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THE MOLECULAR BASIS OF DRUG ACTION

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I. INTRODUCTION

It has been postulated for a long time that many drugs exert their actions after being attached to specific receptors. However, only very recently has it become possible to hope that drug-receptor interactions may eventually be visualized as the interaction of 3-dimensionally defined molecular entities. This hope is based on the rapid advances made in elucidating the structure of the biopolymers involved in many of the functions of the body. Until recently, enzymes and nucleic acids had to be discussed primarily in terms of the biological functions they exerted, while little information about their structure was available. Today, in some cases, we think of proteins, polynucleotides and some other biopolymers as molecules of defined molecular weight, sequence of monomeric building blocks and even 3-dimensional structure. Enzyme chemists, once known as "people, who if they spill sulfuric acid on a table will call it tablease and feel that the matter has been explained" can now consider the interaction of some enzymes with their substrates in terms of clearly defined molecules interacting in a fashion to be understood in terms of organic reaction mechanisms. It may be hoped that the more difficult problem of drug-receptor interactions will also reach the point of being understood in molecular terms, with the result that fully rational drug design will become a reality rather than a dream.

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II. DRUG RECEPTORS

It has been assumed for many years that most drugs exert their actions by being attached to specific, chemically defined portions of the tissue on which they act. This concept was based on the high structural specificity and stereospecificity in the action of many drugs and the dose-response relationships in the action of mutually antagonistic drugs.

After studying the antagonistic effects of pilocarpine and atropine, Langley (81) postulated that "there is some 'receptor' substance . . . with which both atropine and pilocarpine are capable of forming compounds . . . according to some law in which their relative mass and chemical affinity for the substance are factors." Ehrlich (43) in discussing the action of toxins said in 1900: "The protoplasm is equipped with certain atom groups, whose function especially consists in fixing to themselves certain food stuffs of importance to cell life. Adopting the nomenclature of organic chemistry these groups may be designated side chains. . . . As these side chains have the office of attaching to themselves certain food stuffs, we must also assume an atom grouping in these food stuffs themselves, every group uniting with a corresponding combining group of a side chain. The relationship of the corresponding groups, i.e. those of the food stuffs and those of the cell, must be specific. The groups must be adapted to one another, e.g. as male and female screw (Pasteur) or as lock and key (E. Fischer). . . . The toxins possess a haptophore (binding) group corresponding to that of a food stuff. Alongside of the binding haptophore group which condiditions their union to the protoplasm, the toxins are possessed of a second 'toxophoric' group, which, in regard to the cell, is not only useless but actually injurious."

On considering the antagonistic actions of atropine and pilocarpine, Ehrlich (44) came to the conclusion that the interaction between the alkaloids and their receptor does not involve the formation of a chemical bond, but is "loose and reversible." He further postulated that active agent and antagonist must have similar "anchoring groups" permitting attachment to the identical receptor and that differences in the "toxophoric groups" of the 2 alkaloids are responsible for the differences in the physiological effects they exert.

In an attempt to quantitate drug-receptor interactions, Clark (36) applied to drug action the Langmuir isotherm dealing with the adsorption of gases to polished metal surfaces. The assumption was made that drug molecules combine with receptors at a rate proportional to the concentration of free drug and to the number of free receptors. Thus, the rate of complex formation between receptor and drug would be $k_1 A(1- y)$ and the rate of dissociation of the complex would be $k_2 y$, where k_1 and k_2 are constants, A is the drug concentration in solution, and y the degree of saturation of the receptors. At equilibrium, where $k_1/k_2 = K$, Clark used the relationship $KA = y/1 - y$ to relate the concentration of drug to the proportion of receptors occupied. By assuming response to be proportional to y, Clark then used the latter equation to relate drug concentration to response.

It should be noted that Clark himself questioned this relationship since it seemed unlikely to him that all drug receptors would be equally accessible to

the drug and that there should be a direct relationship between the number of receptors occupied by a drug and the intensity of action of that drug.

In spite of these misgivings, Clark's relationships were accepted generally. For many years considerations of drug-receptor interactions were dominated by 4 basic assumptions:

1. Intensity of response is directly related to the number of receptors occupied by an active drug (agonist).
2. At each receptor an "all or none" drug response is elicited by an agonist.
3. Drug and receptor stand in a rigid "lock and key" relationship.
4. Occupation of one receptor does not alter the tendency of other receptors to to occupied.

The first of these assumptions was shaken by the investigations of Furchgott (51, 52) and Nickerson (108), who used pretreatment with irreversible blocking agents as a means of studying the degree of receptor occupancy involved in drug action. For instance (108), pretreatment with GD-121 (an alkylating agent related to nitrogen mustard) caused a progressive shift of the log concentration-response curve for histamine by 2 log units (100-fold) to the right before causing any significant decrease in either the slope or the maximum of the curve. This finding indicated that, in the normal tissue, receptor occupancy was not directly related to drug action and that maximal drug effect could be seen when only 1 % of the available receptors was occupied. A similar although smaller shift of the log concentration-response curve for epinephrine was noted (51, 52) in the case of strips of rabbit aorta pretreated with the alkylating agent dibenamine.

The second of Clark's assumptions was challenged by Arïens (5) and by Furchgott (52). Somewhat later, on reinvestigating Clark's dose-response relationships, Stephenson (131) introduced the following hypotheses into receptor theory:

1. A maximum effect can be produced by an agonist when only a small proportion of the receptors is occupied.
2. The response is not linearly proportional to the number of receptors occupied.
3. Different drugs may have different capacities to induce a response and consequently occupy different proportions of the receptors when producing equal responses. This property will be referred to as the efficacy of the drug.

Thus, in drug action it is necessary to consider both "affinity," the ability to combine with a receptor, and "efficacy," the ability to induce a response subsequent to receptor occupation (Fig. 1*a*). Before Stephenson's work, Arïens (5) had introduced the somewhat related concept of "intrinsic activity," with the assumption that response was proportional to the concentration of receptor-agonist complex formed. More recently, van Rossum and Arïens (136) have redefined "intrinsic activity" so as to make it virtually identical with Stephenson's "efficacy." It should perhaps be added that the concept of the separation of "affinity" and "efficacy" was implicit in the ideas proposed by Ehrlich (43, 44) at the turn of the century. The current status of receptor theory has recently been reviewed by Furchgott (53).

The concepts of "affinity" and "efficacy" are particularly useful in differen-

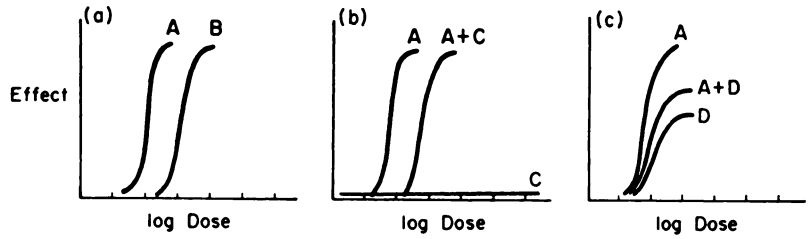


FIG. 1. Examples of dose-response curves of agonists and partial agonists. (*a*) Drugs A and B are agonists; they have equal efficacies, but A has 10 times the affinity of B. (*b*) Drug C is a competitive antagonist of drug A. It may have a high affinity but exhibits negligible efficacy. The degree of displacement of the curve for A in the presence of C depends on the concentration of C relative to its dissociation constant. (*c*) Drug D is a partial agonist. It may have an affinity of the same order of magnitude as that of agonist A but its efficacy is less than that of A. The intermediate curve results if a dose of a mixture of A and D is administered. The exact shape and position of the intermediate curve depends on the ratio of the concentrations of A and D.

tiating the actions of "agonists," "partial agonists," and "antagonists" exerting their effects at identical receptors. It was noted some time ago that in studying analogs of physiologically active compounds one occasionally encountered substances which acted both as agonist and as antagonist. Thus, some analogs of *p*-aminobenzoic acid could partly replace the normal metabolite, while, at the same time, acting as sulfanilamide-like antimetabolites. Similarly, some quaternary ammonium compounds can either act as weak agonists in certain isolated muscle preparations, or antagonize agonists of greater efficacy. For compounds of this type Stephenson (131) coined the name "partial agonists." Whereas agonists have high efficacy at a given receptor, competitive antagonists have zero efficacy, and partial agonists exhibit low efficacy. Partial agonists may, therefore, be defined (131) as compounds that induce submaximal responses even when occupying nearly all receptors, but, because of their ability to occupy receptors, diminish the action of agonists with high efficacy when the latter are added simultaneously (Fig. 1*c*).

Before considering possible interpretations of "affinity" and "efficacy" in molecular terms, another theory of drug-receptor interaction, Paton's rate theory (111), must be considered. This hypothesis states that drug effects are not related to the proportion of receptors occupied, but are proportional to the rate of drug-receptor combination; each drug-receptor association would provide one "quantum" of excitation.

One of the bases for criticism of the rate theory has been the "fade phenomenon." If the rate of drug-receptor occupation were related to the intensity of drug action, then the response should be maximal immediately after the application of the drug, and should then decrease to an equilibrium value. This is often the case. Furchgott (53) suggested that the fade phenomenon might be due to effects other than those ascribed to it by the rate theory, while Ariëns and Simonis (8) have noted its absence in a system in which it would be expected to occur in experiments with depolarizing agents in the single cell electroplax preparation (69),

in which the lack of fade presumably cannot be ascribed to slow diffusion of the agonist (fig. 2).

It has been noted (6, 11) that if rate of occupation of receptors were an essential factor in drug action, then competitive antagonists such as tubocurarine and atropine should induce a response before acting as blocking agents of cholinergic agonists [provided that the rate of diffusion of such antagonists in reaching the active site is not too slow (111a)].

It should be pointed out that there are some classes of drugs, notably general anesthetics and hypnotics, the action of which is structurally relatively nonspecific and does not appear to involve drug attachment to specific receptors.

III. SITES OF DRUG ACTION

The elucidation of drug action in molecular terms is complicated by the fact that such compounds may exert their effects by affecting totally different target sites. A majority of drugs can be classified as follows: (1) drugs that inhibit enzyme systems or alter the specificity of enzymes; (2) drugs that alter the permeability of biological membranes; and (3) drugs that alter template molecules.

In all these cases drugs act by affecting the structure of essential biopolymers. However, it is not necessary that this effect must be brought about by drugs being attached to specific receptor sites. Changes in the structure of enzymes, membranes, receptor proteins, or nucleic acids may be brought about either by attachment of drug molecules to these entities (an attachment that, in some cases, may involve interaction with metal ions) or by a drug-induced modification of the solvent structure surrounding the biopolymers (99, 112). Only in the former case would drugs exerting a given physiological effect be expected to exhibit high structural specificity or stereospecificity. Therefore, drugs can be subdivided into

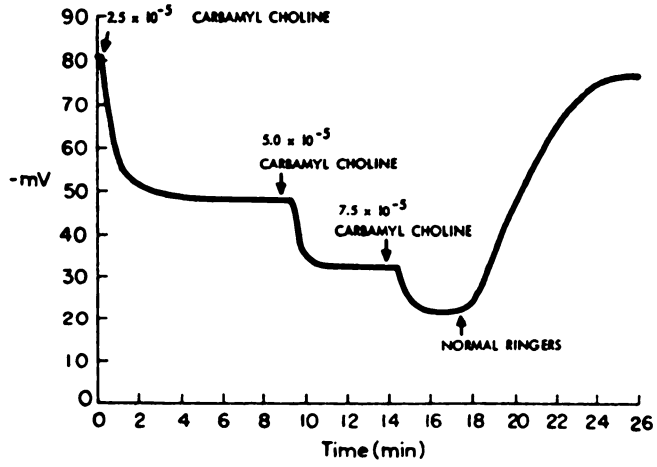


FIG. 2. Depolarization by carbamylcholine of the monocellular electroplax preparation. (From Higman, Podleski, and Bartels, Biochem. Biophys. Acta. 75: 187, 1963, with permission.)

2 major classes: (1) drugs that modify biopolymers by interacting with specific receptors; and (2) drugs that modify biopolymers by changing the structure of surrounding solvent without interacting with specific receptors.

It may be predicted that the elucidation, in molecular terms, of the mechanisms of action of the former group of compounds will be less difficult than those for the nonspecific agents.

IV. PHYSICAL NATURE OF RECEPTORS

In all cases in which drug action involves drug-receptor complex formation, the following questions have to be answered:

1. What is the primary, secondary, tertiary and quaternary structure of the molecule containing the receptor?
2. Which part of the drug molecule interacts with the receptor?
3. Which part of the receptor interacts with the drug molecule?
4. What kind of bonding holds the drug molecule to the receptor?
5. How does attachment of the drug modify the molecule to which it is bound?

The first problem to be solved, the purification of the polymer containing the receptor, will be relatively easy if the macromolecule affected by the drug "does something" readily measurable. Thus, the elucidation of the molecular function of drugs which inhibit specific enzymes, such as the phosphate esters that block acetylcholinesterase (1) or the folic acid antagonists that block dihydrofolic acid reductase (142), becomes simply a problem of enzyme-substrate analog interaction. Here, the purification of the macromolecules containing the receptor will be greatly facilitated by the increase in enzymic activity parallelling increase in the degree of purity during procedures aimed at obtaining the receptor protein. The isolation and purification of receptor proteins without enzymic or other readily measured activity present a much more difficult problem since here no convenient criterion of purity exists. This is unfortunate since, obviously, for drug-receptor interaction to be visualized in molecular terms, both these entities must be available in analytically pure form. The additional problem that the "receptor polymer" may be only a part of a multicomponent system will be discussed later.

Since, at this time, more is known about the precise structure of proteins than about that of other biological macromolecules, the discussion of drug-receptor interaction will be centered primarily on changes in protein structure, although other biopolymers can, undoubtedly, serve as targets of drug action.

A. Primary protein structure

In discussing the structure of proteins, it is convenient to accept the classification into primary, secondary, and tertiary structure first proposed by Linderstrøm-Lang (84). Primary structure refers to the fixed amino acid sequence of the polypeptide chains making up the backbone of the molecule. Secondary structure refers to the helical coiling of polypeptide chains stabilized by hydrogen bonds between amide groups. Tertiary structure refers to the overall conformation of the protein molecules induced by side-chain interactions as well as by interaction with the solvent in which the protein is dissolved. It

should be mentioned that in many cases it is difficult to differentiate the secondary from the tertiary structure. In recent years, the term quaternary structure has been introduced to describe the arrangement of subunits within macromolecules.

Since Sanger (123) and his group succeeded in solving the primary structure of insulin, the primary structures of a large number of polypeptides and proteins of physiological importance have been elucidated (32).

In determining primary structure it is first necessary to destroy the secondary and tertiary structure of the protein being investigated, with the aim of making all peptide groups equally accessible to cleavage. The protein is then broken into smaller fragments, preferably by the use of selective reagents, enzymic or nonenzymic (145), capable of attacking the peptide bonds next to specific amino acids. This step is followed by the determination of the structure of each fragment using end-group analysis and stepwise degradation. From the information thus acquired, it should then be possible to reassemble the schematic polypeptide, a process facilitated by the presence of amino acids that occur only once or twice in the protein structure. In recent years, automation has facilitated several phases of the determination of primary structure; the prediction has been made (32) that "the determination of amino acid sequences . . . may eventually become the province of well-trained machine operators."

Because of the irregular coiling of protein molecules brought about by side-chain interactions, it is possible that amino acid residues that are rather close to each other in the catalytic sites of enzymes or the receptor sites of other proteins may be rather far removed from each other in the primary structure of the macromolecules. Realization of this is important since "binding site" and "catalytic site" in enzymes and "binding site" and "effector site" in drug-receptor proteins have often been assumed, tacitly, to be close to each other within the primary as well as the tertiary structure of the protein. As will be seen, antimetabolites have proved to be useful tools for approaching this problem.

Although alteration of the primary structure of proteins can be induced by chemicals that either cleave peptide bonds next to specific amino acids (145) or convert serine to cysteine (150), it seems impossible that drugs with reversible action can act in this fashion. While several drugs are known that react with specific amino acid side chains, such reactions modify secondary and tertiary, but not primary protein structure. No therapeutically used agent appears to be known that modifies primary protein structure in nonlethal doses. There is some evidence, however, that alkylating agents, such as compounds related to nitrogen mustards, may exert their antitumor activity by modifying the primary structure of nucleic acids. These compounds alkylate nucleic acid bases, particularly guanine; the alkylation then facilitates the splitting off of the alkylated bases from the polymeric structure (27, 82).

B. Secondary protein structure

Secondary protein structure refers to the helical coiling of the polypeptide chains, stabilized by hydrogen bonds between the chain's amide linkages. Pri-

marily because of the phenomenon of denaturation, and the lack of molecular weight changes during this process, Wu (146) postulated in 1931 that: "the molecule of natural, soluble protein is not a flexible, open chain of polypeptide but has a compact structure. The force of attraction between the polar groups in a single molecule of protein holds them together in orderly ways, just as the force of attraction between different molecules holds molecules together in a crystal. In denaturation and coagulation the compact and orderly structure is disorganized." It is interesting to note that at that time the idea that a protein molecule has a 3-dimensionally defined structure was a novel one.

Wu's postulate that proteins are held in specific configurations by secondary, easily broken bonds was suggested by the fact that denaturation can be brought about by reagents too mild to affect peptide linkages, and by the absence of molecular weight changes in and the reversibility of the denaturation of many proteins. His postulate was supported by the discovery that extended and contracted protein fibers often exhibited different X-ray diffraction patterns.

For many years, scientists tried to determine the manner in which hydrogen bonds hold peptide chains in specific configurations. This work, reviewed lucidly by Low (85), culminated in the discovery by Pauling and co-workers (113) of the α-helix. In this model polypeptides are coiled helically, with each amide group hydrogen-bonded to the third amide group beyond. These interactions result in the formation of 13-membered rings. There are 3.6 amino acid residues per complete turn of the helix. It takes 18 amino acids or 5 turns of the helix for one amino acid residue to be precisely in phase with another one. Thus, in the helical portion of a protein the α-helix possesses a smooth core, with a diameter of 5 Å, surrounded by amino acid side chains projecting radially. The α-helix is depicted in figure 3. The occasional existence of stabilized periodic conformations other than the α-helix, and the existence of stabilized nonperiodic conformations should at least be mentioned.

While the presence of the α-helix in some proteins is well established, it soon became apparent that many proteins contain major nonhelical regions and that interactions other than hydrogen bonding can play a major role in determining the over-all configuration of proteins.

C. Tertiary protein structure

The prediction of Linderstrøm-Lang (84) that side-chain interaction of amino acid residues could result in an over-all protein conformation, the tertiary structure, superimposed on the secondary structure of the helical regions of the molecule, has recently been confirmed by the studies of Kendrew (74) and of Perutz (114) leading to the complete elucidation of the structure of myoglobin and hemoglobin. This work was the culmination of many years of effort in using X-ray diffraction to resolve the structures of crystalline biopolymers, work that was initiated by the discovery of Bernal and Crowfoot-Hodgkin (21) that protein crystals could yield sharp X-ray diffraction patterns.

In X-ray analysis a small crystal of the sample being investigated is mounted on a glass capillary and rotated in certain ways while being illuminated by a nar-

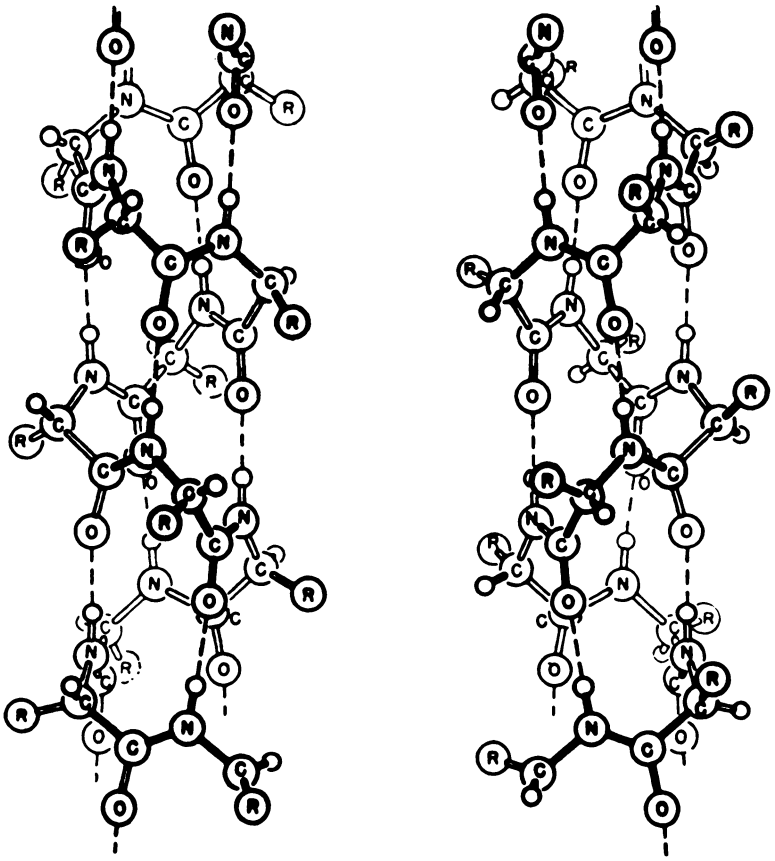


FIG. 3. Left-handed and right-handed α-helices. (From Green, Currents in Biochemical Research, Interscience Publishers, New York, N.Y., 1956, p. 398, with permission.)

row beam of X-rays. This procedure results in a regular pattern of diffracted X-rays being produced on a photographic plate located behind the crystal. The spots formed lie at the corners of a regular lattice bearing a reciprocal relationship to the arrangement of molecules in the crystal, while the intensity of each spot is determined, in part, by the atomic arrangement inside the molecule.

X-ray analysis is concerned with the task of obtaining the 3-dimensional arrangement of atoms in a molecule from the X-ray diffraction pattern produced. This is accomplished by applying a Fourier synthesis, a process in which a complex wave is reconstructed from a series of sine waves of the proper relative amplitude in the proper relative phases. Needless to say, for complex molecules, this process is immensely complicated and requires the extensive use of high-speed computers.

One of the serious obstacles in X-ray analysis was the "phase problem." X-ray diffraction patterns provide information only about the wavelengths and amplitudes, but not about the phase of the harmonics from which the total structure is

to be derived. This problem can now be attacked by the "isomorphous replacement method" (58). This procedure compares the X-ray diffraction patterns of crystals of protein with the X-ray diffraction patterns of crystals of the same protein containing heavy atoms in various positions. The presence of heavy atoms produces measurable changes in the diffraction pattern from which information about the magnitude and the signs of the phase angles can be obtained. In the case of hemoglobin, Perutz *et al.* (58) had labelled the 2 free cysteine residues with mercury. In the case of myoglobin, which lacks mercapto groups, the empirical approach of recrystallizing the protein in the presence of various metal ions had to be used.

Three-dimensional models of myoglobin at a resolution of 6 Å and at a resolution of 2 Å are depicted in Figure 4*a* and *b*. It can be seen that the shape of the molecule is completely irregular and lacking in symmetry. Surprisingly, the tertiary conformation of each of the 4 subunits of hemoglobin (fig. 4*b*), in spite of considerable species differences and differences in amino acid composition, closely resembles that of the myoglobin molecule. The 2-Å map of myoglobin (fig. 5)

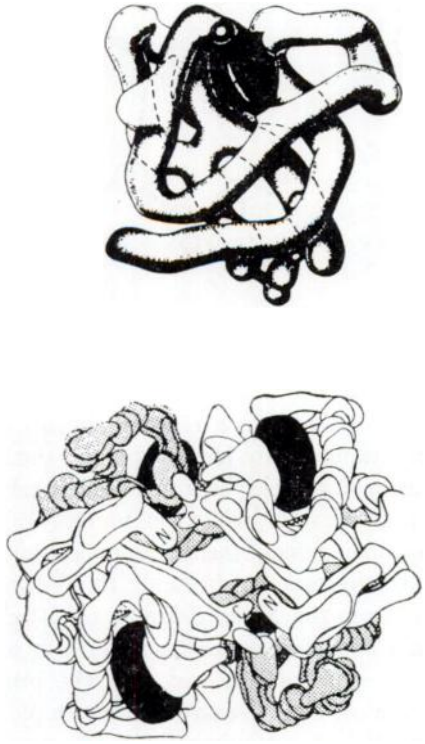


FIG. 4. (*a*) Tertiary structure of myoglobin at a resolution of 6 Å. (*b*) Quaternary structure of hemoglobin at a resolution of 5.5 Å. White: polypeptide α-chains. Gray: polypeptide β-chains. Black disks: heme groups. (From Angew. Chem., **5** (Int. Ed.): 231, 1966, with permission.)

clearly shows the presence of straight, hollow cylinders, the arrangement of atoms in which proved to be identical with that of the α-helix of Pauling *et al.* (113)5. All the helices proved to be right-handed.

From a consideration of the structure of myoglobin the following general terms emerged (73):

1. The molecule is compact, with little water inside and no channels.

2. Almost all the polar groups (lysine, arginine, glutamic acid, aspartic acid, histidine, serine, threonine, tyrosine, and tryptophane) are on the outside of the molecule. The rare exceptions are polar side chains performing special functions within the molecule.

3. The interior of the molecule is made up of nonpolar residues, almost everywhere close-packed in van der Waals contact with their neighbors.

4. The number of van der Waals interactions greatly exceeds the number of hydrogen bonds and salt links; thus, van der Waals interactions are of paramount importance in stabilizing the structure.

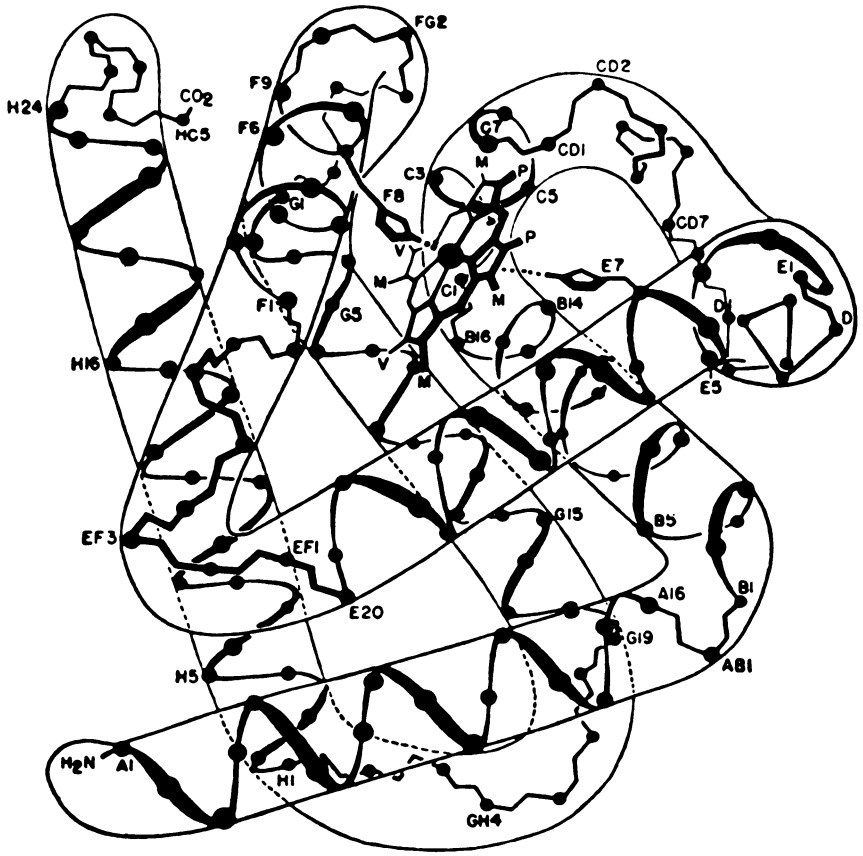


FIG. 5. Tertiary structure of myoglobin at a resolution of 2 Å. (From Neurath, The Proteins, ed. 2, vol. 2, p. 634, 1964, with permission.)

5. Bound water molecules are attached to all polar groups at the surface.

Since the publication of the work on myoglobin and hemoglobin, X-ray analysis of several other proteins has reached an advanced stage. The structure of hen egg-white lysozyme, at a resolution of 2 Å, was elucidated recently (24). The configuration of this enzyme proved to be even more complicated than that of myoglobin. Again, the protein proved to have a predominantly hydrophobic interior and a predominantly hydrophilic exterior.

The extreme complexity of the unique tertiary structure of proteins has raised the question whether the formation of these intricate folding patterns requires template mechanisms or whether they are formed spontaneously. This question has been attacked by Anfinsen and his group through studies of the reduction and reoxidation of ribonuclease (3, 62), while similar studies have been carried out with other proteins in recent years. Ribonuclease is a complex molecule, the single peptide chain of which, in its native form, contains 4 disulfide bonds. After reduction, the probability of 8 mercapto groups recombining to form the original set of disulfides is only 1 in 104, yet the reduced protein readily undergoes spontaneous reoxidation to reform the native enzyme. If the reoxidation is permitted to take place in urea or guanidine, which interfere with hydrogen bonding and hydrophobic bonding, inactive molecules, containing "incorrectly" paired half-cystine residues, are obtained. When these oxidation products are incubated with mercaptoethanol or other thiols, disulfide exchange occurs, and a large fraction of the initial enzymic activity is regained. These results imply that the tertiary structure of native ribonuclease is overwhelmingly favored thermodynamically and that the major factor in the formation of disulfide bonds in this protein lies in the concerted interaction of amino acid side chains. It seems likely, therefore, that polypeptide chains with a given amino acid sequence will spontaneously assume that conformation in which the solvent-polymer system has a low free energy and a high entropy. In other words, the tertiary structure of most proteins is completely defined by their primary structure (3). It should be added, however, that in the maintenance of the structure of proteins, their aqueous environment plays a vital role.

D. Water structure

Before considering the problem of the interaction of biopolymers and water, it is necessary to consider the structure of water. Röntgen (121) in 1892 came to the conclusion that water at room temperature should be considered a mixture of "liquid" water and of water in an "ice-like" form. This idea was based on the observation that the viscosity of water and the temperature of maximum density of water decrease with increasing pressure.

At present, the most popularly accepted model for the structure of water is based on the concepts of Frank and Wen (50), who proposed that charge separation arises between adjacent water molecules when a hydrogen bond is formed between them; this charge separation then induces the formation of further hydrogen bonds with neighboring molecules until, in a cooperative process, a cluster of highly organized, hydrogen-bonded water molecules is formed. In cluster for-

mation and in cluster breakdown, the formation or dissociation of one hydrogen bond can lead to the formation and breakdown of whole groups of bonds. In this model, liquid water is, therefore, considered to contain "flickering clusters" of structured water embedded in and exchanging roles with their nonstructured surroundings. Némethy and Scheraga (106) have calculated the sizes of water clusters at different temperatures and predict these to contain about 90 molecules at 0°C and about 50 molecules at 25°C.

When inert gases or nonpolar solutes are dissolved in water at room temperature, surrounding water structure is altered in the direction of becoming more highly organized; "icebergs" as Frank and Evans (49) call them, are formed. This structure-promoting tendency is responsible for such curious observations as the occasional ability of pipes carrying natural gas to freeze in summer, or the fact that dissolving methane in water liberates 10 times as much heat as dissolving methane in hexane. Stabilization of the "icebergs" is believed to be due to the ability of the inert solute molecules to fill empty spaces within the hydrogen-bonded framework of water clusters. Thus, the ordering of water is promoted and an entropy deficit results (107). This concept is reinforced by the observation that several nonpolar gases and liquids form crystalline hydrates above 0°C (130). X-ray diffraction studies have shown that in these structures water molecules are interconnected by hydrogen bonds and form networks of polyhedra stabilized by the inclusion of solute molecules. It seems reasonable to assume that the Frank-Evans "icebergs" resemble the structure of crystalline hydrates.

In the interaction with water of globular proteins, with their many nonpolar side chains, 2 factors must be considered: the effect of the protein on the structure of the water and the effect of the water on the structure of the protein.

The latter effect has been treated by Kauzmann (72) through the introduction of the concept of hydrophobic bonding. The assumption is made that separated nonpolar groups in an aqueous environment will tend to come out of the water and will tend to associate with each other (fig. 6). This process releases some of the water molecules from "icebergs" around individual nonpolar groups, with a resulting increase in the entropy and decrease in the free energy of the solvent-solute system. The tendency of nonpolar groups to coalesce because of the influence of surrounding water Kauzmann calls hydrophobic bonding. One thus obtains a picture of the macromolecules being passively folded into specific configurations under the pressure of surrounding water molecules, while the structure of the water is, in turn, modified by the presence of the macromolecules. At this time, the molecular nature of the influence of water on macromolecules and of the reciprocal effect of the polymer on water molecules remains uncertain (75). It can be seen that the problem of predicting the tertiary structure of a peptide chain with a given amino acid sequence in a given solvent remains an extremely difficult one.

In view of the delicate balance between solvent structure and protein structure, slight changes in the structure and dielectric constant of the medium in which the macromolecule is located may greatly affect its tertiary structure. Szent-Györgyi (134) said: "Biological functions may actually consist of the building and destruc-

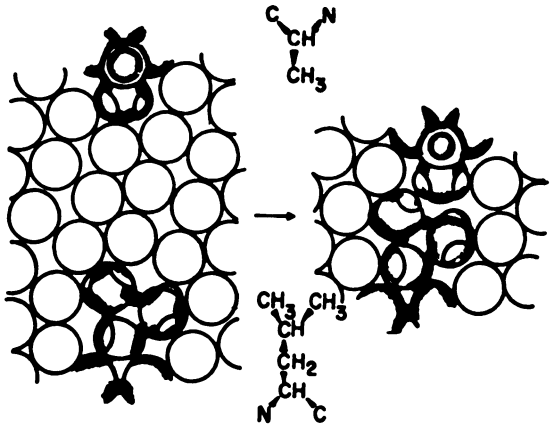


FIG. 6. Schematic representation of a hydrophobic interaction between 2 isolated side chains (alanine and leucine), showing the resulting reduction in the amount of "structured water" as the result of this interaction. (From Neurath, The Proteins, ed. 2, vol. 1, p. 522, 1963, with permission.)

tion of water structures, water being part and parcel of the living machinery and not merely its medium."

It seems a reasonable assumption that many drugs exert their biological effects by modifying the structure and function of biopolymers by affecting the interaction between solvent and macromolecule. The ability of small molecules to affect the structures of proteins and nucleic acids, and the question whether hydrogen bonds or hydrophobic bonds are involved, are now the subject of intensive research in many laboratories. The answers to these questions will be crucial to understanding what the molecular mechanisms of drug action are.

It should be added that the "structuring" of water affects not only the tertiary structure of macromolecules, but also reaction rates. It was noted, for instance, that the hydrolysis of penicillin or the dehydration of 5,6-dihydro-6-hydroxydeoxyuridine are greatly accelerated when occurring in ice as compared to liquid water. Studies by Bruice and Butler (29, 31) led to the conclusion that such acceleration is due partly to an increase in the concentration of the reactants due to freezing and partly due to the ability of ice to function as a general catalyst. If one considers that the active sites of enzymes may be located in a medium of "structured water," one may have a partial explanation for the great rapidity of enzyme reactions as compared to those of model compounds. Whether the sensitivity of many enzyme reactions to the presence of reagents that modify water structure is due to a modification of the tertiary structure of the macromolecule or to a modification of the structure of the solvent remains an open question.

E. Methods of studying conformational changes in macromolecules

It has been recognized by most pharmacologists that drugs acting at specific receptors must not only be able to be attached to that receptor, but must also be

able to bring about some change related to this attachment, the latter process being related to the "efficacy" of the drugs.

Today it is assumed widely that attachment of small molecules to biopolymers can induce conformational changes in the latter. As early as 1937, Meyer (98) postulated that chemical substances should be able to alter the permeability of membranes by bringing about changes in "pore size." In 1953, Nachmansohn (103) presented evidence that suggested that attachment of acetylcholine may induce a conformational change in a receptor protein. Since then, conformational changes in macromolecules have been invoked with increasing frequency to account for the action of certain chemicals either on drug receptors or on enzymes. It might be in order, therefore, to review, briefly, some of the methods used to indicate that such changes are taking place.

Thermodynamic measurements have been used as such a tool. By determining the rate of a reaction at different temperatures, one can readily obtain that reaction's enthalpy of activation and entropy of activation. Energy of activation may be defined as the energy that must be imparted to reactants to enable them to form an activated complex. Entropy of activation is a measure of the freedom from restraint of motion among the reactants, entropy being a measure of the degree of randomness of a system. Entropy effects may also involve changes in the degree of organization of the solvent, a factor of particular importance in the reactions of macromolecules in aqueous solution.

The finding by Vaslow and Doherty (138) that there is a relationship between the catalytic properties of chymotrypsin and the standard enthalpy and entropy changes for the formation of the enzyme-substrate complex, coupled with the observation that the binding of competitive inhibitors of chymotrypsin to that enzyme resulted in an increase in entropy, led to the suggestion (138) that these effects can be related to alterations in the conformation of the enzyme. It was proposed that the active center may consist of 2 parts: (1) an attractive center determining the specificity and orientation of the substrate; and (2) a charge center "activating the labile bond," with the 2 centers not in a rigid framework. Presumably, attachment of the substrate at the first center could then trigger a conformational change bringing it into proximity of the second center.

Similarly, thermodynamic studies of the binding of steroids to serum albumin indicated that binding depended on a large change of entropy. Again, conformational changes in protein structure were invoked to account for this effect (125).

Studies in the thermodynamics of the binding and the hydrolysis of esters related to acetylcholine have been used to suggest conformational changes in that enzyme after the binding of substrate (144). It was noted that the enzymic hydrolysis of acetylcholine proceeds considerably more rapidly than that of 2-dimethylaminoethyl acetate, 2-methylaminoethyl acetate, or 2-aminoethylacetate, even though the enthalpy of activation for the hydrolysis of acetylcholine is much higher than that for the hydrolysis of the nonquaternary analogs. The specificity of the enzyme for the hydrolysis of acetylcholine could be ascribed to a relatively favorable entropy of activation. This finding is particularly striking if one considers that, in the absence of enzyme, at physiological pH, 2-dimethyl-

aminoethyl esters, because of neighboring group catalysis, are hydrolyzed considerably more rapidly than their 2-trimethylammoniumethyl analogs (34). The great difference in the rates of enzymic hydrolysis of acetylcholine and its tertiary analog, coupled with the finding that this difference is entropic in origin, was used as a basis for suggesting (144) that a conformational change of the enzyme takes place during this process.

Difference spectra have also proved to be useful for studying conformational changes. It has been noted that the binding of small molecules may alter the ultraviolet spectra of proteins in the 250 to 310 mμ region, by alterations in the environment of phenylalanyl, tyrosyl, and tryptophanyl side chains. Since changes in the environment of molecules containing chromophoric groups can alter their absorption spectra, changes in the ultraviolet spectra of macromolecules have been used as evidence for changes in their internal structure (126). By comparing the optical densities of the solution having an altered spectrum with the optical densities of the solution having the original spectrum, a difference spectrum, that is, a plot of altered extinction *versus* wavelength, may be obtained. Split cells containing separate compartments for protein and for "perturbant," permitting easy mixing of these components (147), have proved very convenient for such measurements, in which usually only very slight spectral changes are induced.

Optical rotatory dispersion and circular dichroism measurements, *i.e.*, studies of rotation as a function of wavelength, have been used widely as a tool for studying alterations in the internal structure of optically active macromolecules (23a, 41, 46). Biopolymers consist of optically active building blocks, put together in an unsymmetrical pattern defined by the secondary and tertiary structure. Since the sign and the magnitude of the optical rotation, at different wavelengths, of such polymers depends not only on the number and kind of optically active units, but also on the spatial relation of the asymmetric units to one another, changes in the secondary and tertiary structure of biopolymers result in measurable changes in their optical rotatory properties.

Studies of synthetic polypeptides led to the development of the Moffitt equation (100), useful for calculating helical content and screw sense of simple α-helical polypeptides; the values obtained are in good agreement with data obtained by other methods. Unfortunately, in applying optical rotatory dispersion measurements to globular proteins, which contain large nonhelical regions, interpretation of measured changes in optical properties as a result of changing temperature or solvent, or as the result of the addition of small molecules, becomes extremely difficult.

It was reported recently that the optical rotatory dispersion spectrum of α-chymotrypsin is altered reversibly when this enzyme binds acetate and irreversibly when it binds diisopropyl fluorophosphate (67). The claim was made that these data provide evidence that the formation of enzyme-substrate complexes is accompanied by conformational changes. Similarly, conformational changes of metmyoglobin and apomyoglobin have been studied by means of circular dichroism measurements (26).

Recently, fluorescence-polarization spectra (141), that is, the set of values of the polarization of fluorescence emitted on excitation with light of different wavelengths, proved useful, in conjunction with difference spectra, to provide evidence for substrate-induced conformational changes in phosphoglucomutase (148). Similarly, synthetic substrates and inhibitors tagged with a "fluorescent label" have been used to provide information about the active sites of chymotrypsin and chymotrypsinogen (39).

X-ray diffraction studies provide a particularly powerful tool for studying conformational changes in polymers brought about by the attachment of small molecules. For instance, Perutz (115) showed that a rearrangement of the 4 subunits of hemoglobin occurs when oxyhemoglobin is formed. The 2 β-chains are displaced in relation to each other, so that the distance between their 2 iron atoms changes by 7 Å. Interpretation of this finding may eventually explain why the rate of combination of the fourth iron atom of hemoglobin with oxygen is greatly accelerated after the first 3 iron atoms have been oxygenated, even though the 4 heme groups of hemoglobin are well separated from each other.

More recently, successful studies of crystalline lysozyme-inhibitor complexes have been carried out (71, 115a). Refinement of such studies will provide further information about conformational changes, as well as about the groups involved in binding substrates and inhibitors.

The problem of studying enzyme-substrate interactions by X-ray diffraction methods has been attacked by Richards (120). Here, ribonuclease crystals were mounted in a saturated ammonium sulfate solution, in which the crystals are insoluble but retain their catalytic activity. Addition and washing out of substrate or substrate analogs produced reversible changes in the X-ray diffraction pattern.

It can be seen that several methods now exist for the study of conformational changes in macromolecules, induced by the attachment of small molecules or the alteration of solvent structure. With the exception of X-ray diffraction studies, in which one has to deal with crystalline, rather than with dissolved polymer, these methods provide data the interpretation of which, unfortunately, is extremely difficult. In spite of these difficulties, enough evidence has been accumulated to suggest that many substrate-receptor interactions in enzyme reactions are mediated through conformational alterations induced by substrate attachment. It may be assumed that drug-receptor interactions also involve conformational alterations of biopolymers induced either by the attachment of small molecules to a receptor, or by modification of the structure of the water surrounding it.

F. Quaternary structure

While conformational changes in biopolymers induced by the attachment of small molecules have been postulated for many years (98, 103), the tacit assumption was made until recently that such a change would involve the tertiary structure of the macromolecule. It was assumed, for instance, that a number of enzymes possess flexible active sites in which the substrate induces a conformational change leading to an altered catalytically favorable realignment of catalytic

groups (76, 77). A schematic representation of such a change is shown in figure 7 (76). Similar conformational changes, as previously noted, have been invoked in drug-induced permeability changes in biological membranes (103).

This view became more complicated when it was discovered that many proteins are made up of subunits. Many enzymes, particularly regulatory enzymes, consist of several polypeptides the arrangement of which with respect to each other is crucial to catalytic activity. The term quaternary structure has been introduced to describe the arrangement of subunits within the structure of macromolecules (77a).

While numerous catalytically active proteins have been shown to contain subunits (28), the oldest and most thoroughly studied example remains hemoglobin. It has been known for a long time that while in hemoglobin 1 molecule of oxygen is bound per atom of iron, the binding of oxygen to hemoglobin follows a sigmoid curve not consistent with binding to identical combining sites, with the affinity of the heme groups for oxygen being cooperative rather than inde-

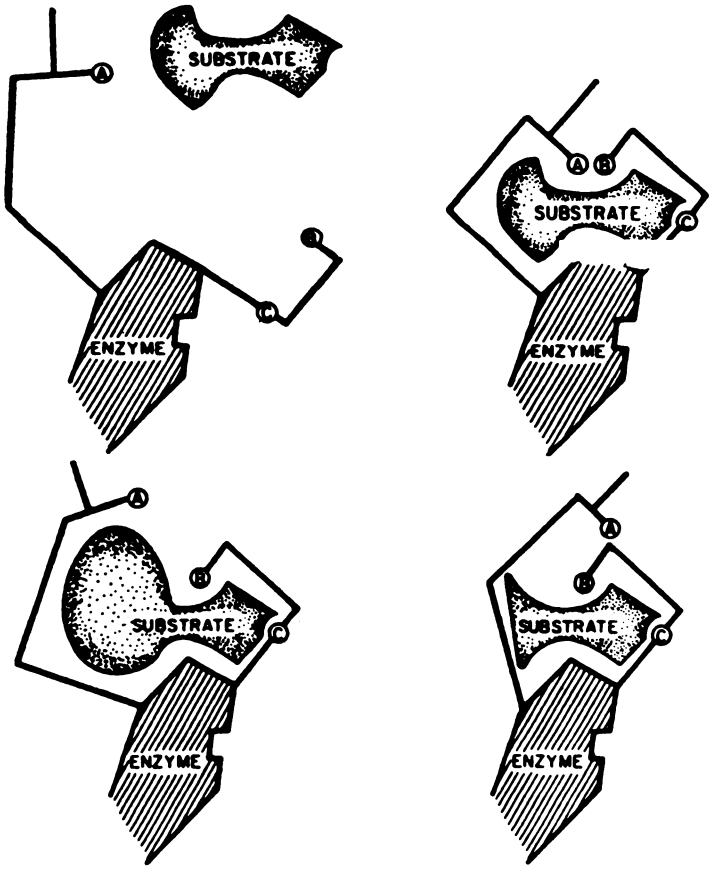


FIG. 7. Schematic model of flexible active site mechanism. From Fedn. Proc. Fedn. Amer. Soc. Exp. Biol. 23: 5, 1964, with permission.

pendent. As already noted, it could be shown that the 4 iron atoms in hemoglobin, although not vicinal, are capable of interacting with each other. The combination of any 3 iron atoms with oxygen greatly accelerates the combination of the fourth with oxygen. The observation by Haurowitz (66) that hemoglobin and oxyhemoglobin possess different crystal structures suggested that in this molecule a conformational change is induced by oxygenation, a postulate that was confirmed recently when it was shown that an appreciable rearrangement of the 4 subunits occurs during the formation of oxyhemoglobin (115). This rearrangement is particularly striking if one considers that the 4 heme groups involved are far removed from each other within the molecule while still remaining capable of interacting with each other.

Similarly, good evidence now exists that in many regulatory enzymes inhibition or activation involves the cooperative interaction of subunits within the enzyme molecule. This concept was introduced into enzyme chemistry by the discovery of "feedback" or "end-product inhibition" by Novick and Szilard (109), a phenomenon where the first enzyme of a sequence is inhibited by the end product of the entire pathway. The finding of feedback inhibition led Jacob and Monod (70) to postulate that in metabolically interconnected proteins, a metabolite may act as a physiological signal rather than as a chemically necessary component of the reaction. This theory, in turn, led to the proposal that such proteins contain at least 2 stereospecifically different, non-overlapping receptor sites. One of these, the active site, binds the substrate and initiates the biological activity of the protein. The other site, the allosteric site, is complementary to the structure of the product of the reaction, which it binds specifically and reversibly (101). The formation of the enzyme-allosteric effector complex brings about a discrete reversible alteration, an allosteric transition, in the structure of the protein, which modifies the properties of the active site and "shuts off" the reaction (Fig. 8). It is important to note that the allosteric effector needs little structural similarity to the initial substrate of the reaction.

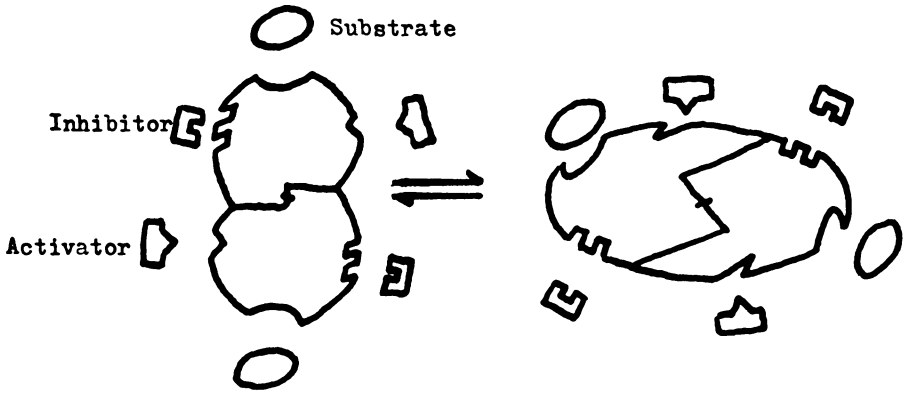


FIG. 8. Schematic representation of regulatory changes in an allosteric molecule. (Redrawn from Sci. Amer., Apr. 1965, p. 43, with permission.)

Allosteric effects have been classified as: "homotropic effects," *i.e.*, interactions involving identical ligands, and "heterotropic effects," *i.e.*, interactions involving different ligands. The general properties of allosteric systems have been summarized recently (102):

1. Most allosteric proteins are polymers or oligomers composed of several identical units.
2. Allosteric interactions frequently appear to be correlated with alterations of the bonding between subunits.
3. While heterotropic effects may be either cooperative or antagonistic, homotropic effects appear to be always cooperative.
4. Few, if any, allosteric systems exhibiting only heterotropic effects are known.
5. Changes that alter heterotropic interactions simultaneously alter homotropic interactions.

Much of the literature dealing with allosteric proteins and the evidence for allosteric transitions is reviewed in the above paper. It should be noted that the model presented here specifically assumes the subunits of an allosteric protein to be identical, an assumption which even with the comparatively simple example of hemoglobin, with its α-chains and β-chains, is not valid. Another example of an allosteric protein, aspartate transcarbamylase, can be dissociated into 2 types of subunits, a protein of high molecular weight with catalytic activity and a protein of lower molecular weight with regulatory activity (54). Models with nonidentical subunits have been discussed recently by Koshland and his co-workers (78).

A still more complicated situation exists in multienzyme complexes. For instance, it could be shown in the case of fatty acid synthetase that the synthetic sequence from acetyl coenzyme A and malonyl coenzyme A to palmityl or stearyl coenzyme A is carried out by a complex consisting of 6 or 7 functionally interrelated enzyme components grouped around a central carrier protein (86). The central sulfhydryl group carries the acyl group until butyryl coenzyme A is formed when the now lengthened acyl chain is transferred to a peripheral mercapto group which carries it until palmityl or stearyl coenzyme A are formed (Fig. 9). Thus, one is dealing with an allosterically related, unidirectional assembly line into which building blocks are supplied from outside and from which only fully assembled products will depart. Similarly, it has been proposed by Green and Järnefelt (57) that processes intimately bound up with subcellular

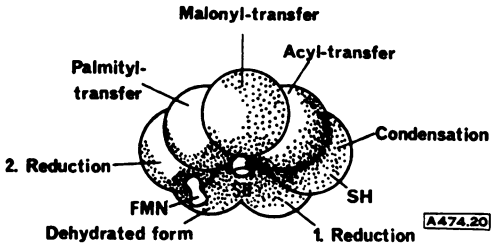


FIG. 9. Hypothetical structure of multienzyme complex for fatty acid synthesis. (From Angew. Chem. 77: 929, 1965, with permission.)

structure, such as electron transport, photosynthesis, or oxidative phosphorylation are carried out by the cooperation of structurally related units in multienzyme complexes. The quaternary structure of complexes made up of nonidentical components carrying out an interrelated chain of nonidentical functions, while remaining capable of allosteric interactions, must be considered not only in enzyme processes but also in membrane processes, and, presumably, in complexes involving both membrane and enzyme components. It should be noted that some evidence exists that some membranes are made up of subunits the arrangement of which with respect to each other or with respect to ancillary enzyme systems can be altered (56). It seems possible, as in the case of nerve conduction, to consider allosteric interactions involving both the membrane, in which permeability changes are being induced, and enzymes responsible for the formation and hydrolysis of the compound responsible for the permeability change (104).

Still further complexity was introduced into the understanding of the machinery of living systems by the finding that apoenzymes, in contrast to coenzymes, may possess different structures even when they exert identical functions. As early as 1895 Fischer (48) cautioned that: "the various maltases that undoubtedly exist should be termed corn, yeast, etc. maltases, depending on their origin." As methods of separating macromolecules were increasingly refined, it became apparent that enzymes that have the same function may show differences in both physical and biochemical characteristics when isolated from different organs of the same animal (143). For different forms of the same enzyme the name isozyme has been proposed (88). For instance, lactate dehydrogenase could be fractionated into 5 different forms, the relative amounts of which differed in different tissues of the same animal (88). Later it was demonstrated that lactate dehydrogenases were composed of 4 subunits which could be separated by the use of either guanidine or surface-active agents (4, 40). Binding of coenzyme I (DPNH) "locks" the subunits together and prevents dissociation by the addition of detergent, possibly through the induction of a "protective" conformational change in the protein (40). It could also be shown that 2 different kinds of subunit (M and H) exist in this enzyme and that different isozymes are formed depending on which subunits interact to form what tetramer (M_4, M_3H, M_2H_2, MH_3, H_4), while oxygen tension could regulate what proportion of M and H subunits was synthesized (38, 55). Thus, the cellular environment can regulate which subunits are synthesized and determines what isozyme will be prevalent in what tissue. Several other examples of the molecular heterogeneity of individual enzymes have been noted in recent years (143).

The pertinence of the discovery of isozymes to the understanding of drug action is emphasized by the observation that it is possible to design compounds which inhibit M-lactate dehydrogenase irreversibly without inhibiting H-lactate dehydrogenase, while other compounds exhibit the opposite specificity (11).

G. The problem of receptor structure

The body of evidence accumulated in recent years that attachment of small molecules may trigger conformational changes affecting the tertiary structure of

biopolymers and the quaternary structure of subunit aggregations, coupled with the demonstration of allosteric interactions, multienzyme complexes, and isozymes, has introduced entirely new complexities into the understanding of drug-receptor interactions on a molecular level.

It used to be fashionable to assume that a receptor can be visualized by making a Bakelite mold of molecules capable of being attached to it. The tacit assumption was also made that receptors interacting with a given molecule would be identical throughout the body. Both of these assumptions seem dubious in the extreme, a point which, with respect to acetylcholine receptors, was raised many years ago by Welch and Roepke (141a).

If a drug is to act through inducing a configurational change, it is necessary to differentiate clearly between the portion of the molecule which is attached to the receptor (responsible for "affinity") and the portion of the molecule triggering a conformational change (responsible for "efficacy"), while recognizing that, at the same time, the configuration of the receptor before drug attachment may be quite different from the configuration of the receptor after drug attachment. It is tempting to reclassify drugs that interact with receptors as follows:

Agonists—drugs which through attachment to a receptor induce a maximal effective configurational change

Antagonists—drugs which through attachment to a receptor cannot induce an effective configurational change

Partial agonists—drugs which through attachment to a receptor can induce an effective configurational change less than that induced by the agonist

This definition implies that not only 2 conformations, "resting" and "folded," as in the case of most theoretical models of subunit interactions, but a number of intermediate "partially folded" conformations, must be considered as receptor sites for a series of related molecules exerting similar pharmacological effects.

While lack of purified receptor proteins leaves the above postulates in the realm of speculation, it seems likely that tools for proving or disproving this theory will become available in the not too distant future. It should be noted that the possibility exists that, in some cases, the same portion of a molecule might be responsible for both "affinity" and "efficacy."

Consideration of flexible drug receptors also shakes the assumption that stereospecific, optically active drugs may serve as template models of stereospecific receptor sites (16, 17). Rather than assuming rigid 3-point attachment to a rigid receptor, one can just as readily visualize 2-point (or even 1-point) attachment to an "affinity site" on a receptor, resulting in a stereospecific configurational change of the biopolymer, which may be either helped or hindered by the other portions of the drug enantiomer (Fig. 10). It would even seem conceivable that for "partial agonists" inducing only a partial configurational change, stereospecificity might be different than for agonists inducing a more extensive configurational change, since the stereospecificities of "partly folded" and "fully folded" polymer would not necessarily have to be the same. This explanation might account for the observation that in some series of optically active drug molecules the ratio of the relative activities of enantiomers is progressively altered as the structure of the molecule is modified and that even inversion of the

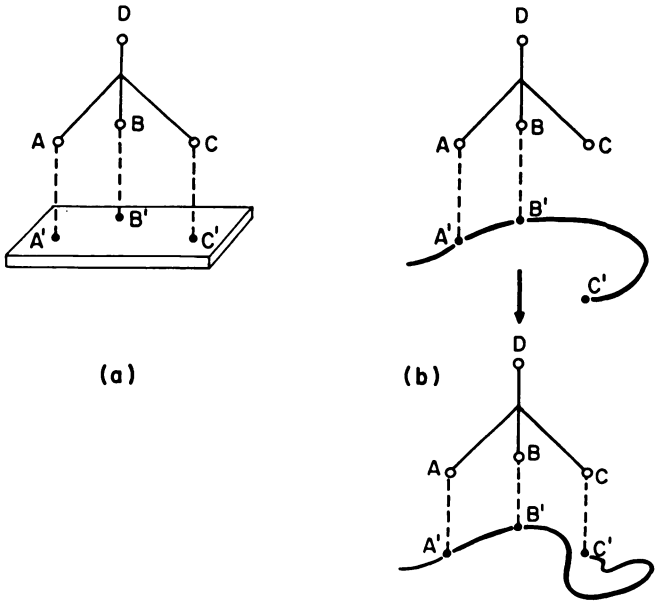


FIG. 10. Schematic representation of "3-point attachment" of a small molecule to a non-flexible receptor and "2-point attachment" to a flexible receptor.

absolute configuration of the more active drug may be observed. For instance, a series of analgesics related to phenampromide was reported to have the opposite absolute configuration from that of most other analgesics (117). Similarly, the pharmacologically more active enantiomer of muscarone has the opposite absolute configuration from the more active enantiomer of muscarine (139).

Very likely, just as it has become necessary for biochemists to deal with "isozymes," it will be necessary for medicinal chemists to deal with "isoreceptors." In view of the previously discussed observation that environment, in some cases, can determine what isozyme is found in what tissue, it would seem surprising that receptors for a given drug should prove to be identical throughout the body. It is well known that the structure of membranes is extremely complex. The common structural feature is believed to be either a complex "molecular sandwich" involving the incorporation of proteins into bimolecular layers of polar lipids (63), or, as suggested recently, a polymer of lipoprotein subunits (19), stabilized by specific hydrophobic protein-lipid interactions. In either case, one would deal with a flexible structure probably forming a complex organized unit with associated enzymes; the tertiary and quaternary structure of such complexes would be likely to be subject to alteration depending on the environment in which they are located. In view of the knowledge available regarding the structure of enzymes and of membranes, the prime targets of drug action, it seems unreasonable to talk about receptors, as in the case of "muscarinic receptors" or "analgesic receptor sites," as if these entities were rigid and identical throughout the living systems in which they are located.

The concept of isoreceptors may also be useful for explaining why, even, though

basic metabolic patterns are very similar throughout the animal kingdom, there is such extensive species specificity and organ specificity in the abilities of drug molecules to affect these patterns.

In view of the possibility of allosteric interactions, it is also important to consider whether as drug-receptors are being saturated, their affinities may not be altered as well.

It should be emphasized that the concept that drug action may involve the induction of conformational changes in the biopolymer molecules on which the receptor sites are located, is not particularly novel. This idea was introduced by Nachmansohn (103) and Meyer (98) and has recently been revived by Belleau (18), Portoghese (118) and others. It is necessary also to point out that while conformational changes in some enzymes appear well established, conformational changes in nonenzymic receptor-containing polymers have had to be inferred indirectly from stereochemical (18) and thermodynamic (103) considerations. Although all the facts available point to this postulate being correct, it will be necessary for receptor-polymers to be isolated and purified to an extent which will permit the application of physical measurements (such as circular dichroism, optical rotatory dispersion measurements, or X-ray diffraction), in order to provide direct proof that the attachment of drug molecules does indeed alter the receptor molecule's conformation.

V. ANTIMETABOLITES AS TOOLS FOR ELUCIDATING DRUG-RECEPTOR INTERACTIONS

A. Types of antimetabolite

Antimetabolites may be defined as compounds bearing structural resemblance to metabolites and capable of being attached to the same receptors; however, while under physiological conditions attachment of metabolites to receptors results in an event compatible with the normal functioning of the cell on which the receptors are located, attachment of antimetabolites does not. By this definition, many drugs that are attached to specific receptors may be considered to be antimetabolites; for instance, atropine would be an antimetabolite of acetylcholine. It should be noted that in this paper the term metabolite is not limited to compounds metabolized during the course of their action, but is to include all compounds normally found in the body which through attachment to biopolymers, can exert a functional or regulatory role. On the other hand, the term antimetabolite is to include compounds with "efficacies" different from those of the metabolites with which they compete for common receptors. The term antimetabolite is not to be restricted to compounds with zero efficacy. Receptors on which these compounds act are not to be limited to active sites of enzymes, but, for the purpose of this discussion, are also to include the active sites of receptor polymers devoid of enzymic activity.

It is not within the scope of this review to discuss the very numerous medicinally useful compounds discovered through the systematic synthesis and screening of antimetabolites. It should be pointed out, however, that the study of analogs of metabolites provides a particularly useful approach to the study of the

structure of receptor molecules and, potentially, to the isolation and purification of the polymers on which receptors are located.

It has already been noted that in both metabolite and antimetabolite molecules it is necessary to differentiate between the part of the molecule that is attached to a receptor and the part of the molecule that "does something" through attachment taking place. This consideration led to the proposal that antimetabolites can be classified as:

1. Endotoxic antimetabolites—Compounds in which only those groups of the metabolite are modified which are involved in binding to the receptor.
2. Toxophoric antimetabolites—Compounds carrying reactive groups adjacent to the area of the molecule responsible for attachment to the receptor. Here, once antimetabolite-receptor interaction has taken place, nearby areas of the biopolymer are attacked irreversibly (90).

A similar proposal was made independently by Baker (10), who classified antimetabolites as "endo-alkylating agents" and "exo-alkylating agents." It should be noted that "endo-alkylating agents" (10) were defined as binding irreversibly to the "affinity-site" of the biopolymer with formation of a covalent bond, while "endo-toxic antimetabolites" were defined more flexibly as binding more strongly to a receptor than the analogous metabolites, without covalent bond formation necessarily taking place, and with the possibility existing that the strength of binding will be pH dependent (90).

It might seem desirable to consider a third type of antimetabolite:

3. Agonistomimetic antimetabolites—Compounds capable of normal attachment to "affinity sites," but carrying groups adjacent to the part of the molecule responsible for attachment to this site, which will interact with nearby areas of the biopolymer in a fashion different from the interaction induced by the metabolite. For example, partial agonists with cholinergic activity could be considered to be agonistomimetic antimetabolites of acetylcholine. This class of antimetabolites should, however, include not only compounds with "efficacies" lower but also compounds with "efficacies" higher than those of the metabolites with which they compete. For this class of compounds, the term "partial agonists," familiar to most pharmacologists, but not to most medicinal chemists, could also be used.

B. Problems in using antimetabolites as molecular probes

The most useful approach to the elucidation of drug-receptor interactions in molecular terms remains the study of the effects of metabolite analogs in which systematic modifications of functional groups have been carried out. Such studies may provide information about: (1) the portion of the metabolite responsible for affinity; (2) the forces involved in metabolite-affinity site interactions; (3) the portion of the metabolite responsible for efficacy; and (4) the detailed structure of the receptor.

Potentially, suitably designed antimetabolites should also be useful tools for the isolation and purification of receptor proteins. This problem will have to be solved before detailed studies of the mechanisms by which drugs exert their activity can be undertaken.

In theory, to determine which part of a molecule is responsible for affinity and which part for efficacy is comparatively simple. In practice, this problem is far from simple. When the effects of various metabolites and antimetabolites are measured and compared in biological preparations, the assumption is made generally that these compounds behave differently only at the observed receptor site, but that, in terms of metabolism, ability to be carried to the site of action, ability to cross permeability barriers, or effect unrelated to the observed site of action, these compounds will behave in very similar fashion. This assumption, unfortunately, is hardly ever valid, since most molecular modifications will affect not only the molecule's ability to interact with a receptor, but many other parameters as well.

Another problem that must be faced may be termed the "stick toy fallacy." The assumption is made frequently that an atomic alteration of a given molecule will be localized at the point of the molecule where it is carried out, without affecting the rest of the molecule. It should, of course, be realized that molecules are not stick toys and that the substitution of even a single atom by another, particularly in unsaturated compounds, may affect electron distribution and conformation throughout even complex molecules and thus alter the chemical reactivity and binding capacity of groups far removed from the point being modified.

It is, therefore, very important in studying the effects of series of antimetabolites to consider to what extent each replacement of one group or atom by another affects electron distribution and conformation throughout the molecule being modified. Thus, in molecules possessing several ionizable groups, it is important to determine to what extent any structural alteration affects the acid and base strengths of all of these.

In recent years, molecular-orbital calculations have been widely used to visualize electron distribution in biologically active molecules and to predict to what extent changes in the structures of such compounds will alter their electron distributions (119). Such calculations have proved to be useful in many problems of biological interest and have the advantage of requiring comparatively simple mathematical procedures (119, 132). It must, however, be remembered that while molecular orbital calculations are useful in interpreting data and in making predictions, they cannot take the place of physical measurements, particularly since molecular orbital calculations, especially in heterocyclic compounds, tend to provide only a rough, over-all picture of electron distribution. It remains desirable that in each series of analogs of biologically active compounds being studied, all possible information about electron distribution in each member of the series should be gathered, whether by spectroscopic or dipole moment measurements or by the application of newer tools such as nuclear magnetic resonance, electron spin resonance, or mass spectrometry (25, 30, 124). It is unrealistic to hope that study of a series of antimetabolites can yield much information about the receptor on which these compounds act, unless electron distribution in each of the compounds can be visualized fairly clearly.

Ideally, compounds should be tested in biological systems in which both affinity and efficacy can be determined quantitatively, in other words, in systems in which

precise information can be obtained, not only about the dose of compound required to induce a response, but also about the maximal response that each given compound can induce. These systems should be as well defined as possible, so that differences in drug metabolism or differences in the ability of the drug molecules to be carried to the site of action do not contribute in a major way to differences in the response induced by different compounds. At the same time, it should be possible to make meaningful correlations between observations based on the test systems and observations made in whole animals. Such test systems are hard to find. At this time, the single cell electroplax preparation (127), a monocellular test system in which drug-induced depolarization can be measured with an accuracy of $\pm$ 5% over a wide pH range, can probably serve as an ideal model test system suitable for making structure-activity correlations of many types of depolarizing and antidepolarizing agents. Ideally, of course, even in this area of pharmacology, structure-activity correlations require a wide range of test systems of varying complexity. Unfortunately, in many important areas of pharmacology, satisfactory test systems, in which differences in drug action can be interpreted meaningfully, are not available at this time.

It is not surprising that since the early days of chemotherapy numerous attempts have been made to correlate differences in the action of structurally related compounds to differences in readily measured physical parameters of such compounds. For instance, Meyer (97), Overton (110) and, more recently Ferguson (47) and Hansch and co-workers (64, 65) have tried to relate partition coefficients to biological potency. Ferguson's studies indicated that the action of some drugs was related to the equilibrium between the concentration of drug in the external phase and its concentration in the biophase; this observation enabled Ferguson to postulate that: "substances which are present at the same proportional saturation in a given medium have the same degree of biological action." It should be noted that this rule applies only to drugs exerting nonstereospecific activity (2); within this limitation it has proved to be useful for predicting the activity of homologous, biologically active compounds.

Recently, Hansch (64, 65) introduced the use of a substituent constant, π, equal to log (P_X/P_H), relating the partition coefficient of a biologically active compound (P_H) to that of a derivative (P_X). This substituent constant was defined as a measure of the hydrophobic bonding power of a substituent and claimed to be useful for predicting how given substituents would affect biological activity of related derivatives in a wide variety of biological systems.

While this approach may prove to be useful for predicting whether a given analog is worth synthesizing once the biological activities and partition coefficients of several related analogs have been established, the knowledge of "thermodynamic activity" (47) or "substitution constants" (64) is only of empirical value and provides little information, at this time, about the molecular mechanisms of drug action. The recent statement (65): " 'The lock and key' theory does not seem to be so demanding that it precludes many good correlations with a simple two parameter equation," raises the question: "What molecular significance do these correlations have?"

Certainly, it seems unrealistic to think of drug-receptor interactions in terms

of simple lock and key relationships. It seems much more likely that drug attachment to a receptor may affect a whole sequence of functionally related, conformationally mobile biopolymers, similar to a simple stimulus setting in motion a complex "Rube Goldberg contraption."

Rube Goldberg (and in England, W. Heath Robinson) drew cartoons of somewhat anthropomorphic parodies of intricate machinery. Thus, a man might light a flame, which would burn through a string holding a weight; the weight dropping would tip a board, spilling a jug, the spilled water tipping a balance, *etc.* A "Rube Goldberg contraption" would, therefore, represent an essentially unidirectional assembly, each component of which would go from a high energy to a low energy state during the operation of the contraption. Each component would, for repetition of the action of the machine, have to have its potential energy restored by processes different from those involved in the functioning of the machine as a whole; the vast importance of entropy factors in the operation of a "Rube Goldberg contraption" is obvious. In view of what little is known about biological functionally related multicomponent systems, the analogy to such a contraption is a tempting one.

Much more work with well defined polymers will be required before it will be possible to interpret the physical meaning of relationships between drug action and partition coefficients. It seems well established that hydrophobic bonding capacity plays an important role in the effects of small molecules on polymers. However, in the field of drug-receptor interactions, little information is available whether hydrophobic bonding can be related to affinity or to efficacy, or whether drugs act by modifying water structure or by introducing hydrophobic regions, with altered dielectric constant, into the environment of macromolecules, thus affecting side-chain interactions.

C. The use of antimetabolites as molecular probes

In spite of the limitations of the antimetabolite approach outlined above, the use of rationally designed analogs of biologically active compounds can yield a great deal of information about the receptors on which they act.

Particularly useful for this purpose have been acylating and alkylating antimetabolites, the "endotoxic" and "toxophoric" antimetabolites (90) noted previously. This author prefers these terms to Baker's (10) terminology of "endo-alkylating" and "exo-alkylating agents," since compounds of both types can form covalent bonds by means of reactions other than alkylation. A lucid review of the use of such antimetabolites for receptor elucidation has been written by Baker (12). While the use of compounds of this type for labelling receptor sites is comparatively recent [for a review with key references see Singer and Doolittle (129)], the concept of "toxophoric antimetabolites" is not particularly novel; certainly, amino acid mustards (20), pyrimidine mustards (87), purine mustards (33), carbohydrate mustards (137), and the numerous other examples of biologically active compounds carrying mustard groupings may be considered to be antimetabolites of this type.

In designing "active-site-directed irreversible inhibitors" (12) it should be

remembered that while such compounds must carry reactive groupings capable of covalent bond formation, these groupings should not be so reactive as to result in the antimetabolite reacting before its destination is reached. Such premature reaction would result in a loss of specificity. It is also important to consider all the information available regarding the specificity of the binding site before deciding to which portion of the metabolite or antimetabolite the reactive grouping should be attached. If the compound is to be a "toxophoric antimetabolite" then it should be capable of being attached to the affinity site of its target before covalent bond formation occurs. For instance, on the basis of such considerations, Baker and his co-workers (13), succeeded in designing glutamic dehydrogenase inhibitors capable of irreversibly inhibiting this enzyme after being attached to it. Similar rationale was followed in the design of purine mustards designed to cause inter-helical cross-linking of deoxyribonucleic acid (33).

A particularly interesting example of this approach in enzymology is represented by the use of Lawson and Schramm (83) of *p*-nitrophenyl bromoacetyl-α-aminoisobutyrate as a bifunctional reagent for the elucidation of the active site of chymotrypsin. Like other nitrophenyl esters (96), this compound specifically acylates the serine residue at the active site thus forming an acyl enzyme that carries a bromoacetyl group, capable of alkylating a methionine residue in the vicinity of the serine. Hydrolysis of the acyl enzyme frees the serine and releases partly inactivated chymotrypsin with 1 of its 2 methionine residues alkylated. That this "bridging reaction" takes place within the active site of the enzyme is suggested by the observation that the above bromoacetyl reagent is incapable of alkylating the enzyme's methionine if the serine residue is blocked previously with diisopropyl fluorophosphate. Thus intramolecular alkylation rather than random alkylation is occurring (Fig. 11).

Since amino acids vicinal to each other in the receptor site of a protein are not necessarily close to each other within the primary structure of this protein, reagents such as the one used by Lawson and Schramm (83) provide a very valuable tool for the determination of which amino acid residue is near the affinity site to which substrate—or drug—is attached. For instance, it proved possible for Lawson and Schramm, by use of the methionine peptide cleavage reaction (59), to show which of the 2 methionine residues had been alkylated and so indicate which methionine is close to the serine of the active site of chymotrypsin.

It may be expected that the use of antimetabolites capable of covalent bond formation will have many applications both in enzyme chemistry and in medicinal chemistry. In particular, compounds combining groups conferring specificity with alkylating or acylating groups should be very useful for the labelling, isolation, and purification of the biopolymers on which drugs act.

It was noted earlier that the isolation and purification of receptor proteins without enzymic or other readily measured activity is peculiarly difficult since no convenient, biologically correlatable criterion of purity exists. Attempts have been made to isolate receptor proteins by precipitating these with compounds known to be receptor inhibitors. For instance, the claim has been made and later

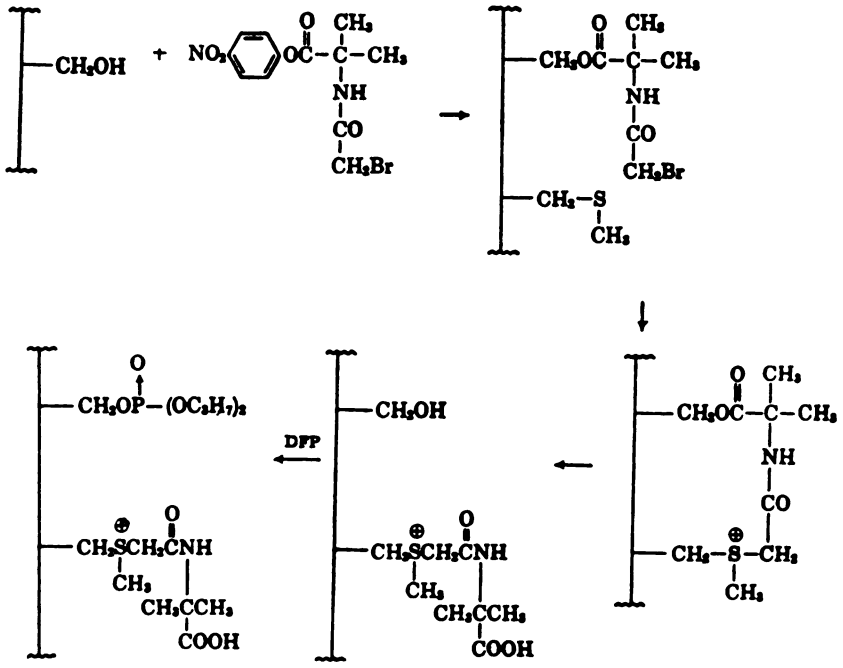


FIG. 11. Reaction of chymotrypsin with *p*-nitrophenyl bromoacetyl-α-aminoisobutyrate. (From J. Pharm. Sci., 53: 351, 1964, with permission.)

amended by Ehrenpreis (42, 42a) that the acetylcholine-receptor protein of the electroplax preparation could be obtained in electrophoretically pure form by precipitation with curare, a receptor inhibitor. Recently Beychok (23) showed the "receptor protein" to be quite nonhomogeneous. The problems encountered here emphasize the importance and the difficulty of showing that reagents used in receptor protein purification are truly specific in their action.

It would seem that toxophoric antimetabolites should be very useful for solving problems of this kind. Alkylating or acylating compounds, radioactively labelled, carrying groups capable of specific receptor attachment, should through their ability to form covalent bonds, serve as labels useful for the isolation and purification of biopolymers to which these compounds are attached. It would be desirable for the alkylating and acylating groups to be carried by compounds the action of which is stereospecific. If only the toxophoric antimetabolite based on the biologically active enantiomer would be attached to the polymer to be purified, one could be certain that the acylation or alkylation is really receptor-specific.

In addition to the use of antimetabolites capable of covalent bond formation, antimetabolites that are attached reversibly to receptors can also be very useful for obtaining information about metabolite-receptor or drug-receptor interactions.

Isosteric antimetabolites (*i.e.*, molecules sterically identical to the metabolites with which they compete) have been particularly useful for this purpose. Obvi-

ously, if metabolite and antimetabolite have the same size and shape, differences in biological activity are attributable not to differences in the ability to fit receptor sites, but rather to differences in the ability to bind to receptor sites or to differences in the ability to exert intrinsic activity after being bound. As a cautionary note it should be added that the possibility exists that some isosteric molecules might be capable of being hydrated to greatly different degrees and, thus, would not necessarily be isosteric in solution.

It is not within the scope of this article to review the voluminous literature dealing with isosteric compounds (9), but a few examples of the use of isosteres as molecular probes should be cited.

Isosteric but non-isoelectronic analogs of biologically active compounds can be used to obtain information about the forces within the drug-receptor complex. For instance, it is known that deuterium bonds, whether with nitrogen, oxygen, or carbon, are more stable than corresponding hydrogen bonds (37, 80). Since deuteration has only a negligible effect on the size and shape of groups being deuterated, differences in the binding of deuterated and nondeuterated analogs to receptor sites (45) should be particularly useful for determining what interactions are taking place within the drug-receptor complex. It would, of course, be important for the deuterium not to be readily exchangeable in aqueous solution. Thus, as in the example of work comparing morphine with N-trideuteromethylmorphine (45), this approach would have to be limited to the deuteration of lipophilic groups.

Similarly, the observation that sulfur forms weaker hydrogen bonds than does oxygen, whether it is acting as a proton donor (116) or as a proton acceptor (79), while having a greater ability to form hydrophobic bonds than oxygen (79, 94), has proved useful in making decisions about bonding forces in drug-receptor complexes. It may be assumed that in those cases where sulfur compounds are bound to receptors more strongly than their oxygen analogs, hydrophobic bonding or chelation rather than hydrogen-bonding is taking place. Thus, the postulate by Zakrzewski (149) that folate and folate antagonists are held to dihydrofolic reductase by hydrogen bonds was made unlikely when it was found that 4-thiopyrimidines, 4-thiopteridines, and 6-thiopurines (22), as well as a sulfur compound related to an open chain analog of tetrahydrofolic acid (14), were all bound to the enzyme more strongly than the corresponding oxygen analogs. On the other hand, the demonstration by Baker and co-workers (15) that thymidine is bound to thymidine kinase more effectively than 4-thiothymidine indicates that, in this case, the 4-oxo group of the pyrimidine is hydrogen-bound to the enzyme.

A series of isosteres of particular interest to our laboratory have been isologous oxygen, sulfur, and selenium compounds. While the atomic radii of oxygen and sulfur are slightly different, the radii of sulfur and selenium are so similar that sulfur and selenium isologs have identical crystal structures, (89, 135), identical conformations and identical packing within the crystal lattice (128a), and form the same hydrates. The similarity of their partition coefficients (94) makes unlikely any major differences in the abilities of such compounds to be solvated.

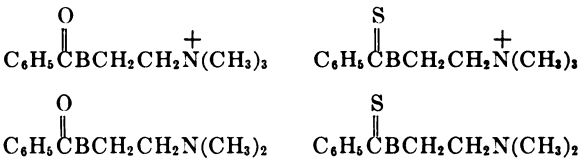
On the other hand, electron distribution in isologous oxygen, sulfur, and selenium compounds differs greatly as shown by spectroscopic and dipole moment measurements (34, 79, 89, 95), with the result that the reactivities of sulfur and selenium compounds differ both in nonenzymic (34, 91, 93), and enzymic (61) systems.

In recent studies with acylcholine isologs in which both the side chain oxygen and the carbonyl oxygen have been replaced systematically with sulfur and selenium (35, 60), we have been dealing with isologs that are not only isosteric but also electronically defined, since it has been possible by the application of physical measurements to collect a great deal of information about electron distribution in each of the compounds being investigated. Studies of these isologs in the electroplax and in other preparations have shown striking differences in the abilities of such isosteres either to induce depolarization or to block the induction of depolarization (92, 122, 128).

That acetylthiolcholine and acetylselenolcholine are truly isosteric was demonstrated recently by X-ray diffraction measurements (128a), which indicate that these compounds possess not only very similar dimensions but also identical conformations and identical packing patterns within the crystal, slightly different from those of acetylcholine. In spite of the steric similarity of these compounds and in spite of the fact that they are hydrolyzed by acetylcholinesterase at very similar rates, replacement of oxygen by sulfur or selenium in these isologs greatly alters their biological activities. In the electroplax, as well as in the guinea pig ileum and frog rectus abdominis preparations, replacement of the ether oxygen of acetylcholine by sulfur and selenium progressively decreases depolarizing activity. In the presence of an acetylcholinesterase inhibitor, acetylselenolcholine (in contrast to acetylthiolcholine) cannot induce full depolarization no matter how high the concentration (92).

The decrease in activity noted in the above series as the result of the introduction of sulfur and selenium stands in striking contrast to the depolarizing activities of their hydrolysis products. Cholinethiol is a more potent depolarizing agent than choline, which even at a concentration of 10^{-1} M depolarizes only very slightly. Cholineselenol is too oxidizable for reproducible values to be obtained. By carrying out measurements over a wide pH range, it could be shown that cholinethiol acts in the mercaptan and not in the mercaptide form. Methylation of choline, cholinethiol, and cholineselenol increased the depolarizing activities of these compounds. From these and related findings the conclusion was drawn that whereas the receptor of acetylcholinesterase possesses an "anionic" and an "esteratic" site (105), the receptor of the depolarizing membrane possesses an "anionic" and what is tentatively referred to as a "hydrophobic bonding" site (92).

While so far it has not proved possible to synthesize acetylthionocholine and acetylselenonocholine, the following compounds related to benzoylcholine have been prepared:



where B = O,S,Se.

Comparison of the antidepolarizing blocking actions of the above analogs in both the electroplax preparation and the giant axon of the squid, in all cases, led to the same relative order of activity, reinforcing the postulate that similar mechanisms are involved in the permeability changes during electrical activity of the membranes of axons and of synapses (103a, 122, 122a).

In the electroplax the weak depolarizing activity of the carbonyl compounds was altered little, but the antidepolarizing blocking activity increased as the side chain oxygen was replaced by sulfur and by selenium. On the other hand, replacement of the carbonyl oxygen by sulfur turned agents with a mixed effect into pure blocking agents.

Through dipole moment and spectroscopic measurements, relative electron distribution of esters, thiolesters, selenolesters, thionoesters, thionothiolesters, and thionoselenolesters can be visualized fairly precisely. Since the above compounds may be assumed to be essentially isosteric, it is tempting to extrapolate from the electron distribution of each active compound to the electron distribution of the receptors on which this series of compounds acts. In view of the complexity, already discussed, of membrane receptors, such an extrapolation seems premature. However, it seems likely that isosteric, nonisoelectronic antimetabolites will eventually prove to be valuable tools for obtaining information about the receptors on which they act as well as about the physical events which their attachment induces.

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