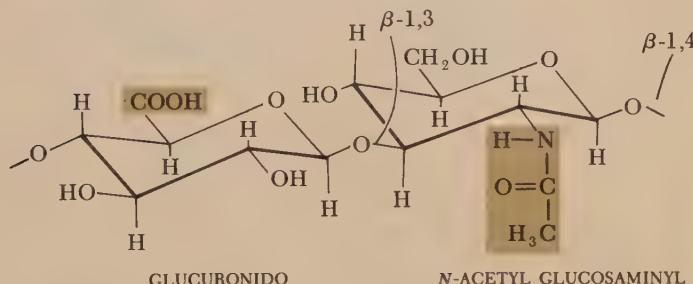
In 1955 K. Meyer said,

*From a functional point of view the cellular elements of the connective tissues play a more important role than either ground substance or formed elements. It would be useful to consider connective tissue as a highly integrated organ, in quantity the largest in the mammalian body, whose maintenance and function depends on cellular metabolism like any other organ.\**

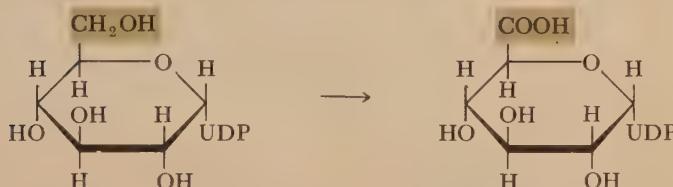
\* By permission of Academic Press Inc. From K. Meyer, *Harvey Lectures*, 1955-1956, p. 88.

For many years the task of identifying the components have required the energies of many competent investigators. Only recently have the metabolic origins of some of the components been revealed.

The bulk of the nonprotein ground substance consists of nonsulfated acid mucopolysaccharides, hyaluronic acid, and chondroitin. The chondroitin sulfates form the "cement" substance of these tissues. The repeating unit of hyaluronic acid is



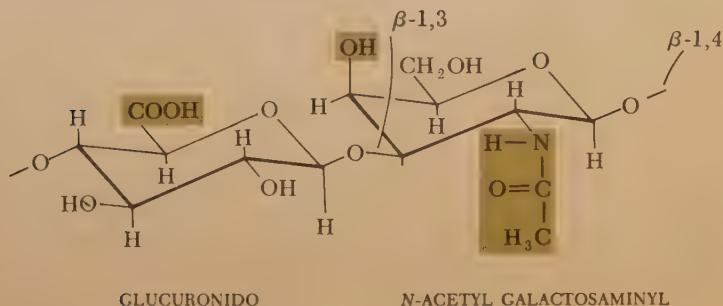
The glucuronic portion of this heteropolysaccharide may arise by either of two mechanisms. In animals and in bacteria a dehydrogenase (1.1.1.22) catalyzes the oxidation of UDP glucose.



In rat liver there is a separate system that catalyzes glucuronate formation from *meso*-inositol.

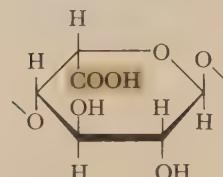


The repeating unit of chondroitin is



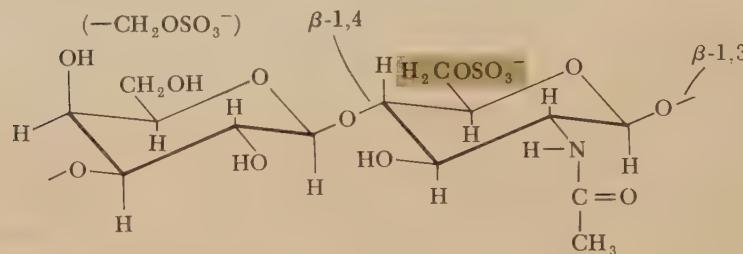
Various substituents may be present as in

- Chondroitin sulfate A. The  $-\text{OH}$  on carbon 4 of galactosamine is replaced by  $-\text{OSO}_3^-$ .
- Chondroitin sulfate B. Like A except that glucuronido is iduronido.



- Chondroitin sulfate C. The  $-\text{OH}$  on carbon 6 of galactosamine is replaced by  $-\text{OSO}_3^-$ .
- Chondroitin sulfate D. The  $-\text{OH}$  groups on both carbons, 4 and 6, are replaced by  $-\text{OSO}_3^-$  groups.

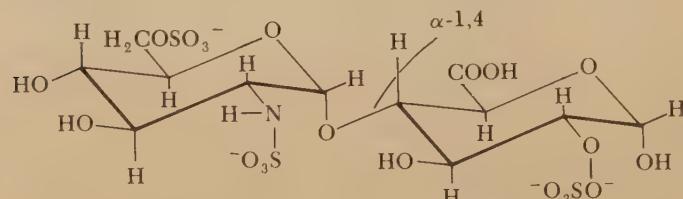
Another variant of the theme is kerato sulfate, which is less widely occurring.



GALACTOSIDO

N-ACETYL GLUCOSAMINYL  
DERIVATIVE

Probably the best studied of all such compounds is a natural anticoagulant present in blood, heparin. The structure almost certainly is



GLUCOSAMINE DERIVATIVE

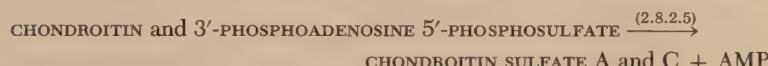
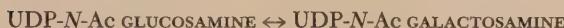
GLUCURONIC DERVATIVE

A mere catalog of such compounds is of little interest, but many formulas are included here in order to show the variability and the various combinations and permutations in this family of negatively charged polymers. The functions, as well as the biosynthetic patterns, are far from being well documented.

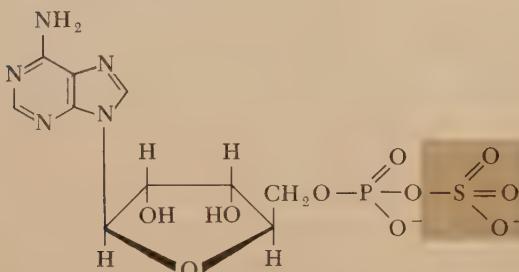
The synthesis of hyaluronic acid has been demonstrated both in streptococcal extracts and in embryonic rat skin. It was found that streptococcal

plasma membranes, free of DNA and RNA, retained the ability to synthesize hyaluronic acid. When UDP derivatives of glucuronic acid and *N*-acetyl glucosamine were used as substrates, they were joined in an alternating linear pattern in a product of high molecular weight. It seems likely that the A-B-A-B- pattern results from the intermittent action of enzymes whose specificities "demand" A when B is to be added and "demand" B when A is to be added.

Recent evidence for chondroitin sulfate synthesis in cell-free extracts of chick embryo epiphyses supports the following suggestion.



The sulfating agent, phosphoadenosine phosphosulfate (PAPS), is formed by the following reactions. Inorganic sulfate resulting from the metabolism of methionine, cysteine, or other sulfur-containing compounds can react (2.7.7.4) with ATP to form



Further phosphorylation (2.7.1.25) with ATP results in PAPS.

It is known that some of the polysaccharides are attached to proteins. Specifically, in cartilage, chondroitin sulfates are bonded covalently to protein, and it has been found that the protein and polysaccharide portions have roughly the same rate of turnover. It is not certain whether the syntheses of the two types of compounds are coupled in any way. However, there is an intriguing observation that mucopolysaccharide synthesis is modified by thyroid hormone and pituitary growth hormone. Many aspects of connective tissue metabolism are of more than usual interest. Several of the crippling diseases difficult to treat, such as rheumatoid arthritis, involve pathology of connective tissue.

### sugar inter-conversions

As investigators have continued to study the complex saccharides of tissues, more and more kinds of sugars have been shown to occur naturally. Most of these sugars have their origin in glucose, and many of the interconversions involve sugar derivatives of purine or pyrimidine nucleoside diphosphates.

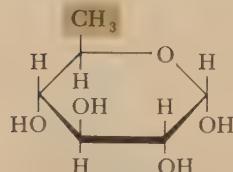
The coding processes, and the control processes involved in sugar intercon-

versions, are being documented. Energetically, sugars do not differ from each other very much; a wide variety of structures are, thermodynamically, at about the same energy level. As noted, glucose is readily phosphorylated and glucose-1-P readily interacts with UTP in the presence of a pyrophosphorylase to yield UDP glucose. This compound is the precursor of UDP galactose through the action of an epimerase (5.1.3.2), or of UDP glucuronic acid in the presence of a dehydrogenase. Note that UDP N-Ac glucosamine derives from a glucosamine-1-P formed from glucose-6-P.

In plants it has been observed that each of the major homopolymers of glucose seems to have as a precursor a characteristic nucleoside diphosphoglucoside.

Callose	linear polyglucose	$\beta$ -1,3 bonds from uridine diphospho glucose
Amylose	linear polyglucose	$\alpha$ -1,4 bonds from adenosine diphospho glucose
Cellulose	linear polyglucose	$\beta$ -1,4 bonds from guanosine diphospho glucose

In some organisms thymidine diphospho glucose is a precursor for rhamnose.



In other organisms the reaction pattern in Fig. 30.9 has been demonstrated.

**FIGURE 30.9**  
formation of thymidine diphosphate hexoside

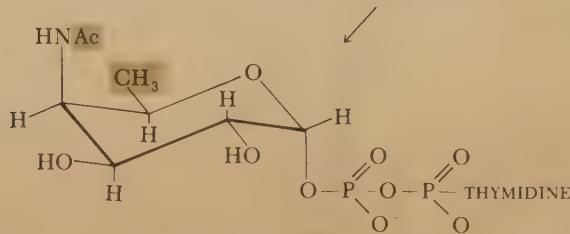
TDP



TDP 4-KETO 6-DEOXY GLUCOSE + Glu + PYRIDOXAL P

+

AcCoA



THYMIDINE DIPHOSPHATE 4-ACETAMIDO-4,6-DIDEOXY HEXOSE

No exhaustive list of such compounds will be given, since it is possible that for every sugar found in nature there is at least one such nucleoside derivative. Probably several such derivatives exist for each major sugar.

**comment**

The investigation of bacterial cell wall structure is a relatively recent endeavor. Without minimizing present knowledge it can be concluded that only the barest outlines of the topic have been recognized. One substantial bar to progress in the study of complex polysaccharides, from whatever source, is the lack of methods for structure determination. Until very recently the study of carbohydrate structure has employed primarily organic techniques, mostly "classical" in nature. Such techniques usually do not have the specificity requisite for the chemical dissection of these complex structures. In the case of protein structure, advances followed rapidly upon the development of enzymic techniques, and the same advances may be expected in the case of complex polysaccharides. There is no doubt that the availability of homogeneous carbohydrases will improve our ability to discern the structural complexities of polysaccharides.

It has been recognized for a long time that polysaccharides are heterodisperse in the ultracentrifuge, and the prevalent idea is that a polysaccharide preparation is a hopeless mixture containing a wide range of molecular sizes. Recent observations suggest that the size spectrum may not be so wide and that there are regularities to be discovered. In the discussion of glycogen it was emphasized that the unit could hardly be called a molecule, but must be thought of as a molecular network. Some of the cell wall structures may be considered as similar systems. For such systems, size must reflect the topology of the cell whose metabolism furnishes its substance. In contrast, complex polysaccharides, such as chondroitin or hyaluronic acid, that eventually are confined to the extracellular spaces might be expected to reflect the synthetic process and to exhibit a narrower range of molecular size.

The presumption, at the moment, is that the formation of complex polysaccharides is not closely monitored by the nucleus as is the synthesis of proteins and nucleic acids. This implies that the genetic directive terminates in the production of enzymes and that synthesis occurs whenever adequate substrate concentrations are generated by the metabolic ensemble. If this is actually the situation, then we may expect to find some interesting control mechanisms that couple such syntheses to the epigenetic system.

Our knowledge of membranes is also in an early stage of development. It is agreed that lipoproteins and complex saccharides form an important portion of these structures, but they are the very macromolecules that are difficult to characterize and are the source of experimental difficulties. We know little about the synthesis of lipoproteins, or glycoproteins, and still less about their orderly incorporation into biological structures.

It is also quite unclear how sugar interconversions are controlled. The intermediate, uridine diphosphoglucose, is the precursor of several different carbohydrates, but not all the carbohydrates are formed in the same tissue. Part of the control owes simply to the presence or absence of an enzyme, the

expression of potentiality. But part of the control process must be correlated with other aspects of metabolism. Some of the possibilities are outlined in the next, and last, section of this book.

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# 31

## metabolic networks and mechanisms for control

That the control of reaction rates in cells is of critical importance has been recognized by biochemists as a basic working hypothesis. It has been assumed that there are in the cell no uncontrolled reactions. Thus, for many years, biochemists have been isolating one metabolic transformation process after another and have accumulated a vast fund of data about the various intermediary reactions in metabolism. Until such data were collected it seemed premature to inquire into the coupling mechanisms. Moreover, questions about control often became questions about protein structure.

It is likely that the biochemist should refrain from extending his inquiry to interactions among cells and should leave this quest to the physiologists or other biologists. For example, how can a biochemist react when it is demonstrated that a desert plant excretes a substance that inhibits the germination of seeds? Or that bacteria produce substances that ensure a favorable environment? Or that insects over several generations develop colors that blend with the environment? How exceedingly difficult it is to avoid an invocation of purpose! The working hypotheses of the biochemist and the molecular biologist do not include the recognition of purposive action, nor do they embrace the notion that the activities of a collection of cells are, in total, greater than the sum of the parts. The classical biologist is not so bound and hence has reserved to him a fascinating array of problems that may yield only to approaches different from, and newer than, those of biochemistry and molecular biology.

Many now believe that the main lines of metabolism are known. We also know in, or on, what structures various metabolic reactions are occurring. It is reasonable that energy should now be directed toward the elucidation of control mechanisms.

### **evolutionary changes**

It is convenient to group control processes by the time scale involved. Processes involving the very longest time spans are evolutionary and presumably unidirectional;<sup>°</sup> others have time spans of hours or days and are rhythmic or oscillatory. The latter may be a response to light, such as the flowering of plants or the mating of birds. Again there are rhythmic changes that seem to derive from the organism itself and that are often referred to as the phenomenon of the biological clock. Included in this time group are the unidirectional processes of differentiation. The control processes of the epigenetic

<sup>°</sup> In his *Cybernetics*, Wiener noted that Bergson emphasized the reversible nature of time in classical physics—time in which nothing new happens—and the irreversible time of biology in which something new is always happening.

Somewhat similarly, in chemistry true equilibria, in which quite reversible processes can be described, are attained. In biochemistry, equilibria are the result of separate, opposing, unidirectional reactions.

system, which include the biosynthesis, distribution, and interaction of macromolecules, require time periods of seconds to hours. Finally, metabolic processes, the most rapid of all, consist of reactions requiring no more than micro- or millimicroseconds. Even oscillatory phenomena here probably have periods of less than a few minutes.

At the present time evolutionary processes seem beyond the proper concern of biochemists. Simpson has noted that:

*DNA is not subject to feedback within individuals . . . changes in individual expression . . . do not affect the message itself. The necessary message-construction feedback is not here but in a system of higher order: in the population and not in the individual. It operates through natural selection, which operates in populations, just as populations are what really evolve. Thus through a different approach we come again to natural selection and now see it as the most truly causative element in the adaptive system. Viewed in this way, it is the composer of the genetic message and DNA, RNA, enzymes and the other molecules in the system are successively its messengers.<sup>9</sup>*

Darlington has also called attention to the fact that the discovery of crossing over enables us to recognize two processes. One of them embraces chemical determination and the character of individuals; the other is the process of meiosis that sometimes seems indeterminate but really is a function of natural selection.

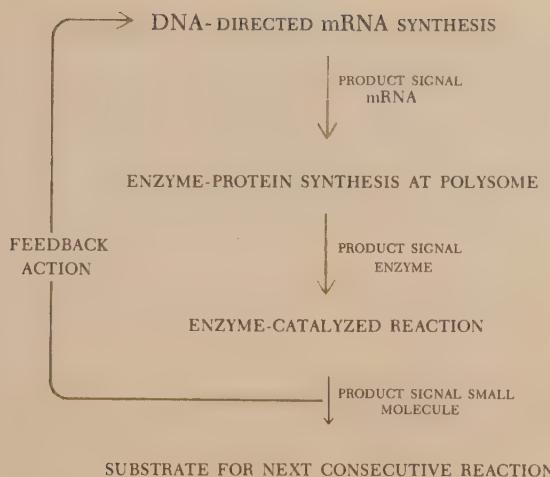
#### **feedback**

The term "feedback" is derived from engineering but has gained very wide usage in biology recently.† At present it is used so loosely that it has lost the inherent precision of meaning and also implies a simplicity that seldom exists. In the case of the epigenetic system we may consider a very simplified model such as that in Fig. 31.1. This model implies that the object of control is to maintain a steady-state concentration of the small molecule that is the product of the enzyme-catalyzed reaction. It implies that a rise in the concentration of the small molecule will inhibit mRNA synthesis, but it does not specify a mechanism. It may, or may not, imply that a decline in the concentration of the small molecule will increase mRNA synthesis. If mRNA synthesis is a poised system, then the feedback effect may be either positive or negative. But if it is a poised system, we must imagine that a normally present repressor is actually the target in the case of a positive effect. The model also implies that small molecules from the environment will have an effect on the synthetic processes producing large molecules. To some extent, the model demands

<sup>9</sup>By permission of G. G. Simpson and *Science*. From *Science*, 146, 1535 (1964). Copyright 1964 by the American Association for the Advancement of Science.

†The discussion of feedback here is aimed at the clarification of biochemical problems. Perhaps it should be mentioned that much of physiology is a description of feedback mechanisms in the animal as a whole. For example, a lowering of the environmental temperature will cause peripheral vasoconstriction and, eventually, shivering. If the temperature drop is not too great, the body temperature will be unchanged. Wound healing, and many other processes, may also be thought of as feedback mechanisms.

**FIGURE 31.1**  
epigenetic feedback



protein turnover. If there were no turnover, the control would be very poor unless marked product inhibition occurred in the enzyme-catalyzed reaction.

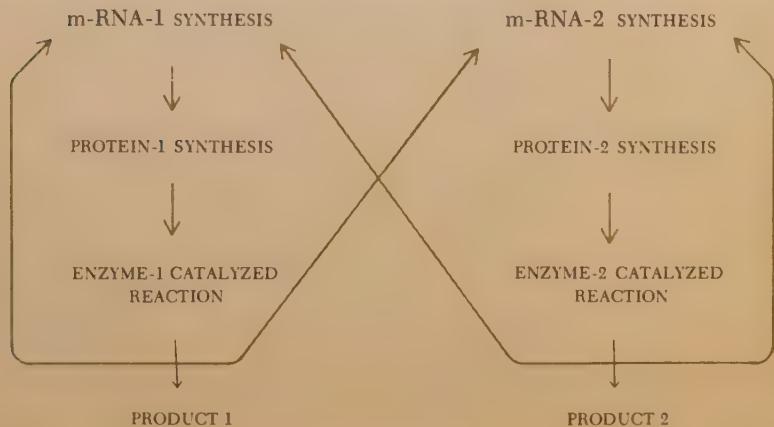
Of course, more complicated relations can be imagined. For example, a small-molecule product may affect more than one synthesis site, as illustrated in Fig. 31.2.

In addition to systems of this type, others, involving only consecutive reactions, are possible.



Most of the metabolic sequences studied are of this kind. Obviously, some step will be rate-limiting and, for control purposes, inhibition of the first step will slow or stop all subsequent steps to the point at which an alternate reaction may occur. If substrate E, for example, inhibits enzyme  $\alpha$  in the above sequence, there will be negative feedback providing a measure of control even if the steady-state concentrations of enzymes  $\alpha$ ,  $\beta$ , . . . are invariant or are varied too slowly. The reactions have been written as nonequilibrium reac-

**FIGURE 31.2**  
multiple feedback



tions because a catalyst has no effect on a system at equilibrium. Biochemical control mechanisms operate only in open systems. The feedback target in this case is the catalyst, and it may be a competitive inhibitor or an inhibitor that reversibly changes the enzyme in some other fashion. The time constant for such a control mechanism would be much smaller and peculiarly adapted to cells that are in as rapid growth as bacteria, whereas, the epigenetic apparatus might be expected in cells that are resting or in a steady state.

### Pasteur effect

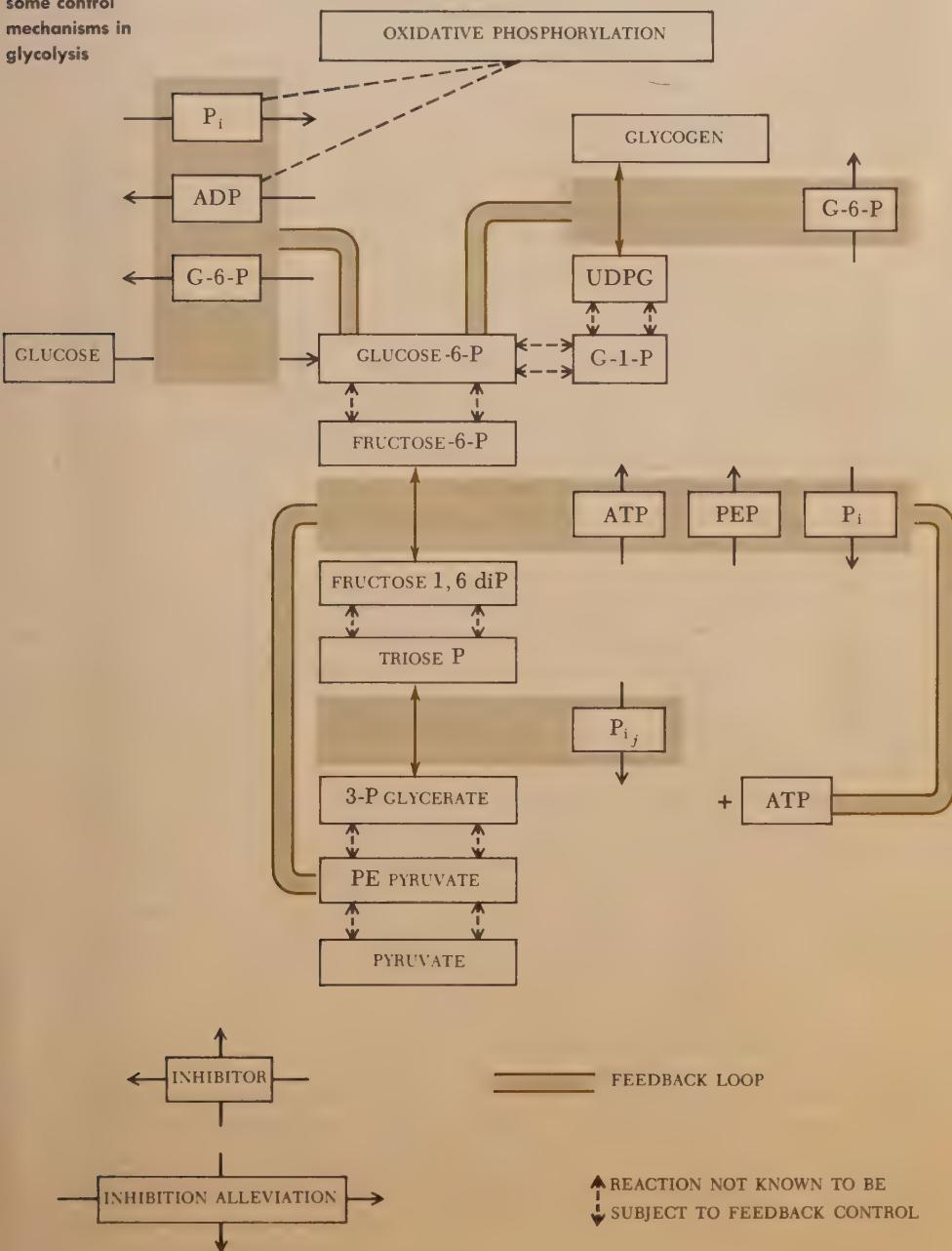
The search for an explanation, or description, of control processes arose early in the development of biochemistry. One of the classic problems is known as the "Pasteur effect." In "Etudes sur la bière," published in 1876, Pasteur noted that fermentation is inhibited by respiration. In an aerobic environment no alcohol is produced and the rate of sugar utilization drops appreciably. A reasonable working hypothesis finally formulated rested on two basic investigations: the discovery by Harden and Young (1905) that fermentation of glucose requires inorganic phosphate and the demonstration by Warburg and Christian (1939) that the oxidative reaction of glycolysis, catalyzed by glyceraldehyde 3-phosphate dehydrogenase, involves inorganic phosphate. The hypothesis was that oxidative phosphorylation in aerobiosis competes for inorganic phosphate, and hence limits the supply for glycolysis. Moreover, it is assumed that the ATP generated by oxidative phosphorylation is largely restricted to the mitochondria and hence little is available for the hexokinase reaction, necessary as the first step in glycolysis. Experiments on tumor cells have supported the idea that control is a function of the coupling of two processes, occurring in separate cell compartments, through the common intermediate, inorganic phosphate. In this case inorganic phosphate may be considered the "feedback agent."

In Fig. 31.3 are shown a number of the relationships now known. For clarity the diagram is highly simplified. It illustrates the generality that only reactions whose products and reactants are not in an equilibrium relation are subject to feedback control. An impressive array of evidence indicates phosphofructokinase as a key controlling enzyme susceptible to a glycolysis intermediate (PEP), substrate inhibition (ATP), and an allosteric effect ( $P_i$ ). The latter, together with the adenine nucleotides, provides for the coupling to the process of oxidative phosphorylation. Again, glucose-6-P is seen as a regulatory agent at a "branch point." An increase in concentration is seen to inhibit hexokinase (except in yeast) and to activate glycogen synthetase. The scheme presented is based on experiments performed with a reconstituted system and is not valid for all cells. In any case, control in the living cell includes not only such relations as these but also effects due to compartmentation and modification, in many cases, by insulin or other hormones.

The change from anaerobic to aerobic metabolism evidently involves many metabolic "fine control" adjustments. Studies of yeast, even of cell-free extracts, show that such a change involves damped oscillations in the NAD/NADH ratio as well as cyclic changes in the concentrations of glycolytic intermediates.

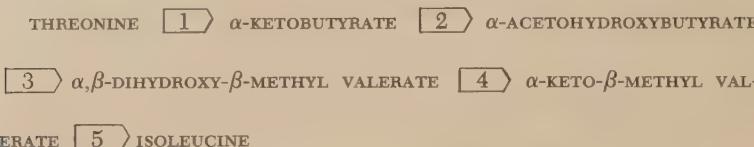
As already noted in Sec. 4, oxaloacetate is an inhibitor of succinic dehydrogenase. The level of this metabolite must be an important factor in balancing several metabolic rates. A number of instances of stimulation as well as inhibition have been reported. For instance, AMP acts as an "effector," and

**FIGURE 31.3**  
some control  
mechanisms in  
glycolysis



thus the flux of adenine nucleotides not only reflects the several activities of enzymes but acts as a control device as well.

Another example of metabolic regulation is that of amino acid synthesis. One of the first to be studied was the series of reactions leading from threonine to isoleucine (Sec. 13).



When the concentration of isoleucine rises above a certain level, it begins to inhibit the enzyme catalyzing reaction 1. The increase in concentration may be effected by the artificial addition of isoleucine to the medium or it may result from metabolic events within the cell. Either way, there is a competitive inhibition of the first enzyme in the reaction series. In other cases, more than one step may be inhibited. Another variant described in the literature is the coordinate repression in "branched metabolic pathways." In *E. coli* the biosynthesis of threonine (Fig. 11.6), methionine (Figs. 12.2 and 12.3), and lysine involves the common intermediate, aspartyl phosphate. Two separate kinases (2.7.2.4) have been demonstrated to be involved in its synthesis. One is noncompetitively inhibited by lysine, the other competitively inhibited by threonine. Thus feedback control from one branch does not affect that in the others.

As long as the product is somewhat similar to the initial substrate, there is little difficulty in understanding the mechanism that is operating. However, in another well-studied case the inhibiting product is quite dissimilar. It was found that ASPARTATE CARBAMOYL TRANSFERASE (TRANSCARBAMYLASE) (2.1.3.2), which catalyzes an initial step in pyrimidine synthesis (Sec. 17), is inhibited by the product, cytidine triphosphate. Here there is no obvious similarity between the substrate and the inhibitor, and the assumption was made that two quite different sites on the protein were involved. Further experimentation showed that the protein can be treated in such a way that the inhibition phenomenon is abolished without destroying the catalytic activity. Evidence is now at hand that this enzyme contains two kinds of subunits, catalytic and regulatory. Kinetic evidence leads to the view that the catalytic subunit is not affected by the inhibitor, CTP, but that the regulatory subunit responds. When the "intact" enzyme is exposed to CTP, the overall rate is affected by the inhibitor acting through the regulatory unit, presumably by causing structural change.

**steric effects** Two ideas are currently being used as working hypotheses in such cases. One is the postulation of an allosteric effect. A competitive inhibitor is assumed to be isosteric; an inhibitor of quite different form would be allosteric. The assumption is that binding of an allosteric substance causes a change in the conformation of the enzyme as the substrate itself may. Just how many meta-

bolic intermediates have allosteric effects<sup>\*</sup> remains to be seen. The other idea is that the catalytic activity of an enzyme varies with the state of aggregation. In this specific case there is evidence that aspartate induces changes making for more activity and the CTP induces an opposite change. Thus the enzyme itself becomes a poised system. Whether this will prove to be generally correct is a development we must await. It is obvious that the second idea is a corollary of the first, so that the generality of the allosteric effect is very great. Unfortunately, ideas that are so general do not serve as precise guides to experimentation. Many examples in which isosteric, or allosteric, effects seem to provide control of metabolic substances in the cell can now be cited. Most of these examples originate from studies on microorganisms, but some have been described for multicellular forms, including mammals.

If we examine systems in which not only the concentrations of small molecules vary but also the concentration of biopolymers can change, we must consider the epigenetic systems recently mentioned.

### **epigenetic system**

The experimentation in this subject is currently being dictated largely by the idea cluster of the operon hypothesis, briefly referred to at the end of Sec. 20. To recapitulate, base-coded "information" for the synthesis of a protein resides in a section of DNA. This information is "transcribed" in the 5' → 3' direction by a "promoter" enzyme into a base sequence code in a single-stranded messenger RNA complementary to one of the DNA strands. The "translation" of the base sequence code in the 5' → 3' direction into an amino acid sequence (codon sequence) occurs in the polysome complex and results in a few polypeptide chains (probably more than one but not a large number). Conditions where the DNA-RNA transcription is prevented have been described, and this phenomenon has been termed "repression." Referring now to the sketch in Sec. 20, postulate that the operator site is adjacent to this operon in a "regulator" gene, or site, whose product is a cytoplasmic factor, a "repressor," perhaps a protein, activated by small molecules. Invocation of the allosteric effect allows the postulate that such a protein may have an effective or an ineffective conformation depending on the kind and concentration of small molecules ("effectors"). The repressor† may be assumed to interact with the operator site. Mutation may modify the regulator,‡ the operator, or the promoter.

Here it is in order to present, not extensive genetic evidence, but only the model derived from the concerns of genetics. In the genetic sense, each gene,

\*Reactions catalyzed by allosteric enzyme systems exhibit kinetic behavior different from that described in Sec. 2. When plots of velocity vs. substrate concentration are made, sigmoid instead of hyperbolic curves are obtained if an inhibitory conformation is present. Thus multimolecular kinetics reinforces the view that substrate and inhibitor are bound at different sites of the protein; for example, see reference 11.

† A repressor may act on more than one operator.

‡ Note that the regulator gene is defined as that segment in which mutations will not affect the structure of protein but will affect the conditions and rate of synthesis of the correlated proteins.

or DNA cistron, in the operon<sup>\*</sup> is associated closely with a common gene, the operator. Each cistron represents a specific succession of codons. The operator can be either "open" or "closed." When open, the operator does not prevent cistron transcription; when closed, it does. The operator is closed by combination with the repressor. The effectiveness of the repressor is determined by effectors (where biochemistry and metabolism enter the picture). As yet, the repressor is not an isolable substance but is an abstraction, a postulation. It has been postulated to correlate experimental results.

The study of histidine synthesis in *Salmonella typhimurium* by genetic methods has resulted in some additional conclusions about the operon model. The conversion of PRPP and ATP to histidine by the serial action of ten enzymes has already been delineated in Sec. 14. The structural genes for these enzymes form the "histidine operon," which is divided into 15 cistrons. In some cases, enzymes are determined by more than one cistron. The operator gene appears to be at one end of the operon, and the length of the operon has been estimated to be that of 13,000 nucleotides. It has been found that histidine represses the formation of all ten enzymes (coordinate repression), and it has been proposed that a single messenger RNA carries the code message of the entire operon. The existence of a high-molecular-weight polycistronic messenger RNA seems likely. Moreover, a polarity appears to exist in the whole process from DNA code to protein. As noted, the evidence indicates that the "reading" of the polycistronic mRNA code begins at the operator end and that enzyme proteins are synthesized in a serial order. Present results allow the belief that the quantity of each enzyme decreases as the code position of the enzyme is farther removed from the operator end.

Many biochemical questions in relation to the operon hypothesis are yet to be resolved. No experiments yet performed allow us to decide whether regulation is achieved by blocking the operator gene, or the operator site, on the polycistronic RNA or by interfering with the transcription process. Despite such uncertainties, there is little doubt that experimentation will continue in an effort to validate this model.

#### tissue cultures

In multicellular creatures it is expected that control mechanisms of the kinds just described are operative and that, in addition, there are intercellular actions that activate or inhibit. The extensive studies on mammalian cells cultured in vitro furnish evidence on control mechanisms. Apparently such cells are relatively insensitive to environmental variations in small-molecule concentrations and to end-product inhibition.

A variety of intercellular effects have been observed. Some substances are lost to the medium at a rate greater than that of their synthesis. In dense populations this does not occur. Again, some cells exhibit contact inhibition in which DNA-RNA-protein synthesis declines as more and more of the cell surface contacts other cells. If these cells are parted, synthesis begins again. Perhaps this phenomenon is not unrelated to the regulation of DNA synthesis

\*An operon may contain one or several structural genes.

in bacteria. Here first replication occurs, followed by a cell surface event that ends by separation of cells, which again initiates DNA replication.

Recent developments hold the promise that cultured animal cells may be hybridized, and if this promise is fulfilled, the methods of biochemical genetics will surely be applied. Thus it will be possible to evaluate directly the relative importance of epigenetic events and intercellular vectors. However, the fact that protein turnover (synthesis and degradation) is a well-defined feature of such cells strongly points to a constant epigenetic activity.

One of the characteristics of the cells in multicellular organisms is specialization. Cytodifferentiation must be a process integrated with, and derivative from, the regulatory processes considered. Many intriguing facts lie in the record, but as yet no satisfactory model has been offered. Differentiation, in a kinetic sense, is a series of discontinuities, and it is quite difficult to envisage the on-off mechanism that is operating.

#### **hormonal control**

A long-studied and well-recognized form of regulation in both plants and animals is that due to hormones. As has been seen, these compounds may be relatively small (e.g., indoleacetate), polypeptide, sterols, or proteins, such as insulin. In short, a whole variety of compounds function as hormones, and, in general, they are noncompetitive. They often have different actions on different tissues, so that they would not, seemingly, bear a "message." Until recently there has been small success in demonstrating the direct action of a hormone on a chemical reaction. Hormones seemed to have little effect on enzymes or, for that matter, on *in vitro* systems in general. Now evidence is accumulating concerning the influence of hormones on RNA synthesis and on protein synthesis. It is far too early to state whether this is the chief acceptor site or not, but the present search is exciting.

#### **comment**

Without any depreciation of past efforts to understand metabolic control it may be said that the quest has acquired a new luster. The possibility of describing control in terms of protein structure is particularly exciting. As Atkinson has noted, the modulation of enzyme kinetics by effectors resembles the modulation of plate current by the voltage on the grid of a vacuum tube. If the analogy is apt, we may anticipate a marked expansion in our comprehension.

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## epilogue

Those who have been patient enough to work through the topics in this book will now appreciate that they have merely been introduced to biochemistry. A wide variety of topics have been touched upon; hardly any have been penetrated in depth.

For a long time the academic peck order has ordered scientific preoccupations vertically. The physical sciences were always above the biological sciences. Physics, somewhere near the top, consists of inquiries into the fundamental nature of all matter. Far-reaching and powerful as the methods of physics are, they are limited to a certain type of descriptive process and to very simple theoretical models. In contrast, biology, somewhere near the bottom, has consisted of a mass of description and a minimum of theory. Physicists have ignored living tissue; biologists have seemed to be quite preoccupied with its reproduction. Molecular biology, in good analogy, has largely consisted of research on reproduction at the molecular level. Pure and applied sex, as it were.

This book is a testament to the idea that the concepts of biochemistry lie somewhere between physics and biology, bowing to the strictures of physics, borrowing models from chemistry, and drawing problems from biology. Biophysics is no less broad but has had fewer practitioners.

The bulk of experimentation has been devoted to puzzling out metabolism, and there are now signs that other Big Problems are needed. I have attempted to point to a few during the narrative. Now that we have a brief chemical description of biological structure, and some notion of the molecular and biological reproductive process, it is perhaps in order to ask questions, in an answerable form, about the relations of cells to the environment. How do they react to light, to radiation in general, to temperature changes, to each other? How is the transduction of energy accomplished? Almost all the interesting questions remain to be answered.

# catalogue of enzymes referred to in this volume

The numbering, classification, and description of the enzymes listed is that of the 1964 recommendations, Report of the Commission on Enzymes, International Union of Biochemistry. A comparison of this partial list with the complete one will provide one measure of the coverage of this book.

<i>page</i>	<i>number</i>	<i>systematic name</i>	<i>recommended trivial name</i>
1		Oxidoreductases	
1.1		Acting on the CH—OH groups of donor	
1.1.1		With NAD or NADP as acceptor	
14	(1.1.1.1)	Alcohol:NAD oxidoreductase	Alcohol dehydrogenase
33			[Aldehyde reductase]
292			
118	(1.1.1.3)	L-Homoserine:NAD oxidoreductase	Homoserine dehydrogenase
60	(1.1.1.8)	L-Glycerol-3-phosphate:NAD oxidoreductase	Glycerol-3-phosphate dehydrogenase
282			
319	(1.1.1.19)	L-Gulonate:NADP oxidoreductase	Glucuronate reductase
319	(1.1.1.20)	L-Gulono- $\gamma$ -lactone:NADP oxidoreductase	Glucuronolactone reductase
320			
319	(1.1.1.22)	UDP glucose:NAD oxidoreductase	UDPG dehydrogenase
333			
148	(1.1.1.23)	L-Histidinol:NAD oxidoreductase	Histidinol dehydrogenase
156	(1.1.1.25)	Shikimate:NADP oxidoreductase	Shikimate dehydrogenase

Some enzyme names (in brackets) now in use are considered improper by the Commission. In time, usage will become more uniform, but in the interim this table provides a guide to terminology used in the present literature.

One-, two-, and three-digit entries (in black) are categories; four-digit entries (in parentheses) represent well-characterized enzymic activities, and any proteins exhibiting these activities may be designated by the digital system.

To facilitate reference to the more complete Commission report, the nomenclature used in that report has been retained. Note, for example, that  $\alpha$ -ketoglutarate is designated as 2-oxoglutarate and that  $\alpha$ -keto acid is 2-oxo-acid. The singularities, however, are relatively minor.

reaction	notes on specificity and other comments
Alcohol + NAD = aldehyde or ketone + reduced NAD	Acts on primary or secondary alcohols or hemiacetals; the animal, but not the yeast, enzyme acts also on cyclic secondary alcohols
L-Homoserine + NAD = L-aspartate $\beta$ -semialdehyde + reduced NAD	NADP also acts, more slowly
L-Glycerol-3-phosphate + NAD = dihydroxyacetone phosphate + reduced NAD	Acts also on 1,2-propanediol phosphate
L-Gulonate + NADP = D-glucuronate + reduced NADP	Also reduces D-galacturonate
L-Gulono- $\gamma$ -lactone + NADP = D-glucurono- $\gamma$ -lactone + reduced NADP	
UDP glucose + 2NAD + H <sub>2</sub> O = UDP glucuronate + 2 reduced NAD	
L-Histidinol + 2 NAD = L-histidine + 2 reduced NAD	Also oxidizes L-histidinal
Shikimate + NADP = 5-dehydroshikimate + reduced NADP	

<i>page</i>	<i>number</i>	<i>systematic name</i>	<i>recommended trivial name</i>
34	(1.1.1.27)	L-Lactate:NAD oxidoreductase	Lactate dehydrogenase
50			
247			
249			
323			
133	(1.1.1.31)	3-Hydroxyisobutyrate:NAD oxidoreductase	3-Hydroxyisobutyrate dehydrogenase
268	(1.1.1.34)	Mevalonate:NADP oxidoreductase (acylating CoA)	Hydroxymethylglutaryl-CoA reductase
93	(1.1.1.35)	L-3-Hydroxyacyl-CoA:NAD oxidoreductase	3-Hydroxyacyl-CoA dehydrogenase [ $\beta$ -Hydroxyacyl dehydrogenase, $\beta$ -ketoreductase]
23	(1.1.1.37)	L-Malate:NAD oxidoreductase	Malate dehydrogenase
40			
323			
69	(1.1.1.49)	D-Glucose-6-phosphate:NADP oxidoreductase	Glucose-6-phosphate dehydrogenase [Zwischenferment]
323			
126	(1.1.1.68)	5-Methyltetrahydrofolate:NAD oxidoreductase	5,10-Methylene-tetrahydrofolate reductase
1.1.3		With oxygen as acceptor	
319	(1.1.3.8)	L-Gulono- $\gamma$ -lactone:oxygen oxidoreductase	L-Gulonolactone oxidase
1.1.99		With other acceptors	
92	(1.1.99.5)	L-Glycerol-3-phosphate:(acceptor) oxidoreductase	Glycerolphosphate dehydrogenase
1.2		Oxidoreductases acting on the aldehyde or keto group of donors	
1.2.1		With NAD or NADP as acceptor	
118	(1.2.1.11)	L-Aspartate- $\beta$ -semialdehyde:NADP oxidoreductase (phosphorylating)	Aspartate semialdehyde dehydrogenase

<i>reaction</i>	<i>notes on specificity and other comments</i>
$\text{L-Lactate} + \text{NAD} =$ $\text{pyruvate} + \text{reduced NAD}$	Also oxidizes other L-2-hydroxymonocarboxylic acids; NADP also acts, more slowly
$\text{3-Hydroxyisobutyrate} + \text{NAD} =$ $\text{methylmalonate semialdehyde} +$ $\text{reduced NAD}$	
$\text{Mevalonate} + \text{CoA} + 2\text{NADP} =$ $3\text{-hydroxy-3-methylglutaryl-CoA} +$ $2 \text{ reduced NADP}$	
$\text{L-3-Hydroxyacyl-CoA} + \text{NAD} =$ $3\text{-oxo-acyl-CoA} + \text{reduced NAD}$	Also oxidizes S-3-hydroxyacyl-N-acylthioethanolamine and S-3-hydroxyacylhdrolipoate
$\text{L-Malate} + \text{NAD} =$ $\text{oxalacetate} + \text{reduced NAD}$	Also oxidizes some other 2-hydroxy-dicarboxylic acids
$\text{D-Glucose-6-phosphate} + \text{NADP} =$ $\text{D-glucono-6-lactone 6-phosphate} +$ $\text{reduced NADP}$	Also acts slowly on $\beta$ -D-glucose and other sugars. Certain bacterial preparations also reduce NAD as well as NADP
$\text{5-Methyltetrahydrofolate} + \text{NAD} =$ $5,10\text{-methylenetetrahydrofolate} + \text{reduced NAD}$	
$\text{L-Gulono-}\gamma\text{-lactone} + \text{O}_2 =$ $\text{L-xylohexulonolactone} + \text{H}_2\text{O}_2$	The product spontaneously isomerizes to L-ascorbate
$\text{L-Glycerol-3-phosphate} + \text{acceptor} =$ $\text{dihydroxyacetone phosphate} + \text{reduced}$ $\text{acceptor}$	Formerly EC 1.1.2.1
$\text{L-Aspartate-}\beta\text{-semialdehyde} + \text{phosphate} + \text{NADP} =$ $\text{L-}\beta\text{-aspartylphosphate} + \text{reduced NADP}$	

<i>page</i>	<i>number</i>	<i>systematic name</i>	<i>recommended trivial name</i>
34 59	(1.2.1.12)	D-Glyceraldehyde-3-phosphate: NAD oxidoreductase (phosphorylating)	Glyceraldehydophosphate dehydrogenase, triosephosphate dehydrogenase
175	(1.2.1.14)	IMP:NAD oxidoreductase	IMP dehydrogenase
	1.2.4	With lipoate as acceptor	
44	(1.2.4.1)	Pyruvate:lipoate oxidoreductase (acceptor-acetylating)	Pyruvate dehydrogenase
	1.3	Acting on the CH—CH group of donors	
	1.3.3	With oxygen as acceptor	
177	(1.3.3.1)	L-4,5-Dihydro-orotate:oxygen oxidoreductase	Dihydro-orotate dehydrogenase
	1.3.99	With other acceptors	
25 323	(1.3.99.1)	Succinate:(acceptor) oxidoreductase	Succinate dehydrogenase
93	(1.3.99.2)	Butyryl-CoA:(acceptor) oxidoreductase	Butyryl-CoA dehydrogenase [Butyryl dehydrogenase, ethylene reductase]
	1.4	Oxidoreductases acting on the CH—NH <sub>2</sub> group of donors	
	1.4.1	With NAD or NADP as acceptor	
138 249	(1.4.1.3)	L-Glutamate:NAD(P) oxidoreductase (deaminating)	Glutamate dehydrogenase [NAD(P)]
	1.4.3	With oxygen as acceptor	
112 114	(1.4.3.2)	L-Amino-acid:oxygen oxidoreductase (deaminating)	L-Amino-acid oxidase [Ophio-amino-acid oxidase (for the snake enzyme only)]
	1.5	Oxidoreductases acting on the C—NH group of donors	

reaction	notes on specificity and other comments
$\text{D-Glyceraldehyde-3-phosphate} + \text{phosphate} + \text{NAD} =$ $1,3\text{-diphospho-D-glyceric acid} + \text{reduced NAD}$	Also acts very slowly on D-glyceraldehyde and some other aldehydes; thiols can replace phosphate
$\text{IMP} + \text{NAD} + \text{H}_2\text{O} = \text{xanthosine 5'-phosphate} + \text{reduced NAD}$	
$\text{Pyruvate} + \text{oxidized lipoate} =$ $6\text{-S-acetylhydrolipoate} + \text{CO}_2$	Requires thiamine pyrophosphate; possibly a system
$\text{L-4,5-Dihydro-orotate} + \text{O}_2 =$ $\text{orotate} + \text{H}_2\text{O}_2(?)$	A flavoprotein containing 1 FAD and 1 FMN group per molecule. NAD can replace O <sub>2</sub> ; reduced NAD can replace dihydro-orotate
$\text{Succinate} + \text{acceptor} =$ $\text{fumarate} + \text{reduced acceptor}$	A flavoprotein
$\text{Butyryl-CoA} + \text{acceptor} =$ $\text{crotonyl-CoA} + \text{reduced acceptor}$	A flavoprotein; forms with another flavoprotein ("electron-transferring flavoprotein") a system reducing cytochrome c and other acceptors
$\text{L-Glutamate} + \text{H}_2\text{O} + \text{NAD(P)} =$ $2\text{-oxoglutarate} + \text{NH}_3 + \text{reduced NAD(P)}$	A zinc protein
$\text{An L-amino acid} + \text{H}_2\text{O} + \text{O}_2 =$ $\text{a 2-oxo-acid} + \text{NH}_3 + \text{H}_2\text{O}_2$	A flavoprotein. The enzyme from liver and kidney also oxidizes 2-hydroxy-acids; that from snake venom does not

<i>page</i>	<i>number</i>	<i>systematic name</i>	<i>recommended trivial name</i>
	1.5.1	With NAD or NADP as acceptor	
141	(1.5.1.2)	L-Proline:NAD(P) 5-oxidoreductase	Pyrroline-5-carboxylate reductase
312	(1.5.1.3)	5,6,7,8-Tetrahydrofolate:NADP oxidoreductase	Tetrahydrofolate dehydrogenase [Folic acid reductase]
110	(1.5.1.5)	5,10-Methylenetetrahydro- folate:NADP oxidoreductase	Methylenetetrahydrofolate dehydrogenase
	1.6	Oxidoreductases acting on reduced NAD or NADP	
	1.6.1	With NAD or NADP as acceptor	
73	(1.6.1.1)	Reduced NADP:NAD oxidoreductase	NAD(P) transhydrogenase [Pyridine nucleotide transhydrogenase, transhydrogenase]
	1.6.4	With a disulfide compound as acceptor	
40	(1.6.4.3)	Reduced NAD:lipoamide oxidoreductase	Lipoamide dehydrogenase [Diaphorase, lipoyl dehydrogenase]
	1.6.99	With other acceptors	
	(1.6.99.3)	Reduced NAD:(acceptor) oxidoreductase	Reduced NAD dehydrogenase [Cytochrome c reductase]
	1.10	Oxidoreductases acting on diphenols and related substances as donors	
	1.10.3	With oxygen as acceptor	
160	(1.10.3.1)	<i>o</i> -Diphenol:oxygen oxidoreductase	<i>o</i> -Diphenol oxidase [Catechol oxidase, polyphenol oxidase, phenolase, tyrosinase]
	1.11	Oxidoreductases acting on hydrogen peroxide as acceptor	
296	(1.11.1.6)	Hydrogen peroxide:hydrogen peroxide oxidoreductase	Catalase
304			

*reaction* *notes on specificity and other comments*

**L-Proline + NAD(P) =**  
 **$\Delta'$ -pyrroline-5-carboxylate +**  
**reduced NAD(P)**

Also reduces  $\Delta'$ -pyrroline-3-hydroxy-5-carboxylate to L-hydroxyproline

5,6,7,8-Tetrahydrofolate + NADP = Also slowly oxidizes 7,8-dihydrofolate to folate  
7,8-dihydrofolate + reduced NADP

$$5,10\text{-Methylenetetrahydrofolate} + \text{H}^+ + \text{NADP} = \\ 5,10\text{-methenyltetrahydrofolate} + \text{H}_2\text{O} + \text{reduced NADP}$$

Reduced NADP + NAD = NADP + reduced NAD  
Also acts with deaminocoenzymes

Reduced NAD + lipoamide = A flavoprotein  
NAD + dihydrolipoamide

Reduced NAD + acceptor =  
NAD + reduced acceptor

A flavoprotein. After preparations have been subjected to certain treatments, cytochrome c may act as acceptor

*2o*-Diphenol + O<sub>2</sub> = Contains Cu. Acts on various *o*-quinols;  
*2o*-quinone + 2H<sub>2</sub>O monophenols also undergo oxidations in the system

$\text{H}_2\text{O}_2 + \text{H}_2\text{O}_2 = \text{O}_2 + 2\text{H}_2\text{O}$

A hemoprotein. Several organic substances, especially ethanol, can act as hydrogen donor.

<i>page</i>	<i>number</i>	<i>systematic name</i>	<i>recommended trivial name</i>
304	(1.11.1.7)	Donor:hydrogen peroxide oxidoreductase	Peroxidase
	1.12	Oxidoreductases acting on hydrogen as donor	
104	(1.12.1.1)	Hydrogen:ferredoxin oxidoreductase	Hydrogenase
	1.13	Oxidoreductases acting on single donors with incorporation of oxygen (oxygenases)	
159	(1.13.1.5)	Homogentisate:oxygen oxidoreductase	Homogentisate oxygenase [Homogentisicase]
153	(1.13.1.6)	3-Hydroxyanthranilate:oxygen oxidoreductase	3-Hydroxyanthranilate oxygenase
153	(1.13.1.12)	L-Tryptophan:oxygen oxidoreductase	Tryptophan oxygenase [Tryptophan pyrolase]
	1.14	Oxidoreductases acting on paired donors with incorporation of oxygen into one donor (hydroxylases)	
	1.14.1	With reduced NAD or NADP as one donor	
153	(1.14.1.2)	L-Kynurenone, reduced-NADP:oxygen oxidoreductase (3-hydroxylating)	Kynurenone 3-hydroxylase
269	(1.14.1.3)	Squalene, reduced NADP:oxygen oxidoreductase (hydroxylating)	Squalene hydroxylase [Squalene oxydocyclase]
	1.14.2	With ascorbate as one donor	
160	(1.14.2.1)	3,4-Dihydroxyphenylethylamine, ascorbate:oxygen oxidoreductase (hydroxylating)	Dopamine hydroxylase
	1.14.3	With reduced pteridine as one donor	

<i>reaction</i>	<i>notes on specificity and other comments</i>
Donor + H <sub>2</sub> O <sub>2</sub> = oxidized donor + 2H <sub>2</sub> O	A hemoprotein
H <sub>2</sub> + 2 ferredoxin = 2 reduced ferredoxin	Contains Fe. Uses molecular hydrogen for the reduction of a variety of substances
Homogentisate + O <sub>2</sub> = 4-maleylacetoacetate	Needs ferrous ions
3-Hydroxyanthranilate + O <sub>2</sub> = 2-amino-3-carboxymuconate semialdehyde	Needs ferrous ions
L-Tryptophan + O <sub>2</sub> = L-formylkynurenone	A hemoprotein
L-Kynurenone + reduced NADP + O <sub>2</sub> = 3-hydroxy-L-kynurenone + NADP + H <sub>2</sub> O	
Squalene + reduced NADP + O <sub>2</sub> = lanosterol + NADP + H <sub>2</sub> O	
3,4-Dihydroxyphenylethylamine + ascorbate + O <sub>2</sub> = noradrenaline + dehydroascorbate + H <sub>2</sub> O	

<i>page</i>	<i>number</i>	<i>systematic name</i>	<i>recommended trivial name</i>
159	(1.14.3.1)	L-Phenylalanine, tetrahydropteridine: oxygen oxidoreductase (4-hydroxylating)	Phenylalanine 4-hydroxylase [Phenylalaninase]
	2	Transferases	
	2.1	Transferring one-carbon groups	
126	2.1.1	Methyltransferases	
	2.1.2	Hydroxymethyl-, formyl-, and related transferases	
109	(2.1.2.1)	L-Serine : tetrahydrofolate 5,10-hydroxymethyltransferase	Serine hydroxymethyltransferase [Serine aldolase, serine hydroxymethylase]
168	(2.1.2.2)	5'-Phosphoribosyl-N-formyl-glycineamide : tetrahydrofolate 5,10-formyltransferase	Phosphoribosylglycineamide formyltransferase
169	(2.1.2.3)	5'-Phosphoribosyl-5-formamido-4-imidazolecarboxamide : tetrahydrofolate 10-formyltransferase	Phosphoribosyl-amino-imidazole-carboxamide formyltransferase
	2.1.3	Carboxyl- and carbamoyltransferases	
133	(2.1.3.1)	Methylmalonyl-CoA : pyruvate carboxyltransferase	Methylmalonyl-CoA carboxyltransferase
177	(2.1.3.2)	Carbamoylphosphate : L-aspartate carbamoyltransferase	Aspartate carbamoyltransferase
344			
142	(2.1.3.3)	Carbamoylphosphate : L-ornithine carbamoyltransferase	Ornithine carbamoyltransferase
	2.1.4	Amidinotransferases	
144	(2.1.4.1)	L-Arginine : glycine amidinotransferase	Glycine amidinotransferase
	2.2	Transferring aldehyde or ketonic residues	

reaction	notes on specificity and other comments
L-Phenylalanine + tetrahydropteridine + O <sub>2</sub> = L-tyrosine + dihydropteridine + H <sub>2</sub> O	A number of reduced pteridine derivatives can act as donor
L-Serine + tetrahydrofolate = glycine + 5,10-methylenetetrahydrofolate	A pyridoxal-phosphate protein
5'-Phosphoribosyl-N-formylglycineamide + tetrahydrofolate = 5'-phosphoribosylglycineamide + 5,10-methylenetetrahydrofolate + H <sub>2</sub> O	
5'-Phosphoribosyl-5-formamido-4-imidazole-carboxamide + tetrahydrofolate = 5'-phosphoribosyl-5-amino-4-imidazole-carboxamide + 10-formyltetrahydrofolate	
Methylmalonyl-CoA + pyruvate = propionyl-CoA + oxaloacetate	Contains biotin
Carbamoylphosphate + L-aspartate = orthophosphate + N-carbamoyl-L-aspartate	
Carbamoylphosphate + L-ornithine = orthophosphate + L-citrulline	
L-Arginine + glycine = L-ornithine + guanidinoacetate	Canavanine can act instead of arginine

<i>page</i>	<i>number</i>	<i>systematic name</i>	<i>recommended trivial name</i>
71	(2.2.1.1)	Sedoheptulose-7-phosphate: D-glyceraldehyde-3-phosphate glycolaldehydetransferase	Transketolase, glycolaldehyde transferase
71	(2.2.1.2)	Sedoheptulose-7-phosphate: D-glyceraldehyde-3-phosphate dihydroxyacetone transferase	Transaldolase, dihydroxyacetone transferase
	2.3	Acyltransferases	
	2.3.1	Acyltransferases	
328	(2.3.1.3)	Acetyl-CoA : 2-amino-2-deoxy- D-glucose N-acetyltransferase	Glucosamine acetyltransferase [Glucosamine acetylase]
332	(2.3.1.4)	Acetyl-CoA : 2-amino-2-deoxy- D-glucose 6-phosphate N-acetyl- transferase	Glucosamine-phosphate acetyltransferase [Phosphoglucosamine transacetylase]
112	(2.3.1.5)	Acetyl-CoA : arylamine N-acetyltransferase	Arylamine acetyltransferase [Arylamine acetylase]
101	(2.3.1.7)	Acetyl-CoA : choline O-acetyltransferase	Carnitine acetyltransferase
92	(2.3.1.15)	Acyl-CoA : L-glycerol-3- phosphate O-acyltransferase	Glycerolphosphate acyltransferase
282			
93	(2.3.1.16)	Acyl-CoA : acetyl-CoA C-acyltransferase	Acetyl-CoA acyltransferase, 3-ketoacyl-CoA thiolase [ $\beta$ -Ketothiolase]
92	(2.3.1.20)	Acyl-CoA : 1,2-diglyceride O-acyltransferase	Diglyceride acyltransferase
	2.4	Glycosyltransferases	
	2.4.1	Hexosyltransferases	

reaction	notes on specificity and other comments
Sedoheptulose-7-phosphate + D-glyceraldehyde-3-phosphate = D-ribose 5-phosphate + D-xylulose 5-phosphate	Needs thiamine pyrophosphate. Wide specificity for both reactants, e.g., converts hydroxypyruvate and RCHO into CO <sub>2</sub> and RCHOHCOCH <sub>2</sub> OH
Sedoheptulose-7-phosphate + D-glyceraldehyde-3-phosphate = D-erythrose 4-phosphate + D-fructose 6-phosphate	
Acetyl-CoA + 2-amino-2-deoxy-D-glucose = CoA + 2-acetamido-2-deoxy-D-glucose	
Acetyl-CoA + 2-amino-2-deoxy-D-glucose 6-phosphate = CoA + 2-acetamido-2-deoxy-D-glucose 6-phosphate	
Acetyl-CoA + arylamine = CoA + N-acetylarylamine	Wide specificity for aromatic amines, including serotonin; also catalyzes acetyl transfer between arylamines without CoA
Acetyl-CoA + carnitine = CoA + O-acetylcarnitine	
Acyl-CoA + L-glycerol-3-phosphate = CoA + monoglyceride phosphate	Acts only with CoA derivatives of fatty acids of chain length above C <sub>10</sub> . Also forms di-glyceride phosphates
Acyl-CoA + acetyl-CoA = CoA + 3-oxoacyl-CoA	
Acyl-CoA + a 1,2-diglyceride = CoA + a triglyceride	

<i>page</i>	<i>number</i>	<i>systematic name</i>	<i>recommended trivial name</i>
63 241	(2.4.1.1)	$\alpha$ -1,4-Glucan:orthophosphate glucosyltransferase	$\alpha$ -Glucan phosphorylase, glycogen phosphorylase [P-enzyme (only for the plant enzyme); muscle phosphorylase a]
65 342	(2.4.1.11)	UDP glucose:glycogen $\alpha$ -4-glucosyltransferase	UDP glucose-glycogen glucosyltransferase, glycogen-UDP glucosyltransferase
65	(2.4.1.18)	$\alpha$ -1,4-Glucan: $\alpha$ -1,4-glucan 6-glycosyltransferase	$\alpha$ -Glucan-branched glycosyltransferase [Q-enzyme branching factor]
	2.4.2	Pentosyltransferases	
176	(2.4.2.7)	AMP:pyrophosphate phosphoribosyltransferase	Adenine phosphoribosyltransferase, AMP pyrophosphorylase
177	(2.4.2.10)	Orotidine-5'-phosphate:pyrophosphate phosphoribosyltransferase	Orotate phosphoribosyltransferase, orotidine-5'-phosphate pyrophosphorylase [Orotidylate acid phosphorylase]
168	(2.4.2.14)	Ribosylamine 5-phosphate:pyrophosphate phosphoribosyltransferase (glutamate-amidating)	Amidophosphoribosyltransferase, phosphoribosylpyrophosphate amidotransferase
	2.5	Transferases transferring alkyl or related group	
268	(2.5.1.1)	Dimethylallylpyrophosphate:isopentenylpyrophosphate dimethylallyltransferase	Dimethylallyltransferase, phenyltransferase [Farnesyl pyro phosphate synthetase]
314	(2.5.1.3)	2-Methyl-4-amino-5-hydroxymethyl-pyrimidine-pyrophosphate:4-methyl-5-(2'-phosphoethyl)-thiazole 2-methyl-4-aminopyrimidine-5-methenyltransferase	Thiaminephosphate pyrophosphorylase
126	(2.5.1.6)	ATP:L-methionine S-adenosyltransferase	Methionine adenosyltransferase
	2.6	Transferases transferring nitrogenous group	

<i>reaction</i>	<i>notes on specificity and other comments</i>
$(\alpha\text{-}1,4\text{-Glucosyl})_n + \text{orthophosphate} = (\alpha\text{-}1,4\text{-glucosyl})_{n-1} + \alpha\text{-D-glucose 1-phosphate}$	The mammalian enzyme contains pyridoxal phosphate
$\text{UDP glucose} + (\text{glycogen})_n = \text{UDP} + (\text{glycogen})_{n+1}$	Activated by D-glucose 6-phosphate and other hexose phosphates
Transfers part of a 1,4-glucan chain from a 4 to a 6 position	Converts amylose into amylopectin
$\text{AMP} + \text{pyrophosphate} = \text{adenine} + 5\text{-phospho-}\alpha\text{-D-ribosylpyrophosphate}$	5-Amino-4-imidazolecarboxamide can replace adenine
$\text{Orotidine-5'-phosphate} + \text{pyrophosphate} = \text{orotate} + 5\text{-phospho-}\alpha\text{-D-ribosylpyrophosphate}$	
$\beta\text{-D-Ribosylamine 5-phosphate} + \text{pyrophosphate} + \text{L-glutamate} = \text{L-glutamine} + 5\text{-phospho-}\alpha\text{-D-ribosylpyrophosphate} + \text{H}_2\text{O}$	
$\text{Dimethylallylpyrophosphate} + \text{isopentenyl pyrophosphate} = \text{pyrophosphate} + \text{geranyl pyrophosphate}$	Also transfers geranyl and farnesyl residues
$2\text{-Methyl-4-amino-5-hydroxymethyl-pyrimidine-pyrophosphate} + 4\text{-methyl-5-(2'-phosphoethyl)-thiazole} = \text{pyrophosphate} + \text{thiamine monophosphate}$	
$\text{ATP} + \text{L-methionine} + \text{H}_2\text{O} = \text{orthophosphate} + \text{pyrophosphate} + \text{S-adenosylmethionine}$	

<i>page</i>	<i>number</i>	<i>systematic name</i>	<i>recommended trivial name</i>
	2.6.1	Aminotransferases	
113	(2.6.1.1)	L-Aspartate : 2-oxoglutarate aminotransferase	Aspartate aminotransferase [Glutamic-oxaloacetic transaminase, glutamic-aspartic transaminase]
113	(2.6.1.2)	L-Alanine : 2-oxoglutarate aminotransferase	Alanine aminotransferase [Glutamic-pyruvic transaminase, glutamic-alanine transaminase]
148	(2.6.1.9)	L-Histidinolphosphate : 2-oxo-glutarate aminotransferase	Histidinolphosphate aminotransferase
112	(2.6.1.12)	L-Alanine : 2-oxoacid aminotransferase	Alanine-ketoacid aminotransferase
141	(2.6.1.13)	L-Ornithine : 2-oxoacid aminotransferase	Ornithine-ketoacid aminotransferase
	2.7	Transferases transferring phosphorus-containing groups	
	2.7.1	Phosphotransferases with an alcohol group as acceptor	
54	(2.7.1.1)	ATP : D-hexose 6-phosphotransferase	Hexokinase
342			
332	(2.7.1.8)	ATP : 2-amino-2-deoxy-D-glucose phosphotransferase	Glucosamine kinase
56	(2.7.1.11)	ATP : D-fructose-6-phosphate 1-phosphotransferase	Phosphofructokinase [Phosphohexokinase]
342			
81	(2.7.1.19)	ATP : D-ribulose-5-phosphate 1-phosphotransferase	Phosphoribulokinase [Phosphopentokinase]
176	(2.7.1.20)	ATP : adenosine 5'-phosphotransferase	Adenosine kinase
316	(2.7.1.24)	ATP : dephospho-CoA 3'-phosphotransferase	Dephospho-CoA kinase

L-Aspartate + 2-oxoglutarate =  
oxaloacetate + L-glutamate

A pyridoxal-phosphate protein

L-Alanine + 2-oxoglutarate =  
pyruvate + L-glutamate

A pyridoxal-phosphate protein. 2-Aminobutyrate acts slowly instead of alanine

L-Histidinol phosphate + 2-oxoglutarate =  
imidazolacetyl phosphate + L-glutamate

A pyridoxal-phosphate protein

L-Alanine + a 2-oxoacid =  
pyruvate + an L-amino acid

A pyridoxal-phosphate protein

L-Ornithine + a 2-oxoacid =  
L-glutamate  $\gamma$ -semialdehyde +  
an L-amino acid

A pyridoxal-phosphate protein

ATP + D-hexose =  
ADP + D-hexose 6-phosphate

D-Glucose, D-mannose, D-fructose, and D-glucosamine can act as acceptor; ITP and deoxy-ATP can act as donor

ATP + 2-amino-2-deoxy-D-glucose =  
ADP + 2-amino-2-deoxy-D-glucose  
phosphate

D-Tagatose 6-phosphate can act as acceptor;  
UTP, CTP, and ITP can act as donor

ATP + D-ribulose-5-phosphate =  
ADP + D-ribulose 1,5-diphosphate

ATP + adenosine =  
ADP + AMP

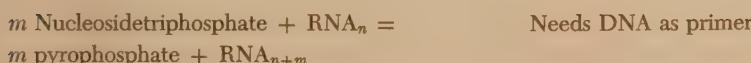
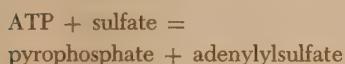
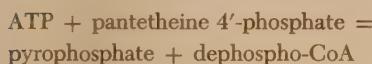
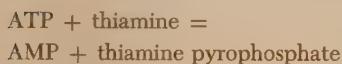
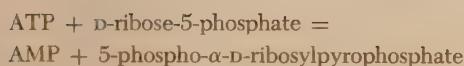
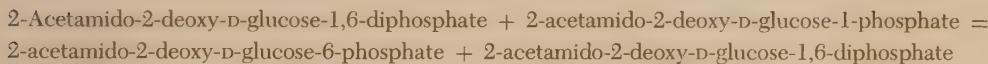
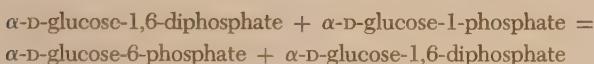
2-Aminoadenosine can also act as acceptor

ATP + dephospho-CoA =  
ADP + CoA

<i>page</i>	<i>number</i>	<i>systematic name</i>	<i>recommended trivial name</i>
335	(2.7.1.25)	ATP:adenylylsulfate 3'-phosphotransferase	Adenylylsulfate kinase [APS kinase]
310	(2.7.1.26)	ATP:riboflavin 5'-phosphotransferase	Riboflavin kinase [Flavokinase]
282	(2.7.1.32)	ATP:choline phospho- transferase	Choline kinase
316	(2.7.1.34)	ATP:pantetheine 4'-phosphotransferase	Pantetheine kinase
268	(2.7.1.36)	ATP:mevalonate 5-phosphotransferase	Mevalonate kinase
118	(2.7.1.39)	ATP:L-homoserine O-phosphotransferase	Homoserine kinase
60	(2.7.1.40)	ATP:pyruvate phospho- transferase	Pyruvate kinase [Phosphoenolpyruvate kinase]
	2.7.2	Phosphotransferases with a carboxyl group as acceptor	
59	(2.7.2.3)	ATP:3-phospho-D-glycerate 1-phosphotransferase	Phosphoglycerate kinase
118	(2.7.2.4)	ATP:L-aspartate 4-phosphotransferase	Aspartate kinase
344			
142	(2.7.2.5)	ATP:carbamate phosphotransferase (dephosphorylating)	Carbamoylphosphate synthase
	2.7.4	Phosphotransferases with a phospho group as acceptor	
268	(2.7.4.2)	ATP:5-phosphomevalonate phosphotransferase	Phosphomevalonate kinase
	2.7.5	Phosphotransferases with regeneration of donors (intramolecular transfers)	

reaction	notes on specificity and other comments
ATP + adenylylsulfate = ADP + 3'-phospho-adenylylsulfate	
ATP + riboflavin = ADP + FMN	
ATP + choline = ADP + phosphocholine	Ethanolamine and its methyl and ethyl derivatives can also act as acceptor
ATP + pantetheine = ADP + pantetheine 4'-phosphate	
ATP + mevalonate = ADP + 5-phosphomevalonate	GTP, CTP, or UTP can also act as donor
ATP + L-homoserine = ADP + O-phospho-L-homoserine	
ATP + pyruvate = ADP + phosphoenolpyruvate	UTP, GTP, CTP, ITP, and d-ATP can also act as donor. Also phosphorylates hydroxylamine and fluoride in the presence of CO <sub>2</sub>
ATP + 3-phospho-D-glycerate = ADP + 1,3-diphospho-D-glyceric acid	
ATP + L-aspartate = ADP + 4-phospho-L-aspartate	
2ATP + NH <sub>3</sub> + CO <sub>2</sub> + H <sub>2</sub> O = 2ADP + phosphate + carbamoylphosphate	May be a system
ATP + 5-phosphomevalonate = ADP + 5-pyrophosphomevalonate	

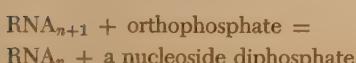
<i>page</i>	<i>number</i>	<i>systematic name</i>	<i>recommended trivial name</i>
63	(2.7.5.1)	$\alpha$ -D-Glucose-1,6-diphosphate : $\alpha$ -D-glucose-1-phosphate phosphotransferase	Phosphoglucomutase, glucose phosphomutase
328	(2.7.5.2)	2-Acetamido-2-deoxy-D- glucose-1,6-diphosphate : 2-acetamido-2-deoxy-D- glucose-1-phosphate phospho- transferase	Acetylglucosamine phosphomutase [Phosphoacetylglucosamine mutase]
	2.7.6	Pyrophosphotransferases	
168	(2.7.6.1)	ATP:D-ribose-5-phosphate pyrophosphotransferase	Ribosephosphate pyrophosphokinase
314	(2.7.6.2)	ATP:thiamine pyrophosphotransferase	Thiamine pyrophosphokinase [Thiamine kinase]
	2.7.7	Nucleotidyltransferases	
310	(2.7.7.2)	ATP:FMN adenylyl- transferase	FMN adenylyltransferase, FAD pyro- phosphorylase
316	(2.7.7.3)	ATP:pantetheine-4'-phos- phate adenylyltransferase	Pantetheinephosphate adenylyltransfer- ase, dephospho-CoA pyrophosphorylase
335	(2.7.7.4)	ATP:sulfate adenylyl- transferase	Sulfate adenylyltransferase [ATP sulfurylase, sulfurylase]
196	(2.7.7.6)	Nucleosidetriphosphate : RNA nucleotidyltransferase	RNA nucleotidyltransferase [RNA polymerase]
190	(2.7.7.7)	Deoxynucleosidetriphosphate : DNA deoxynucleotidyl- transferase	DNA nucleotidyltransferase [DNA polymerase]
198	(2.7.7.8)	Polyribonucleotide :ortho- phosphate nucleotidyltransferase	Polyribonucleotide nucleotidyltransfer- ase, polynucleotide phosphorylase
65	(2.7.7.9)	UTP : $\alpha$ -D-glucose-1-phosphate uridyltransferase	Glucose-1-phosphate uridyltransferase, UDPG pyrophosphorylase

*reaction**notes on specificity and other comments*

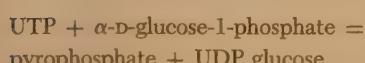
Needs DNA as primer



A DNA chain acts as a primer, and the enzyme forms a complementary chain



ADP, IDP, GDP, UDP, and CDP can act as donor



<i>page</i>	<i>number</i>	<i>systematic name</i>	<i>recommended trivial name</i>
282	(2.7.7.14)	CTP:ethanolaminephosphate cytidyltransferase	Ethanolaminephosphate cytidyltransferase [Phosphorylethanolamine transferase]
282	(2.7.7.15)	CTP:cholinephosphate cytidyltransferase	Cholinephosphate cytidyltransferase [Phosphorylcholine transferase]
324	(2.7.7.16)	Ribonucleate pyrimidine-nucleotido-2'-transferase (cyclizing)	Ribonuclease [RNAase I, RNase]
199	(2.7.7.17)	Ribonucleate nucleotido-2'-transferase (cyclizing)	Ribonuclease
226			
249			
328	(2.7.7.23)	UTP:2-acetamido-2-deoxy- $\alpha$ -D-glucose-1-phosphate uridyltransferase	UDP glucosamine pyrophosphorylase
	2.7.8	Transferase for other substituted phospho groups	
283	(2.7.8.1)	CDP ethanolamine:1,2-diglyceride ethanolamine-phosphotransferase	Ethanolaminephosphotransferase
283	(2.7.8.2)	CDP choline:1,2-diglyceride cholinephosphotransferase	Cholinephosphotransferase [Phosphorylcholineglyceride transferase]
284	(2.7.8.3)	CDP choline:ceramide cholinephosphotransferase	Ceramide cholinephosphotransferase
	2.8	Transferases transferring sulfur-containing groups	
	2.8.2	Sulphotransferases	
335	(2.8.2.5)	3'-Phosphoadenylsulfate:chondroitin sulphotransferase	Chondroitin sulphotransferase

reaction	notes on specificity and other comments
CTP + ethanolaminephosphate = pyrophosphate + CDP ethanolamine	
CTP + cholinephosphate = pyrophosphate + CDP choline	
Transfers the 3' phosphate of a pyrimidine nucleotide residue of a polynucleotide from the 5' position of the adjoining nucleotide to the 2' position of the pyrimidine nucleotide itself, forming a cyclic nucleotide	Also catalyzes the transfer of the phosphate group from the 2' position in the cyclic phosphate to water; the overall reaction brings about the depolymerization of RNA
Acts on polyribonucleotides similarly to 2.7.7.16, but transfers purine nucleotide residues as well as pyrimidine nucleotide residues	Also catalyzes the transfer of the phosphate group from the 2' position in the cyclic phosphate to water; the overall reaction brings about the depolymerization of RNA
UTP + 2-acetamido-2-deoxy-D-glucose-1-phosphate = pyrophosphate + UDP-2-acetamido-2-deoxy-D-glucose	
CDP ethanolamine + 1,2-diglyceride = CMP + a phosphatidylethanolamine	
CDP choline + 1,2-diglyceride = CMP + a phosphatidylcholine	
CDP choline + ceramide = CMP + sphingomyelin	
3'-Phosphoadenylylsulfate + chondroitin = adenosine 3',5'-diphosphate + chondroitin 4-sulfate	Oligo- and polysaccharides containing 2-acetyl-D-galactosamine can act as acceptor

<i>page</i>	<i>number</i>	<i>systematic name</i>	<i>recommended trivial name</i>
	3	Hydrolases	
	3.1	Acting on ester bonds	
	3.1.1	Carboxylic ester hydrolases	
319	(3.1.1.18)	D (or L)-Gulono- $\gamma$ -lactone hydrolase	Aldonolactonase
319	(3.1.1.19)	D-Glucurono- $\delta$ -lactone hydrolase	Uronolactonase
320			
	3.1.2	Thioester hydrolases	
133	(3.1.2.4)	3-Hydroxyisobutyryl-CoA hydrolase	
	3.1.3	Phosphoric monoester hydrolases	
314	(3.1.3.1)	Orthophosphoric monoester phosphohydrolase	Alkaline phosphatase [Alkaline phosphomonoesterase]
324	(3.1.3.2)	Orthophosphoric monoester phosphohydrolase	Acid phosphatase [Acid phosphomonoesterase]
92	(3.1.3.4)	L- $\alpha$ -Phosphatidate phosphohydrolase	Phosphatidate phosphatase
283			
	(3.1.3.5)	5'-Ribonucleotide phosphohydrolase	5'-Nucleotidase
192	(3.1.3.6)	3'-Ribonucleotide phosphohydrolase	3'-Nucleotidase
66	(3.1.3.9)	D-Glucose-6-phosphate phosphohydrolase	Glucose-6-phosphatase
148	(3.1.3.15)	L-Histidinolphosphate phosphohydrolase	Histidinolphosphatase
192	(3.1.4.1)	Orthophosphoric diester phosphohydrolase	Phosphodiesterase

## reaction

## notes on specificity and other comments

D (or L)-Gulono- $\gamma$ -lactone + H<sub>2</sub>O =  
gulonate

D-Glucurono- $\delta$ -lactone + H<sub>2</sub>O =  
D-glucuronate

3-Hydroxyisobutyryl-CoA + H<sub>2</sub>O =  
CoA + 3-hydroxyisobutyrate

Also hydrolyzes 3-hydroxypropionyl-CoA

An orthophosphoric monoester + H<sub>2</sub>O =  
an alcohol + orthophosphate

Wide specificity; also catalyzes transphosphorylations

An orthophosphoric monoester + H<sub>2</sub>O =  
an alcohol + orthophosphate

Wide specificity; also catalyzes transphosphorylations

An L- $\alpha$ -phosphatidate + H<sub>2</sub>O =  
a D-2,3 (or L-1,2)-diglyceride + orthophosphate

A 5'-ribonucleotide + H<sub>2</sub>O =  
a ribonucleoside + orthophosphate

Wide specificity for 5'-nucleotides

A 3'-ribonucleotide + H<sub>2</sub>O =  
a ribonucleoside + orthophosphate

Wide specificity for 3'-nucleotides

D-Glucose-6-phosphate + H<sub>2</sub>O = D-glucose  
+ orthophosphate

Also acts on D-glucosamine-6-phosphate

L-Histidinol phosphate + H<sub>2</sub>O =  
L-histidinol + orthophosphate

A phosphoric diester + H<sub>2</sub>O =  
a phosphoric monoester + an alcohol

Wide specificity, varying with source; the spleen enzyme forms 3'-nucleotides, and the venom enzyme 5'-nucleotides from polynucleotides

<i>page</i>	<i>number</i>	<i>systematic name</i>	<i>recommended trivial name</i>
192	(3.1.4.7)	Ribonuclease (deoxyribonuclease) 3'-nucleotidohydrolase	Micrococcal nuclease
	3.2	Hydrolases acting on glycosyl compounds	
	3.2.1	Glycoside hydrolases	
84	(3.2.1.1)	$\alpha$ -1,4-Glucan 4-glucano- hydrolase	$\alpha$ -Amylase
84	(3.2.1.2)	$\alpha$ -1,4-Glucan maltohydrolase	$\beta$ -Amylase
229	(3.2.1.17)	Mucopeptide	Mucopeptide glucohydrolase, lysozyme
249		<i>N</i> -acetylmuramylhydrolase	[Muramidase]
323			
84	(3.2.1.20)	$\alpha$ -D-Glucoside glucohydrolase	$\alpha$ -Glucosidase [Maltase]
208	(3.2.1.23)	$\beta$ -D-Galactoside galactohydrolase	$\beta$ -Galactosidase [Lactase]
324	(3.2.1.31)	$\beta$ -D-Glucuronide glucuronohydrolase	$\beta$ -Glucuronidase
66	(3.2.1.33)	Dextrin 6-glucanohydrolase	Dextrin-1,6-glucosidase [Amylo-1,6-glucosidase]
	3.3	Hydrolases acting on ether bonds	
	3.3.1	Thioether hydrolases	
126	(3.3.1.1)	S-Adenosyl-L-homocysteine hydrolase	Adenosylhomocysteinase
	3.4	Hydrolases acting on peptide bonds	

reaction	notes on specificity and other comments
Attacks RNA and DNA, forming 3'-nucleotides; DNA is attacked with preference for the adenine-thymine nucleotide pair.	
Hydrolyzes $\alpha$ -1,4-glucan links in polysaccharides containing three or more $\alpha$ -1,4-linked D-glucose units	Acts on starch, glycogen, and related polysaccharides and oligosaccharides in a random manner
Hydrolyzes $\alpha$ -1,4-glucan links in polysaccharides so as to remove successive maltose units from the nonreducing ends of the chain	Acts on starch, glycogen, and related polysaccharides and oligosaccharides, producing $\beta$ -maltose by an inversion
Probably hydrolyzes $\beta$ -1,4-links between N-acetylmuramic acid and 2-acetamido-2-deoxy-D-glucose residues in a mucopolysaccharide or mucopeptide	Dissolves the cell wall substance of certain bacteria; also acts slowly on chitin
An $\alpha$ -D-glucoside + H <sub>2</sub> O = an alcohol + D-glucose	Wide specificity for $\alpha$ -D-glucopyranosides, varying with source; also catalyzes glucotransferase reactions
A $\beta$ -D-galactoside + H <sub>2</sub> O = an alcohol + D-galactose	Also catalyzes galactotransferase reactions
A $\beta$ -D-glucuronide + H <sub>2</sub> O = an alcohol + D-glucuronate	Also catalyzes glucuronotransferase reactions
Hydrolyzes $\alpha$ -1,6-links in dextrans containing short 1,6-linked side chains	
S-Adenosyl-L-homocysteine + H <sub>2</sub> O = adenosine + L-homocysteine	

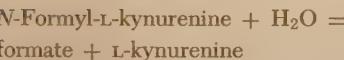
<i>page</i>	<i>number</i>	<i>systematic name</i>	<i>recommended trivial name</i>
	3.4.4	Peptidyl peptide hydrolases	
15 244	(3.4.4.5)		Chymotrypsin A
	3.5	Hydrolases acting on C—N bonds, other than peptide bonds	
	3.5.1	In linear amides	
139	(3.5.1.2)	L-Glutamine amidohydrolase	Glutaminase
199 249	(3.5.1.5)	Urea amidohydrolase	Urease
153	(3.5.1.9)	Aryl-formylamine amidohydrolase	Formamidase [Kynurenine formamidase, formylase]
110 312	(3.5.1.10)	10-Formyltetrahydrofolate amidohydrolase	Formyltetrahydrofolate deformylase
	3.5.2	In cyclic amides	
177	(3.5.2.3)	L-4,5-Dihydro-orotate amidohydrolase	Dihydro-orotase [Carbamyl-aspartic dehydrase]
	3.5.3	In linear amidines	
143	(3.5.3.1)	L-Arginine amidinohydrolase	Arginase
	3.5.4	In cyclic amidines	
110	(3.5.4.9)	5,10-Methenyltetrahydrofolate-5-hydrolase (decyclizing)	Methenyltetrahydrofolate cyclohydrolase
170	(3.5.4.10)	IMP 1,2-hydrolase (decyclizing)	IMP cyclohydrolase
179	(3.5.4.12)	dCMP aminohydrolase	dCMP deaminase
	3.7	Hydrolases acting on C—C bonds	
	3.7.1	In ketonic substances	

## reaction

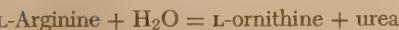
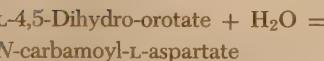
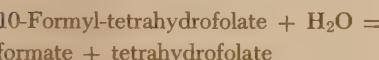
## notes on specificity and other comments

Hydrolyzes peptides, amides, esters, etc., especially at bonds involving the carboxyl groups of aromatic L-amino acids

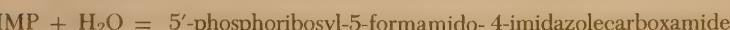
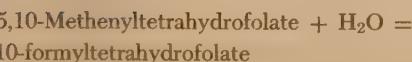
Formed from chymotrypsinogen; a number of chymotrypsins are formed, according to the number of bonds hydrolyzed in the precursor



Also acts on other aromatic formylamines



Also hydrolyzes  $\alpha$ -N-substituted L-arginines and canavanine



Also acts on some 5-substituted dCMPs

<i>page</i>	<i>number</i>	<i>systematic name</i>	<i>recommended trivial name</i>
159	(3.7.1.2)	4-Fumarylacetate fumarylhydrolase	Fumarylacetate [ $\beta$ -Diketonase]
153	(3.7.1.3)	L-Kynurenine hydrolase	Kynureninase
4		Lyases	
	4.1	Carbon-carbon lyases	
	4.1.1	Carboxy-lyases	
138	(4.1.1.15)	L-Glutamate 1-carboxy-lyase	Glutamate decarboxylase
169	(4.1.1.21)	5'-Phosphoribosyl-5-amino-4-imidazolecarboxylate carboxy-lyase	Phosphoribosyl-aminoimidazole carboxylase
148	(4.1.1.22)	L-Histidine carboxylase	
178	(4.1.1.23)	Orotidine-5'-phosphate carboxy-lyase	Orotidine-5'-phosphate decarboxylase
160	(4.1.1.26)	3,4-Dihydroxy-L-phenylalanine carboxy-lyase	DOPA decarboxylase
316	(4.1.1.30)	N-(L-Pantothenoyl) L-cysteine carboxy-lyase	Pantothenoylcysteine decarboxylase
268	(4.1.1.33)	ATP:5-pyrophosphomevalonate carboxy-lyase (dehydrating)	Pyrophosphomevalonate decarboxylase
80	(4.1.1.39)	3-Phospho-D-glycerate carboxy-lyase (dimerizing)	Ribulosediphosphate carboxylase [Carboxydismutase]
	4.1.2	Aldehyde-lyases	

<i>reaction</i>	<i>notes on specificity and other comments</i>
4-Fumarylacetoacetate + H <sub>2</sub> O = acetoacetate + fumarate	Also acts on other 3,5- and 2,4-diketoacids
L-Kynurenine + H <sub>2</sub> O = anthranilate + L-alanine	A pyridoxal-phosphate protein
L-Glutamate = 4-aminobutyrate + CO <sub>2</sub>	A pyridoxal-phosphate protein. The brain enzyme also acts on L-cysteate and L-cysteine sulfinate
5'-phosphoribosyl-5-amino-4-imidazole-carboxylate = 5'-phosphoribosyl-5-aminoimidazole + CO <sub>2</sub>	
L-Histidine = histamine + CO <sub>2</sub>	A pyridoxal-phosphate protein
Orotidine-5'-phosphate = UMP + CO <sub>2</sub>	
3,4-Dihydroxy-L-phenylalanine = dihydroxyphenylethylamine + CO <sub>2</sub>	A pyridoxal-phosphate protein. Also acts on 2-(or 3-)hydroxyphenylalanine and 3-hydroxyphenylserine
N(L-Pantothenoyl)-L-cysteine = pantetheine + CO <sub>2</sub>	
ATP + 5-pyrophosphomevalonate = ADP + orthophosphate + isopentenyl pyrophosphate + CO <sub>2</sub>	
2,3-Phospho-D-glycerate = D-ribulose 1,5-diphosphate + CO <sub>2</sub>	

<i>page</i>	<i>number</i>	<i>systematic name</i>	<i>recommended trivial name</i>
118	(4.1.2.5)	$\text{L}$ -Threonine acetaldehyde-lyase	Threonine aldolase
57	(4.1.2.7)	Ketose-1-phosphate aldehyde-lyase	Ketose-1-phosphate aldolase [Aldolase]
249			
14	(4.1.2.13)	Fructose 1,6-diphosphate- $\text{D}$ -glyceraldehyde-3-phosphate-lyase	Fructosediphosphate aldolase [Zymohexase, aldolase]
57			
249			
	4.1.3	Keto acid lyases	
112	(4.1.3.1)	<i>threo-D</i> <sub>S</sub> -Isocitrate glyoxylate-lyase	Isocitrate lyase [Isocitrase, isocitritase, isocitratase]
112	(4.1.3.2)	$\text{L}$ -Malate glyoxylate-lyase (CoA-acetylating)	Malate synthase [Malate-condensing enzyme, glyoxylate transacetase]
330	(4.1.3.3)	<i>N</i> -Acetylneuraminic acid pyruvate-lyase	<i>N</i> -Acetylneuraminate lyase [ <i>N</i> -Acetylneuraminic acid aldolase]
45	(4.1.3.7)	Citrate oxaloacetate-lyase (CoA-acetylating)	Citrate synthase [Condensing enzyme, citrate-condensing enzyme, citrogenase, oxaloacetate transacetase]
	4.2	Carbon-oxygen lyases	
	4.2.1	Hydro-lyases	
40	(4.2.1.2)	$\text{L}$ -Malate hydro-lyase	Fumarate hydratase [Fumarase]
44			
40	(4.2.1.3)	Citrate (isocitrate) hydro-lyase	Aconitate hydratase [Aconitase]
156	(4.2.1.10)	5-Dehydroquinate hydro-lyase	5-Dehydroquinate dehydratase
115	(4.2.1.13)	$\text{L}$ -Serine hydro-lyase (deaminating)	$\text{L}$ -Serine dehydratase [Serine deaminase, cystathionine synthetase]

<i>reaction</i>	<i>notes on specificity and other comments</i>
L-Threonine = glycine + acetaldehyde	A pyridoxal-phosphate protein
A ketose-1-phosphate = dihydroxyacetone phosphate + an aldehyde	Wide specificity
Fructose-1,6-diphosphate = dihydroxyacetone phosphate + D-glyceraldehyde 3-phosphate	Also acts on ketose monophosphates
<i>threo-Ds</i> -Isocitrate = succinate + glyoxylate	
L-Malate + CoA = acetyl-CoA + H <sub>2</sub> O + glyoxylate	
N-Acetylneuraminate = 2-acetamido-2-deoxy-D-mannose + pyruvate	Also acts on N-glycolyl-neuraminate
Citrate + CoA = acetyl-CoA + H <sub>2</sub> O + oxaloacetate	
L-Malate = fumarate + H <sub>2</sub> O	
Citrate = <i>cis</i> -aconitate + H <sub>2</sub> O	Also converts isocitrate into <i>cis</i> -aconitate
5-Dehydroquinate = 5-dehydroshikimate + H <sub>2</sub> O	
L-Serine + H <sub>2</sub> O = pyruvate + NH <sub>3</sub> + H <sub>2</sub> O	A pyridoxal-phosphate protein. Also forms cystathionine from L-serine and L-homocysteine

<i>page</i>	<i>number</i>	<i>systematic name</i>	<i>recommended trivial name</i>
118	(4.2.1.16)	L-Threonine hydro-lyase (deaminating)	Threonine dehydratase [Threonine deaminase]
93	(4.2.1.17)	L-3-Hydroxyacyl-CoA hydro-lyase	Enoyl-CoA hydratase [Crotonase, enoyl hydrase]
148	(4.2.1.19)	D- <i>erythro</i> - Imidazoleglycerolphosphate hydro-lyase	Imidazoleglycerol- phosphate dehydratase
150	(4.2.1.20)	L-Serine hydro-lyase	Tryptophan synthase
246		(adding indole)	
288	(4.2.1.24)	5-Aminolevulinate hydro- lyase (adding 5-amino- levulinate and cyclizing)	Porphobilinogen synthase, amino- levulinate dehydratase
	4.2.99	Other carbon-oxygen lyases	
118	(4.2.99.2)	O-Phosphohomoserine phospho-lyase (adding water)	Threonine synthase
	4.3	Carbon-nitrogen lyases	
	4.3.2	Amidine lyases	
175	(4.3.2.2)	Adenylosuccinate AMP-lyase	Adenylosuccinate lyase [Adenylosuccinase]
	5	Isomerases	
	5.1	Racemases and epimerases	
	5.1.3	Acting on carbohydrates and derivatives	
71	(5.1.3.1)	D-Ribulose 5-phosphate 3-epimerase	Ribulosephos- phate 3-epimerase [Phosphoribulose epimerase]

reaction	notes on specificity and other comments
L-Threonine + H <sub>2</sub> O = 2-oxobutyrate + NH <sub>3</sub> + H <sub>2</sub> O	A pyridoxal-phosphate protein
An L-3-hydroxyacyl-CoA = a 2,3-(or 3,4)- <i>trans</i> -enoyl-CoA + H <sub>2</sub> O	Also acts (in the reverse reaction) on the cis compounds
D- <i>erythro</i> -Imidazoleglycerol phosphate = imidazoleacetol phosphate + H <sub>2</sub> O	
L-Serine + indole = L-tryptophan + H <sub>2</sub> O	A pyridoxal-phosphate protein. Glyceraldehyde phosphate can act instead of serine, and indoleglycerol phosphate instead of indole
2,5-Aminolevulinate = porphobilinogen + 2H <sub>2</sub> O	
O-Phosphohomoserine + H <sub>2</sub> O = threonine + phosphate	A pyridoxal-phosphate protein
Adenylosuccinate = fumarate + AMP	Also acts on 5'-phosphoribosyl- 4-(N-succinocarboxamide)- 5-aminoimidazole
D-Ribulose 5-phosphate = D-xylulose 5-phosphate	

<i>page</i>	<i>number</i>	<i>systematic name</i>	<i>recommended trivial name</i>
319	(5.1.3.2)	UDP glucose 4-epimerase	UDP glucose epimerase [Galactowaldenase]
336			
328	(5.1.3.7)	UDP-2-acetamido-2-deoxy-D-glucose-4-epimerase	UDP acetylglucosamine epimerase
	5.2	<i>Cis-trans</i> isomerases	
159	(5.2.1.2)	4-Maleylacetoacetate <i>cis-trans</i> -isomerase	Maleylacetoacetate isomerase
292	(5.2.1.3)	all- <i>trans</i> -Retinene 11- <i>cis-trans</i> -isomerase	Retinene isomerase
	5.3	Intramolecular oxidoreductases	
	5.3.1	Interconverting aldoses and ketoses	
58	(5.3.1.1)	D-Glyceraldehyde-3-phosphate ketol-isomerase	Triosephosphate isomerase
71	(5.3.1.6)	D-Ribose-5-phosphate ketol-isomerase	Ribosephosphate isomerase [Phosphopentose isomerase, phosphoribo isomerase]
56	(5.3.1.9)	D-Glucose-6-phosphate ketol-isomerase	Glucosephosphate isomerase [Phosphohexose isomerase, oxoisomerase, hexosephosphate isomerase]
	5.3.3	Transposing C=C bonds	
268	(5.3.3.2)	Isopentenylpyrophosphate $\Delta^3$ - $\Delta^2$ -isomerase	Isopentenylpyrophosphate isomerase
	5.4	Intramolecular transferases	
	5.4.2	Transferring phosphoryl groups	
60	(5.4.2.1)	D-Phosphoglycerate 2,3-phosphomutase	Phosphoglycerate phosphomutase

reaction	notes on specificity and other comments
UDP glucose = UDP galactose	NAD acts as cofactor
UDP-2-acetamido-2-deoxy-D-glucose = UDP-2-acetamido-2-deoxy-D-galactose	
4-Maleylacetoacetate = 4-fumarylacetoacetate	
all-trans-Retinene = 11-cis-retinene	Light shifts the equilibrium toward the cis isomer
D-Glyceraldehyde 3-phosphate = dihydroxyacetone phosphate	
D-Ribose-5-phosphate = D-ribulose-5-phosphate	Also acts on D-ribose-5-pyrophosphate and D-ribose-5-triphosphate
D-Glucose-6-phosphate = D-fructose-6-phosphate	
Dimethylallyl pyrophosphate = isopentenyl pyrophosphate	
2-Phospho-D-glycerate = 3-phospho-D-glycerate	

<i>page</i>	<i>number</i>	<i>systematic name</i>	<i>recommended trivial name</i>
	5.4.99	Transferring other groups	
133	(5.4.99.2)	Methylmalonyl-CoA CoA-carboxylmutase	Methylmalonyl-CoA mutase
	6	Ligases	
	6.3	Forming C—N bonds	
	6.3.1	Acid-ammonia ligases	
139	(6.3.1.2)	L-Glutamate:ammonia ligase (ADP)	Glutamine synthetase
168	(6.3.1.3)	Ribosylamine 5-phosphate: glycine ligase (ADP)	phosphoribosyl glycineamide synthetase [Glycineamide ribonucleotide synthetase]
	6.3.2	Acid-amino acid ligases	
315	(6.3.2.1)	L-Pantoate:β-alanine ligase (AMP)	Pantothenate synthetase [Pantoate activating enzyme]
169	(6.3.2.6)	5'-Phosphoribosyl-4-carboxy- 5-aminoimidazole:L- aspartate ligase (ADP)	Phosphoribosyl aminoimidazole- succinocarboxamide synthetase
	6.3.3	Cyclo-ligases	
169	(6.3.3.1)	5'-Phosphoribosylformyl- glycineamidine cyclo- ligase (ADP)	Phosphoribosyl aminoimidazole synthetase
	6.3.4	Other C—N ligases	
178	(6.3.4.2)	UTP:ammonia ligase (ADP)	CTP synthetase
312	(6.3.4.3)	Formate:tetrahydrofolate ligase (ADP)	Formyltetrahydrofolate synthetase
175	(6.3.4.4)	IMP:L-aspartate ligase (GDP)	Adenylosuccinate synthetase

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*reaction*      *notes on specificity and other comments*

Methylmalonyl-CoA =  
succinyl-CoA

ATP + L-glutamate + NH<sub>3</sub> =  
ADP + orthophosphate + L-glutamine

ATP + glycine + ribosylamine 5-phosphate =  
ADP + orthophosphate + ribosylglycineamide  
5-phosphate

ATP + L-pantoate + β-alanine =  
AMP + pyrophosphate + L-pantothenate

ATP + 5'-phosphoribosyl-4-carboxy-  
5-aminoimidazole + L-aspartate =  
ADP + orthophosphate + 5'-phosphoribosyl-  
4-(N-succinocarboxamide)-5-aminoimidazole

ATP + 5'-phosphoribosyl-formylglycineamidine =  
ADP + orthophosphate + 5'-phospho-  
ribosyl-5-aminoimidazole

ATP + UTP + NH<sub>3</sub> = ADP + orthophosphate + CTP

ATP + formate + tetrahydrofolate =  
ADP + orthophosphate + 10-formyltetrahydrofolate

GTP + IMP + L-aspartate =  
GDP + orthophosphate + adenylosuccinate

<i>page</i>	<i>number</i>	<i>systematic name</i>	<i>recommended trivial name</i>
143	(6.3.4.5)	L-citrulline:L-aspartate ligase (AMP)	Argininosuccinate ligase
	6.3.5	C—N ligases with glutamine as amido-N-donor	
155	(6.3.5.1)	Deamido-NAD:L-glutamine amido-ligase (AMP)	NAD synthetase
176	(6.3.5.2)	Xanthosine 5'-phosphate:L-glutamine amido-ligase (AMP)	GMP synthetase
168	(6.3.5.3)	5'-Phosphoribosyl-formylglycineamide: L-glutamine amido-ligase (ADP)	Phosphoribosyl-formylglycineamidine synthetase
	6.4	Forming C—C bonds	
50	(6.4.1.1)	Pyruvate:carbon dioxide ligase (ADP)	Pyruvate carboxylase
241			
90	(6.4.1.2)	Acetyl-CoA:carbon dioxide ligase (ADP)	Acetyl-CoA carboxylase
135	(6.4.1.3)	Propionyl-CoA:carbon dioxide ligase (ADP)	Propionyl-CoA carboxylase
134	(6.4.1.4)	3-Methylcrotonoyl-CoA:carbon dioxide ligase (ADP)	Methylcrotonoyl-CoA carboxylase

reaction	notes on specificity and other comments
ATP + L-citrulline + L-aspartate = AMP + pyrophosphate + L-argininosuccinate	
ATP + deamido-NAD + L-glutamine + H <sub>2</sub> O = AMP + pyrophosphate + NAD + L-glutamate	NH <sub>3</sub> can act instead of glutamine
ATP + xanthosine 5'-phosphate + L-glutamine + H <sub>2</sub> O = AMP + pyrophosphate + GMP + L-glutamate	
ATP + 5'-phosphoribosyl-formylglycineamide + L-glutamine + H <sub>2</sub> O = ADP + orthophosphate + 5'-phosphoribosyl-formylglycineamidine + L-glutamate	
ATP + pyruvate + CO <sub>2</sub> + H <sub>2</sub> O = ADP + orthophosphate + oxaloacetate	A biotin-protein. The animal enzyme requires acetyl-CoA
ATP + acetyl-CoA + CO <sub>2</sub> + H <sub>2</sub> O = ADP + orthophosphate + malonyl-CoA	A biotin-protein. Also catalyzes transcarboxylation; the plant enzyme also carboxylates propionyl-CoA and butyryl-CoA
ATP + propionyl-CoA + CO <sub>2</sub> + H <sub>2</sub> O = ADP + orthophosphate + methylmalonyl-CoA	A biotin-protein. Also carboxylates butyryl-CoA, and catalyzes transcarboxylation
ATP + 3-methylcrotonyl-CoA + CO <sub>2</sub> + H <sub>2</sub> O = ADP + orthophosphate + 3-methylglutaryl-CoA	A biotin-protein

## APPENDIX



# the determination of molecular weights by ultracentrifugation

The motor-driven ultracentrifuge is standard equipment in biochemistry research laboratories today despite its cost and vulnerability to misuse. By far the most common use is in qualitative and semiquantitative investigations to determine whether a substance is homogeneous and to estimate its molecular weight. The technique employed is the measurement of sedimentation velocity. It is possible to view substances while they are in motion and sedimenting and to make photos at regular time intervals. The sedimentation coefficient can be calculated from the data obtained. This coefficient, usually expressed in Svedbergs ( $10^{-13}$  sec), is proportional to the molecular weight but is not directly convertible without a knowledge of the diffusion coefficient  $D$  and the partial specific volume  $\bar{v}$ . The exact relations between molecular weight  $M$  and the sedimentation coefficient  $s$  are as follows.

We consider some finite volume of solution spinning with an angular velocity  $\omega$  at a distance  $r$  from the center of rotation. For the moment we ignore size and shape of this volume. The centrifugal force on the solute will depend on the number  $N$  of particles, the volume  $\phi$  of particles, and the difference in density  $\rho_P - \rho$  between the solute and the solvent.

$$\text{CENTRIFUGAL FORCE} = N\phi(\rho_P - \rho)\omega^2r$$

Dimensionally

$$[\text{VOL (g/VOL) DISTANCE}] = \text{MASS} \times \text{DISTANCE} = M(1 - \bar{v}\rho)\omega^2r$$

As the particles move through the solvent there must also be a frictional force  $= f dr/dt$ .

$$M(1 - \bar{v}\rho)\omega^2r = f dr/dt$$

For dilute solutions,  $f = RT/D$ , and therefore

$$M = \frac{RT}{D(1 - \bar{v}\rho)} \frac{dr/dt}{\omega^2r}$$

Since  $(dr/dt)/\omega^2r = \text{sedimentation in a unit centrifugal field}$ , this quantity has been designated as  $s$ , and

$$M = \frac{RTs}{D(1 - \bar{v}\rho)}$$

Several things are implicit in this relation, however. The first is that for biological systems the particle is a hydrated one and  $\bar{v}$  must be the partial specific volume of that particle. The diffusion coefficient should be that value extrapolated to zero concentration ( $D^0$ ), and the sedimentation coefficient should also be extrapolated to zero concentration ( $s^0$ ). In order to compare

values,  $s^0$  must be normalized to 20° and the value that would be obtained in water. Only then does one obtain quite comparable numbers.

$$s^0_{20,w} = \frac{MD^0(1 - \bar{v}\rho)}{RT}$$

Since  $D$  and  $s$  may vary independently,  $M$  is not a linear function of  $s$  and cannot be deduced from  $s$  at a glance.

During the last few years, it has become more usual to use sedimentation equilibrium as a technique for determining the molecular weight. This allows the direct determination of  $M_w$ , the weight-average molecular weight, provided the partial specific volume has been determined accurately.

## BIBLIOGRAPHY

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- 2 *Mathematical Theory of Sedimentation Analysis*, H. FUJITA, Academic Press Inc., New York, 1962.

## APPENDIX



# organisms used for biochemical experimentation

**section 5** The classical investigations of glycolysis most frequently employed yeast or muscle preparations. Reference to "yeast" in the biochemical literature is often nonspecific but usually means baker's yeast (*Saccharomyces cerevisiae*) or brewer's yeast (*Saccharomyces carlsbergensis*). Only occasionally have pure strains been used. The success of the Buchners in preparing a yeast juice that fermented sugar stimulated the use of the yeast organism.

**section 6** Much of the earlier study of carbohydrate metabolism was done in medical schools and by biochemists with a medical or physiological background. It was found convenient and satisfactory to use the small laboratory animals: rabbits (*Oryctolagus cuniculus*), white rats (*Rattus rattus*), and guinea pigs (*Cavia porcellus*).

For large-scale preparations of subcellular particles or of enzymes it has often been necessary to use abattoir products. For example, beef heart is often used in the preparation of mitochondria.

Nonmammalian muscle has also been used. Frogs, such as the bull frog (*Rana catesbeiana*), provide a large and readily dissectible leg muscle. At a certain period the breast muscle of the pigeon (*Columba* spp.) was also widely used.

**section 8** *Escherichia coli* is the most intensely studied microorganism. Many strains (K12, B, W, ML) and mutants exist; they differ in metabolic detail. Some strains are motile. *E. coli* is a member of the family *Enterobacteriaceae*, a Gram-negative, aerobic rod about 0.5 by 1 to 3  $\mu$  in size. It ferments sugars to acids, H<sub>2</sub>, and CO<sub>2</sub>. It is normally found in the intestinal tract of animals. As a commonly occurring nonpathogenic organism it is convenient for many studies. Moreover it is susceptible to phage infection. These factors have contributed to its widespread study, and at present the number of papers mentioning *E. coli* is very large indeed.

The *Lactobacilli* form a tribe within the family *Lactobacillaceae*. They are Gram-positive rods about 5 microns long and are nonmotile. Carbohydrates are essential for growth and are fermented to lactic acid. These organisms grow best at low oxygen tensions, and many require specific vitamins for growth. They are commonly found in the intestine of young animals and in milk products.

The *Clostridia*, in the family *Bacillaceae*, are large Gram-positive rods 5 to 8  $\mu$  long, anaerobic, usually motile, and spore-bearing. They are found in soil and in the intestine. Several are pathogenic and include the organisms responsible for tetanus and botulism. Others are used for syntheses in industrial processes.

*Mycobacterium phlei* is an actinomycete, included among the bacteria but showing morphology similar to that of the fungi. It is a Gram-positive, small rod that is nonmotile. It commonly occurs on grasses, in dust, and in the soil.

Bacteria are classified into two orders: *Pseudomonadales* and *Eubacteriales*.

**section 10** The genus *Rhizobium* grows symbiotically in root nodules of leguminous plants. The organisms are small Gram-negative rods. They can be cultured on artificial media.

*Anabaena* and *Nostoc* are filamentous blue-green algae commonly found in the soil and in ditches.

*Rhodospirillum rubrum* is a pseudomonad, family *Athiorhodaceae*. This nonsulfur purple bacterium is an actively motile spiral, 0.6 by 2  $\mu$ , with polar flagella. It can be isolated from mud.

*Bacillus polymyxa*, family *Bacillaceae*, is an aerobic, spore-bearing rod with peritrichous flagella. It is widely distributed in water, soil, and milk.

*Azotobacter vinelandii* is in the sole genus of the family *Azotobacteraceae*. The organism is an oval Gram-negative obligate aerobe. It is free living in soil and in water.

*Chromatium* is a purple sulfur bacterium genus in the family *Thiorhodaceae*. It is photosynthetic and preferentially uses H<sub>2</sub>S as a hydrogen donor. These short rods are found in marine and fresh water.

**section 11** The *Neurospora* are ascomycetes, higher fungi. They are commonly observed as a red mold on bread.

**section 12** The yeasts are also ascomycetes. The non-spore-forming yeast *Candida utilis* is a pseudomycelial organism commonly called "food yeast" and cultivated for that purpose.

**section 13** *Chlorella* is a nonmotile unicellular alga. It can be grown in liquid culture and has been convenient to use in studying photosynthesis. The generation time is relatively slow (8.5 hr) compared with *E. coli* (20 min) or baker's yeast (2 hr).

**section 14** The *Salmonellae* are in the same family as *E. coli*. They are non-spore-forming Gram-negative rods. As experimental material they present the hazard of being pathogenic. Most of the species were originally isolated from infected animals or their metabolic products.

**section 15** *Aerobacter aerogenes* is a widely distributed organism in the same family as *E. coli*. It is a Gram-negative short rod 0.5 by 1.5  $\mu$  in size.

**section 18** Bacteria, insects, plants, and animals are all hosts to self-propagating structures called viruses or phages. The phage or virus is often specific for a cell

type. The bacteriophage  $\phi$ X174 mentioned here has been of particular interest because it contains a single-stranded DNA.

*Bacillus subtilis*, like the related species *B. polymyxa*, occurs widely and was once called the hay bacillus. It is the source of proteases useful in determining protein structure.

The pneumococcus is *Diplococcus pneumoniae*, a streptococcus of the family *Lactobacillaceae*. It is often, but not invariably, the cause of pneumonia. Ordinarily it occurs in pairs, is Gram-positive, and is encapsulated in a type-specific polysaccharide. Many types have been isolated.

**section 24** *Acetabularia* is a marine alga.

**section 25** *Micromonas* is a marine alga.

*Euglena gracilis* is a free-living flagellated protozoan. This species is used because it can be grown relatively easily in culture. The attractive characteristic of this creature is that it photosynthesizes when grown in the light; but when grown in the dark, no chlorophyll is produced. *Euglena* is a kind of plankton and can be found in ponds and lakes.

**section 27** *Chlamydomonas* is a motile unicellular alga that has been classified into hundreds of species. It is capable of photosynthesis but can also live saprophytically and is found in highly polluted water.

*Volvox* is a planktonic creature. It is a colonial form: a hollow sphere of flagellated protozoan cells large enough to be visible without magnification. It is found in both fresh and salt water.

Fruit fly, or *Drosophila*, genetics dominated experimental genetics for many years. With the increasing attention to bacterial and viral genetics, this dominance has receded. From a biochemical standpoint *Drosophila* is less attractive for studying fundamental processes because it is a highly differentiated form. Latterly, however, there have been more concerted efforts to correlate the biochemical and genetic information about these animals.

The coleoptile referred to is the shoot that arises from the sprouting seed. It is sensitive to light; and if it is in an asymmetric light pattern, it will bend toward the light source. The bending is known to be due to asymmetric growth and has been used as an assay for growth hormone.

*Rhodopseudomonas* and *Rhodospirillum* constitute the *Athiorrhodaceae*. The former are spherical or rod-shaped organisms, whereas the latter are spiral.

*Phycomyces blakesleeanus* is one of the lower fungi, a *Zygomycete*. It is nonmotile and has a well-developed mycelium. It is in the same order as *Rhizopus*, the common bread mold, and occurs on decaying organic material.

**section 29** *Clostridium tetani* is the organism associated with tetanus and produces a potent exotoxin. *Clostridium tetanomorphum* is similar in appearance and is widely distributed in the soil.

*Ashbyi* and *Eremothecium* are fungi of the family *Spermophthoraceae*, all of which are plant parasites. These organisms are in the same order as the yeasts and are grown commercially in submerged culture to produce riboflavin.

The diphtheria bacillus is *Corynebacterium diphtheriae*, a member of one of the families in the Eubacterales. It is a Gram-positive rod that produces a very poisonous exotoxin.

**section 30** Probably no organism is more closely associated with man than *Staphylococcus aureus*. It is practically always on the skin, and it is not at all clear why it becomes invasive from time to time. A member of the *Micrococcaceae*, it is a Gram-positive sphere about  $1 \mu$  in diameter.

*Bacillus megaterium* is a Gram-positive rod about 1 by  $5 \mu$  in size. It is widely distributed in soil and water.

*Sarcina lutea* is classified in the *Micrococcaceae*. It is a small sphere about a micron in diameter that tends to divide in three axial directions and occurs in cell clusters. It can be isolated from air, water, soil, and skin surfaces.

*Micrococcus lysodeikticus* is also a member of the *Micrococcaceae*. It is a Gram-positive spherical coccus that occurs in irregular masses.

*Streptococcus albus* is, as a chain-forming streptococcus, in the *Lactobacillaceae*. This species is not common and is uncertain in taxonomic classification.

*Lactobacillus plantarum* commonly occurs in fermenting plant and animal products such as cheese, sauerkraut, and pickles.

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