PHARMACOLOGICAL REVIEWS
Vol. 21, No. 2

Printed in U.S.A.

APPROACHES TO THE MOLECULAR NATURE OF PHARMACOLOGICAL RECEPTORS[1]

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I. INTRODUCTION

It is now almost a decade since the first attempts to isolate and characterize pharmacological receptors were made. In the intervening years, increasing numbers of investigators have been devising ever more sophisticated approaches

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to attack the problem of the molecular nature of receptors. It is our intention to analyze these approaches in a critical fashion, pointing out at least some of the pitfalls and difficulties encountered, in the hope that this may prove constructive for future work. Although definitive answers have not yet appeared, the evidence uncovered especially in recent years has revealed that receptors behave in many ways like other more conventional tissue macromolecules and can be subjected to the same kinds of manipulation. This evidence has done much to dispel the notion that receptors are highly mysterious substances with the property of "shadowy anonymity" (199), and it offers the expectation that before long precise knowledge of the nature of receptors or their active sites will be forthcoming. Such information will provide the basis for defining the molecular mechanism of action of many types of compound.

Several reviews have been written on receptors in this Journal (135, 155, 237) but these have been concerned with other aspects of receptor mechanisms. This review may be considered complementary to the others.

II. DEFINITION OF RECEPTOR

Before considering the various approaches used to obtain information about the nature of receptors, it is essential to define as rigorously as possible the meaning of the term "receptor." The need for this is great because in recent years the term has been widely applied to such a variety of pharmacological systems that it no longer has a precise and useful meaning. This is particularly true if one attempts to deal with the biophysical and molecular aspects of receptors rather than their physiological function.

We propose to restrict the term "pharmacological receptor" to a tissue component that fulfills all the following criteria: 1) A receptor is a macromolecule bearing sites having chemorecognitive properties for a specific natural endogenous molecule or for specific drugs. 2) The specificity of the sites on the receptor macromolecule for a particular endogenous molecule is genetically determined, and the receptor macromolecule has a genetically determined function. 3) Binding of agonists, whether the endogenous molecule or a drug, causes a specific perturbation or change in state of the receptor macromolecule or its immediate environment (the stimulus), or both, which initiates a chain of events leading to a response. 4) The initiation of a response by binding at a receptor site does not depend on the making or breaking of covalent bonds in the agonist. These criteria could encompass certain systems ordinarily not considered as classical pharmacological receptors, *e.g.*, the estrogen-binding protein (128, 129), but these will not be considered in this review.

On the basis of the last criterion, receptor sites are clearly separated from the substrate sites of enzymes. This does not exclude the possibility that a subunit of an enzyme molecule can bear receptor sites. In use, the term "receptor" requires qualification in three respects: the type of endogenous molecule or drug involved, the response initiated, and, if necessary, the specific tissue. It should be emphasized that drugs can combine not only with a particular receptor site but also with other sites in the body. Among these are sites of loss (229), which

result in no response. They may also combine with acceptor sites, reaction with which either initiates a response or modulates an existing response. Acceptor sites may be located at or near the receptor, as defined above, but may also be located on enzymes involved in the synthesis or metabolism of endogenous molecules or on macromolecules involved in their uptake, storage, or release.

Although there are many receptor types, we will limit our attention to studies designed to investigate the nature of pharmacological receptors for acetylcholine (ACh), norepinephrine, serotonin, vasoactive polypeptides, and histamine. The main emphasis of this review, particularly in considering isolation studies, will be on the cholinergic receptor, since most studies in this field involve this system.

III. CRITERIA FOR IDENTIFICATION OF AN ISOLATED RECEPTOR

Before discussing the isolation studies that have been undertaken, we will set forth some of the criteria considered essential for identifying or assaying a given tissue component as a receptor.

1) A receptor candidate must exhibit a high specificity of combination with drugs known to react with the receptor of the particular tissue used.
2) Changes of state of the receptor candidate induced by drugs or other agents should correlate with the effects of these drugs or agents on the tissue from which the receptor originates.
3) The amount of isolated receptor must be consistent with amounts estimated to be present in the tissue used.
4) The receptor candidate must be shown to originate from those regions of the cell where it is demonstrated to exist *in situ*.

For reasons discussed below, the minimal requirement for a receptor candidate would be the fulfillment of all these criteria.

A. Drug interactions

Perhaps the outstanding property of receptors is their ability to combine in specific ways with agonists and antagonists. This is the basis of most receptor bioassays (51–54, 65, 76, 77, 83, 165, 247, 248). Interaction of drugs with receptor candidates is too gross a feature, however, to be the sole criterion for identification since many macromolecules that obviously could not function as receptors combine with highly specific agonists and antagonists (81, 87). In addition, one needs to know that the affinities of the candidate for several compounds, particularly antagonists, parallel the pharmacological potencies of these compounds (76–78, 83). The structure-activity data compiled for several classes of drug, mainly cholinergic and adrenergic agonists and antagonists (7, 146, 227), should greatly facilitate the identification.

1. Difficulties of interpretation. There are many difficulties in extrapolating pharmacological data to test-tube interactions. For example, there is no certainty that drugs react with an isolated receptor candidate in the same manner as they do with the receptor *in situ*. This uncertainty stems from two sources. First, the extraction procedure might change the reactive site of the receptor

molecule, *e.g.*, by denaturation of the protein part, dissociation of a macromolecular complex, or removal of a cofactor or prosthetic group. Second, it is highly likely that receptors are integral parts of membranes, and thus their reactivity after extraction could be very different from what might be observed in solution. *In situ* a receptor may be associated with adjacent membrane macromolecules that could greatly influence the binding site or other aspects of its reaction with drugs. These circumstances could not be readily duplicated in an isolated system unless one had a membrane preparation. Thus the reactivity of membrane-bound cholinesterase (ChE) *in situ* towards a number of inhibitors and substrates may be very different from its reaction in solution (85, 91).

There is some question whether potencies of all compounds having a particular pharmacological action necessarily parallel their affinities for a receptor. This might be true for antagonists, but doubt has been raised that the same holds for agonists (84, 85, 101a, 178, 207).

With regard to affinities of agonists in particular, a number of paradoxes may be discerned in attempting to correlate strength of binding in solution with potency. For example, as the substituents on the nitrogen of ACh or other alkyltrimethylammonium compounds are changed to progressively larger alkyl groups, the apparent affinity for the cholinergic receptor decreases progressively (7), *i.e.*, the potency decreases progressively. This could result from steric hindrance of the interaction of the charged nitrogen with an anionic site on the receptor, yet this kind of chemical modification almost invariably results in compounds having *greater* affinity for most macromolecules, due to the increasing ability to form hydrophobic bonds, a factor that appears to override steric effects. This is the case for the binding of such compounds to cholinesterase (24, 30), chondroitin sulfate (87), other acidic polysaccharides, and nucleic acids (81). Binding of other compounds (*e.g.*, fatty acids and barbiturates) to bovine serum albumin follows the same general rule of increasing affinity with increasing chain length (38), and, as Brodie and Hogben (38) have pointed out, the principles of binding of small molecules to serum proteins would most likely hold for interactions of such molecules with tissue proteins.

Several other comparisons of the relative affinities of ACh and its congeners illustrate the difficulties of using affinities to identify receptor candidates. The lipid-soluble quaternary ammonium compounds noracetylcholine 12, norcholine 12, and pyridine aldoxime dodeciodide have an ACh-like action on electroplax (194), *i.e.*, they depolarize this tissue by activation of the ACh receptor. Their potency as agonists is of about the same order as that of ACh. In most other tissues, these compounds act as antagonists of ACh, particularly at muscarinic sites (86). As inhibitors of ACh action on guinea pig ileum they seem to have significantly lower affinities for the receptor than does ACh (S. Ehrenpreis and M. Bigo-Gullino, unpublished data), *i.e.*, much higher concentrations are required to inhibit ACh than for ACh to stimulate the tissue. Yet, in binding to various macromolecules, the lipid-soluble quaternary ammonium compounds have a much higher affinity than ACh (30, 81).

A somewhat related problem concerns the comparative affinities of ACh and

choline. From their pharmacological activities, one would expect choline to bind far more weakly to the receptor than ACh. In contradistinction, the hydrolysis products of certain selenium analogs of ACh are quite potent, perhaps even more so than the intact molecule (112). Yet ACh and choline would probably bind equally well to isolated macromolecules. Even with ChE, which has a surface highly complementary to that of ACh, the affinities of choline and ACh are similar (13).

Similar difficulties would be encountered in the case of affinities of ACh and tubocurarine to isolated macromolecules, *e.g.*, in relation to their affinities for the receptor of the neuromuscular junction. Both compounds have affinity constants for the receptor in the range of 10^6 M^{-1} (127) (the affinity constant for ACh estimated from its ED50), but tubocurarine is far more strongly bound (100 × to 1000 ×) than ACh to various macromolecules, *e.g.*, cholinesterase (55, 114, 115) and chondroitin sulfate (87). This finding is not unexpected in view of the profound differences in their chemical structure. These differences in binding may well extend to an isolated receptor substance, and if they do, agonist affinity would be of little value for the identification of a receptor substance in solution. It is possible that ACh actually has a relatively low affinity for its receptors and that its high potency depends on a very high efficacy (101a, 207). Alternatively, it is possible that the primary determinant of the potency of an agonist is the rate of receptor occupation, as suggested by Paton (178). Thus the potencies of agonists may not necessarily reflect directly their affinities for a receptor but rather their abilities to initiate the series of events that results in the overt response. Although these parameters of drug combination might possibly be measured with macromolecules, one may be compelled to rely on antagonist interaction for identification of receptors because their pharmacological potency might indeed bear a direct relationship to receptor affinity.

Atropine might thus be considered as an almost ideal compound to assay receptor candidates, particularly since it binds very weakly to well known macromolecules (177), whereas its affinity constant for muscarinic receptors is extremely high, about 10^9 M^{-1} (179). This value is consistent with the binding of labeled atropine to components of intact guinea pig ileum. However, it has been suggested (84, 85) that the great potency of atropine-like compounds arises not from their ability to form a strong complex but rather from the formation of a covalent bond with some highly specific tissue receptor. In fact, there is some evidence that covalent bonding may occur only in the intact tissue (179), and thus it may be futile to use the drug to trace its receptor on the basis of reversible binding in solution.

Many other problems may be recognized in assessing the affinities of compounds for a particular receptor *in vivo*. This includes both agonists and antagonists. Agonists could act indirectly, *i.e.*, by releasing some active substance, most often ACh or norepinephrine (102, 133, 191). This of course must be taken into consideration in calculating affinities. Furthermore, if the first member of a series of compounds is known to act directly, there is no assurance that even simple chemical modification does not result in a change in activity from

direct to indirect, or both. Evidence that this does not occur is very scanty even in the extensive studies of Ariëns (7) and van Rossum (227). These complications arise because of the complexity of all tissue preparations. Smooth muscles contain ganglia and nerve terminals, and striated muscles contain nerve terminals as well as extrajunctional sites of action. Even drugs that classically have been considered to combine solely with postsynaptic receptors, *e.g.*, tubocurarine and succinylcholine, have additional sites of action (182–184, 204, 205).

Another complication may be discerned in structure-activity studies. In the case of drugs in a homologous series, as the nonpolar character increases, so too does lipid solubility as well as the tendency to react nonspecifically with many types of macromolecule. These two factors could change the drug from one that combines with a single receptor to one that reacts with several receptors as well as nonreceptor sites. The latter sites might be masked by lipid barriers and thus not be accessible to members of the series of lower molecular weight, which are poorly lipid-soluble. Norcholine 12, with a dodecyl group on the nitrogen, is a case in point. At low concentrations it readily blocks contractions of rabbit aortic strip to ACh, norepinephrine, histamine, serotonin, and even KCl (86), whereas extremely high concentrations of choline, having only methyl groups on the nitrogen, have little if any effect against these agonists.

2. Methods of studying interactions. Despite these many difficulties, identification ultimately requires the determination of affinity constants of a number of compounds for many types of tissue component. For this purpose, it is necessary to have each component in a purified, homogeneous form. Equilibrium dialysis is probably the method of choice for determining affinity constants, but it has the great disadvantage of requiring large amounts of macromolecule and having large inherent errors.

Several other methods requiring small amounts of material are available for determining various aspects of the interaction of small molecules with macromolecules. The quantitative precipitation method devised by Ehrenpreis (76, 77, 81, 83, 87) requires only about 0.1 the amount of macromolecule needed for equilibrium dialysis, is rapid and highly reproducible, and readily gives relative affinities as well as binding capacity. The influence of pH, ionic strength, temperature, specific ion effects, *etc.*, can be assessed. This method cannot be used to obtain absolute affinity constants but one can correlate strength of binding of a series of drugs with their potency *in vivo*. Although the method is restricted to drugs that precipitate macromolecules, it has recently been adapted for use with compounds forming soluble complexes with the macromolecule by determing their ability to compete with one that precipitates the macromolecule (145). Of course, the method cannot provide information about interactions in the subprecipitating range of concentration. It is known for many proteins that there may be several classes of binding site with very different affinities (see review, 206). The class with the highest affinity may be concerned not with precipitation but rather with the formation of soluble complexes.

Recently, Kruse (145) showed that equilibrium dialysis and precipitation gave complete agreement with respect to the effect of a number of variables on the

tubocurarine-chondroitin sulfate interaction. These findings strengthen the validity of using the precipitation method.

As a modification of the precipitation method, the development of turbidity has been used as a measure of drug-macromolecular interactions (165, 166).

The method of ultraviolet difference spectra has been used to study the binding of atropine to serum albumin (152), the interaction of local anesthetics with nerve extracts (83), and the interaction of catecholamines with various proteins (91, 93). However, there is no simple way to calculate affinity constants or binding capacity from such spectrophotometric measurements. In addition, the method cannot be used with drugs that do not absorb in the ultraviolet unless the drug produces a difference spectrum by causing a change in configuration of the interacting macromolecule or by combining directly with aromatic amino acids of the macromolecule. Alternatively, a nonabsorbing drug could be studied on the basis of its competition with one that does show an ultraviolet difference when it interacts with a given macromolecule.

Dikstein (68) measured drug-induced changes in the surface area-tension relationships of monolayers of stearic acid in an attempt to explain the mechanism of action of drugs that block nerve activity (termed "membrane stabilizers") and those that produce firing of a nerve ("membrane labilizers"). In a sense, interaction of drugs with macromolecules as determined by this method is perhaps more closely related to the reaction of drugs with receptors since these take place also on membrane surfaces or interfaces.

Another promising method involves the use of fluorescence polarization. This has the advantage of detecting interactions at very low concentrations of small molecules, and through its use it was demonstrated that gangliosides interact with a variety of drugs including tubocurarine, chlorpromazine, histamine, and serotonin (1). The drugs were in the concentration range of 10^{-7} M.

Woolley and Campbell (247, 248, 250, 251) devised a novel method for studying the interaction of ACh or serotonin with isolated tissue components. This method was based on the hypothesis that the reaction of certain receptors with agonists renders calcium ions lipid-soluble and so enables this cation to be transferred across a cell membrane to the interior surface, where the calcium is liberated upon enzymatic destruction of the receptor. In their assay, a receptor substance was one which, in conjunction with a neurohormone (ACh or serotonin), enabled Ca to enter a completely nonaqueous phase, *e.g.*, benzene-butanol. It was possible to extract an enzyme from the stomach that destroyed the "receptor" and thereby diminished the transport of calcium (249).

The use of membranes or membrane preparations might represent a most valuable advance in studying interactions. Such studies should be greatly stimulated by the availability of methods for the isolation of intact muscle membranes (15, 157). The interaction of drugs in such a system rather than with soluble components could simulate more closely what occurs with the receptor system of muscles. Fixation of labeled cholinergic compounds to subcellular fractions has recently been explored by de Robertis *et al.* (14, 65). Tobias *et al.* (218) showed that certain drugs can cause artificial protein-phospholipid mem-

branes to undergo some of the changes of ionic permeability exhibited by cell membranes exposed to these drugs, although these authors would not go so far as to claim that the ACh receptor is simply a phospholipoprotein complex. Del Castillo's recent preparation in which enzymes are incorporated into artificial lipid bilayers (63, 64) is of interest in that these systems can generate potentials under the influence of substrates.

B. Induced changes of state of receptors

It is evident that binding of drugs is only the first important step in identifying a given macromolecule as a receptor. In addition, the interaction should perhaps result in specific changes in macromolecular properties; such changes should be different for agonists and antagonists. It is generally agreed that agonist-receptor interaction results in an increase in membrane permeability to ions (110). The molecular basis for this action is a matter of conjecture although it has been postulated that ACh and other quaternary ammonium compounds change the configuration of the receptor (24, 28, 55, 76, 139, 164) and that this change is opposed by an antagonist or receptor inhibitor. Belleau (27) has proposed that structural water changes during receptor reactions, and that this should be manifested by a negative entropy of binding of agonists and a positive entropy of binding of antagonists. Each of these parameters can be measured with isolated components: a change in configuration by optical rotatory dispersion (196) and entropy changes from the thermodynamics of binding (27).

Watkins (236) has suggested that an agonist causes the dissociation of phospholipid from the protein portion of a phospholipid-protein receptor. Ehrenpreis accepted this latter proposition and suggested a mechanism by which antagonists could oppose the effect of agonists (84, 85).

One real indication that rather profound changes can occur in receptor configuration is the well known desensitization that is observed when agonists are applied for relatively long periods (94, 106, 167–169). This desensitization seems to resemble reversible denaturation of proteins, although there are other interpretations of this phenomenon (168).

Other perhaps more drastic methods for altering the state of receptors have recently been explored. These include a determination of the susceptibility of receptors to denaturation by heat and urea and their susceptibility to enzymatic alteration and chemical modification. Such studies, which will be discussed in section V, have provided important information about the molecular properties of receptors that could possibly be measured in isolated macromolecular systems.

C. Amount of receptor candidate isolated

The importance of having some information about the amount of receptor expected to be isolated stems from the fact that interacting drugs lack specificity. Nonreceptor material would certainly constitute the bulk of what is isolated and such elements could have some or perhaps many of the binding characteristics expected of the receptor. The amount of receptor present in a particular tissue could serve as a valuable guide in judging the success of identi-

fication procedures. This would be true whether the direct or indirect method of isolation is used (see below).

The amount of any receptor substance that may be isolated even from the most favorable tissue is almost certainly extremely small. Electric organ of electric eel has an extremely high density of receptor material; a single cell weighing 30 to 50 mg contains 30,000 to 50,000 synapses and consists of only 2% protein. Thus the amount of nonreceptor material is small in this tissue as compared to others. From Waser's (231, 232, 234, 235) data for the number of receptors per endplate, about 10^7, the calculated amount of ACh receptor in electric organ is 10 to 20 mg/kg.[5] It should be noted that Waser's value is based on the assumption that fixation of labeled cholinergic drugs occurs only with specific receptors or acceptors. There is ample evidence that macromolecules that combine with the same drugs are present at other sites, *e.g.*, nerve axon, nerve terminal, conducting membrane, ChE, *etc.* Cholinesterase in particular combines rather strongly with various neuromuscular blocking agents including tubocurarine (55, 114, 115), decamethonium (55, 114, 115, 209), and gallamine triethiodide (55, 114). Thus the figure arrived at by Waser on the basis of the binding of curariform drugs *in situ* may be far too high and the quantity of endplate receptors quoted above would be an upper limit.

When Waser studied depolarizing agents such as muscarone, the apparent uptake was several orders of magnitude greater than that of curarine or toxiferine and even failed to reach equilibrium (234, 235). This finding probably does not reflect a greater binding capacity for muscarone, *i.e.*, a greater number of receptors. The results of Nedergaard and Taylor (170) probably provide an explanation. They showed that in the isolated diaphragm of the guinea pig, uptake of decamethonium increased progressively with time, whereas that of dimethyl-*d*-tubocurarine rapidly reached a constant level. Thus a depolarizing agent appears to penetrate into the intracellular compartment of muscle whereas nondepolarizing blocking agents remain fixed to surface receptors or acceptors. Yet atropine does not depolarize guinea pig ileum but penetrates it (78).

Other estimates for the number of cholinergic and *alpha* adrenergic receptors in smooth muscle have been made by Paton and Rang (179) and by Moran *et al.* (162), respectively. It is indeed surprising that their estimated values were close (10^{12} to 10^{13} receptors/g of tissue) in spite of the use of entirely different methods as well as the fact that two different receptors were examined.

One point to be emphasized is that it is actually not the amount of receptor material which can be determined but rather the total number of binding sites. The values obtained from drug fixation refer to the latter value, not the number of receptor molecules at a junction. There is some evidence, albeit meager, that suggests that the number of binding sites per receptor molecule is very small, perhaps one or two (214, 223, 228) although Inoue (124) contends that the num-

[5] This calculation is equivocal as it makes use of a figure obtained for mouse diaphragm endplates. In addition, the assumption was made that the molecular weight of the receptor is 100,000. In the absence of any directly determined values, this calculation is somewhat useful, in that it provides an order of magnitude for the amount of receptor expected.

ber could be as high as five. If the value is unity, then the total number of receptor molecules and binding sites would be identical.

One important question concerns the number of spare receptors and whether these are accounted for by methods designed to determine the total number of receptors. Evidence for their existence has been postulated on the basis of a variety of pharmacological experiments (11, 12, 172, 207, 228). It is presumed that the values obtained by Waser (231, 232, 234, 235), by Paton and Rang (179) and by Moran *et al.* (162) include such receptors, although this is not a certainty.

D. Localization and distribution of receptor candidate

One must have an adequate method for demonstrating where the proposed receptor candidate is found in the tissue. One possible way is by means of immunohistochemistry, used by Ehrenpreis (78) to localize the drug binding protein of electric organ of electric eel. This method is probably not precise enough to localize a receptor with the accuracy required. The ferritin-labeled antibody technique coupled with electron microscopy might prove more successful.

In considering the distribution of cholinergic receptors in particular, we are confronted with varying and conflicting hypotheses. Thus, Nachmansohn (164) claimed that the receptor is present at postsynaptic membranes, as well as in conducting membranes of nerve and muscle. Evidence for and against this theory has been discussed in great detail elsewhere (82), with the conclusion that the belief in a widespread distribution of ACh receptors, particularly within nerve axons, was not justified. Some of the most definitive evidence against the proposal that an ACh receptor system is involved in conducting membranes appeared more recently. For example, denervation of electroplax markedly decreased the content of choline acetylase whereas ChE remained fairly constant (195). This observation is in agreement with others (95, 118) that only the synthesizing system for ACh as well as ACh itself (118, 143, 208) is predominantly neural. It has now been shown that a highly potent ChE inhibitor penetrates into the axoplasm of squid nerves at a 1000-fold higher concentration than is required to inhibit all of the enzyme, yet the compound had little effect on the action potential of the nerve (122). Also, the restoration by pralidoxime (PAM) of nerve activity after complete block by paraoxon (66) was not accompanied by an increase in ChE activity (92). Moreover, certain sulfur and selenium compounds related to ACh (238) depolarize electroplax by activating junctional receptors whereas these compounds caused a nondepolarized block of axonal conduction (195a). Ritchie and Armett (185, 186) have demonstrated clearly the lack of predictability of Nachmansohn's theory in their studies of the action of cholinergic drugs on nonmyelinated nerves.

There is little doubt that components in conducting membranes can react with many, if not all, of the drugs known to combine with the cholinergic receptor of the postsynaptic membrane. This contention is based on evidence from the direct application of such drugs to the conducting membrane of muscle after denervation (160, 214), to isolated squid nerves which have been treated with venoms or

detergents (192, 193, 194) or to nonmyelinated nerves (185, 186). Components that react with tubocurarine have in fact been isolated from sciatic nerve (83, 171). However, the conclusion (164) that these components represent ACh receptors is questionable in view of the lack of pharmacological evidence for the essentiality of the ACh system in nerve axons. It has been demonstrated by a variety of pharmacological techniques that the nerve terminal is a site of reaction for many cholinergic drugs. Tubocurarine can prevent the release of ACh upon nerve stimulation (19). This is said to be due to a combination of the drug with cholinoceptive sites present in the nerve ending. Waser's autoradiographic studies showed that the denervated diaphragm of the mouse fixed far less curariform drugs than the innervated control (234). Riker and Standaert (184, 204, 205) presented considerable evidence that many types of facilitatory and inhibitory drugs influence nerve terminal activity more readily than neuromuscular transmission. However, it must be emphasized that in their hypothesis the reaction of drugs at the nerve terminal does not involve the ACh system, *i.e.*, cholinesterase or the ACh receptor. Thus tubocurarine is not considered to compete with ACh for a receptor in the terminal, nor are the effects of neostigmine, physostigmine, or isofluorophate (DFP) considered to result from a reaction with ChE. Indeed, it has been suggested that most of the actions of tubocurarine are neural (205).

Recent studies by Hazra (117) involving botulinum toxin have shed light on the question of the major site of action of a number of important cholinergic compounds. On the phrenic nerve-diaphragm preparation, abolition of nerve activity by this agent had little effect on the dose-response curve to injected ACh or on potentiation of injected ACh by isofluorophate. These results are in agreement with earlier work of a more qualitative nature (6, 38–40). Botulinum toxin blocks posttetanic repetition, a phenomenon shown to arise in nerve terminals (205). Hazra's results thus suggest that nerve terminal activity is not essential for initiating the response of exogenously applied ACh or for the potentiating action of isofluorophate.

Studies on denervated skeletal muscles are also in accord with a primary postsynaptic site of action for many types of cholinergic agent. It is well known that such muscles usually retain their sensitivity to ACh whether applied locally (160, 214) or by injection (147). Furthermore, the organophosphorus ChE inhibitor Soman was reported to potentiate ACh contractions of the denervated rat anterior tibial muscle (148). However, the soleus muscle appears to be exceptional in that denervation causes a loss in sensitivity to injected ACh within 24 to 48 hr (176).

Blaber and Karczmar (34) have pointed out the multiplicity of sites of action of cholinergic drugs at junctions. In some respects, their concepts are consonant with those of Riker *et al.* (182–184). However, the important distinction to be made is that Blaber and Karczmar interpreted all their results in terms of the ACh receptor system. They concluded that a receptor for ACh is present within the nerve terminal as well as postsynaptically.

In a related hypothesis, Koelle proposed that ACh liberated upon stimulation

of certain preganglionic nerves reacts at the nerve terminal to liberate additional ACh (140). Although it is not stated explicitly, one may assume that this nerve terminal effect is mediated by an ACh receptor. In a somewhat similar vein, the ability of ACh to liberate norepinephrine from nerve terminals, as suggested by Burn and Rand (41), would be expected to involve an ACh receptor. The question arises whether this release of norepinephrine by ACh actually occurs physiologically. If it does, then the nerve terminal component would be considered a true ACh receptor as defined earlier.

In summary, there seems to be little doubt that macromolecules are present in nerves, particularly in the terminals, that combine with ACh and other cholinergic agents including facilitators and blockers of transmission. What still remains in doubt is whether this represents a true ACh receptor system and if so, whether it is identical to the receptor system of the postsynaptic membrane. On the other hand, the evidence is fairly conclusive that such a receptor system is not present in the nerve axon as distinct from nerve endings.

IV. ATTEMPTS AT ISOLATION

A. Direct attempts

One of the approaches in the identification and isolation of receptors is the tagging of receptors *in situ* by using drugs bearing an irreversibly reacting moiety. This use of site-directed irreversible reagents has been explored for the labeling of active sites on enzymes (29, 245) and antibodies (16, 57, 180, 203). Application of this method to the problem of receptors has some special advantages and also some serious defects which have recently been discussed by Moran and Triggle (163). One of its major advantages is that one can study the relationship between the extent of receptor labeling and the change in degree of response of the tissue. In addition the possibility of labeling receptor molecules *in situ* before the disruption of the tissue obviates many of the problems of studies of reversible drug-binding as outlined in section III.

The most serious problem encountered in this approach is that of specificity of reaction. In the case of enzymes or isolated macromolecules, this problem is minimized because one is dealing with a purified single macromolecular species, but it assumes paramount importance when applied to whole tissues. A high pharmacological potency of the drug used is no guarantee for selective binding to the specific receptor responsible for the response that is measured, because the drug may have a high binding affinity to other sites as well. The problem of nonspecific binding at other sites is intensified by the fact that with reagents forming covalent bonds the interaction is of a nonequilibrium nature, and the amount of labeling depends in part on the rate of binding.

Means of dealing with this problem have been suggested. The first is to use a reagent that reacts in two steps. The initial step, which has a high affinity constant, is a reversible binding of the reagent with the receptor and the subsequent step a covalent reaction which is preferably accelerated by some special circumstances occurring at the site of binding. The second means of reducing nonspecific labeling is the use of a protection experiment in which a drug (agonist or specific

antagonist) protects the specific receptor site while the nonspecific sites react with the unlabeled irreversible reagent. This is followed by application of the radioactive, irreversible agent after wash-out of the protector. Unfortunately such protectors may also protect sites of nonspecific reaction as well as the receptor sites.

The most extensive use of this approach has been made by Takagi *et al.* (210, 211) with labeled Dibenamine as the tag for the ACh receptor of dog intestine. Takagi used atropine as the protector in a type of experiment devised by Furchgott (101). Unlabeled Dibenamine was applied in the presence of atropine to saturate nonspecific sites. After washout of the atropine, labeled Dibenamine was applied and this should now have combined only with those sites initially occupied by the atropine, namely the cholinergic receptor. However, when the tissue was fractionated by differential centrifugation, the label was still found irreversibly bound to components in all seven fractions isolated. Subsequently (211), data were presented to show that two fractions contained a significantly higher specific activity than the others. Since the bulk of this radioactivity was liberated into solution by proteolytic enzymes, it was suggested that the "Dibenamine receptor" is protein in nature. It can be calculated from these data that the number of atropine-protected sites is about 2×10^{15}/g wet weight. This is 10 to 20 times greater than the total number of the ACh receptor sites of guinea pig ileum (179) and suggests that atropine is able to protect a significant number of nonspecific sites against Dibenamine.

One important aspect of this approach is the requirement that a compound that protects the receptor, in this case atropine, is indeed a competitive inhibitor of ACh. Although there is much evidence in support of this mechanism (12, 216), this aspect of atropine's action has been seriously questioned in recent years (10, 67, 84, 85, 163). Furthermore, there is some question of the completeness with which atropine can protect against Dibenamine. Furchgott (101) showed that on rabbit aortic strips, even a high concentration of atropine (2×10^{-7} M) failed to fully protect against 10^{-5} M Dibenamine. However, this finding is subject to revision since it is possible that the residual atropine concentration in the tissue at the time of retesting (1 hr after washout of the atropine-Dibenamine incubation mixture) may have been enough to exert some competitive antagonism on retesting. Thus a greater degree of protection might actually have been achieved (R. F. Furchgott, personal communication). That Dibenamine 10^{-5} M blocks KCl contraction (31) suggests a nonspecific action.

Moran *et al.* (156, 162) attempted to label the adrenergic receptor of rabbit aortic strip and vas deferens with an irreversible, presumably highly specific alkylating agent, S.Y.28 (N-ethyl-N-1-naphthylmethyl-2-bromoethylamine). When this compound was applied to the tissues with the protection procedure, the following results were obtained: 1) No saturation was observed in the uptake nor was there a break in the uptake curve to suggest that different classes of components were binding the compound. 2) Pretreatment with another, short-lasting alkylating *alpha* receptor blocking agent did not materially influence uptake. 3) Pretreatment with high concentrations of norepinephrine also did not

influence uptake. These results suggested that the SY compound is not specific enough to label the receptor, and it was proposed that catecholamine storage sites might be responsible for the greater part of the uptake. Similar proposals to account for the action of this compound were made earlier by Furchgott and Kirpekar (101b) on the basis of purely pharmacological experiments.

Chagas *et al.* (51–54, 113) attempted to label the cholinergic receptor of electric organ of electric eel by using a reversibly binding compound, gallamine triethiodide. The compound was injected into electric eels and allowed to fix to its receptor, and then the electric organ was removed, homogenized, and fractionated. This approach has the disadvantage that upon homogenization of the tissue, the initial drug-receptor equilibrium would be disturbed and the drug would dissociate from its original binding site. Once this occurs, the possibility exists that the highly cationic drug might bind to a variety of macromolecules present in the extract. One particular macromolecule, a polysaccharide resembling hyaluronic acid, bound more gallamine triethiodide than other components extracted. This finding suggested to the Chagas group that this polysaccharide might be identical with the cholinergic receptor. Studies of the competition between various drugs and the binding of gallamine triethiodide strengthened this interpretation (53). However, these experiments were carried out in distilled water and thus are of questionable significance. For example, tetraethylammonium did not compete with gallamine triethiodide, yet this compound has important curariform actions (130) and thus should also bind to a cholinergic receptor in solution.

When binding of cationic drugs to anionic macromolecules is not carried out in buffered solution of fairly high ionic strength, there is a possibility of nonspecific retention within the dialysis bag due to the Donnan effect. This was clearly shown for the interaction of hyaluronic acids with various cholinergic drugs. In buffer of 0.1 ionic strength, the interaction of gallamine triethiodide with the "hyaluronic acid receptors" of electric eel was very weak (affinity constant 5×10^2) (116) despite the apparently strong binding in distilled water. Ehrenpreis and Kellock (90) detected no binding of tubocurarine to purified hyaluronic acid (bovine nasal cartilage) under physiological salt concentration and pH whereas considerable binding of this drug was observed in distilled water. Other quaternary ammonium compounds, including those which are far less potent pharmacologically than tubocurarine, were bound about equally well in distilled water. In other words, such interactions appear to be nonspecific under these conditions. Aside from this, the question remains whether a macromolecule such as hyaluronic acid with its multiple binding sites for cationic molecules can be a reasonable receptor candidate since, as indicated previously, the receptor would most likely have only a limited number of active sites.

In a more recent study, the compound *p*-(trimethylammonium)benzenediazonium fluoroborate (Tdf) irreversibly blocked depolarization of electroplax by carbachol (56). This finding demonstrated the feasibility of tagging this receptor *in situ*. Tubocurarine or phenyltrimethylammonium could prevent the irreversible effect of Tdf. Thus, a typical protection experiment could be performed. The fact that these cationic compounds could protect indicates that the irreversible

drug reacted at the anionic site of the receptor. Although this experiment supports the specific nature of the reaction, the effect of Tdf on KCl depolarization was not determined, and since fairly high concentrations of the reagent were used (10^{-4} M) it is conceivable that part of the block is nonspecific. The carbachol log dose-response curves after progressive exposure to Tdf show nonparallel shifts to the right as well as a progressive decline in the maxima (56). The absence of an initial parallel shift with maintained maximum suggests that in electroplax either there are no spare receptors or Tdf reacts at some other level in blocking the response. In any event, this compound does not seem to have enough specificity to identify the receptor in this tissue as it has been shown to combine irreversibly with ChE at a concentration 10 times lower than that used to block the receptor (245).

An inhibitor resembling a choline ester could prove to be somewhat more specific in labeling cholinergic receptors. Such a derivative, benzilylcholine mustard, has been synthesized by Gill and Rang (105) and is a highly potent, essentially irreversible blocking agent of muscarinic receptors. It is taken up by guinea pig intestinal tissue to an extent at least five times more than by other tissues when applied at a concentration of 3×10^{-9} M (181). Atropine, 3×10^{-8} M, reduced the uptake by about 50%, while nonatropinic drugs (tubocurarine, physostigmine) up to 10^{-6} M did not influence the uptake. Uptake failed to reach saturation even up to 2 hr. It was concluded that despite considerable nonspecific binding, the compound could be of use in tagging muscarinic receptors with the aid of atropine as a protecting agent.

B. Indirect attempts

Ehrenpreis (76, 77) embarked on a series of studies to examine the question of whether the ACh receptor from electric tissue of electric eel could be identified by indirect methods. This should in theory be possible if the high affinity of the receptor for a drug, as demanded by occupation theory, survived disruption of the tissue. In other words, the receptor should be present among those tissue components having a high affinity for cholinergic drugs. The rationale used was to examine essentially all the material in this tissue for receptor activity as indicated by the binding of tubocurarine. This drug had a much higher affinity for macromolecules present in ammonium sulfate precipitates of the extract of this tissue than for the remaining material; for the first time tubocurarine, a potent receptor blocker, was shown to form a precipitate with a macromolecular component from a tissue on which the drug is active. This observation gave hope that among the tubocurarine-binding components was one that could be considered as the receptor in soluble form. By means of the method of tubocurarine precipitation, a phospholipoprotein was isolated and shown to be homogeneous to the extent of 85 to 90% by free electrophoresis at pH 7.5 (77) and gel electrophoresis at a series of pH's (L. Ornstein, personal communication).[6]

[6] Beychok (32) has claimed that this protein was heterogeneous when examined by chromatography on DEAE cellulose. However, the protein is extremely difficult to elute from such an exchanger and the extent of recovery from the column was not indicated.

Studies by equilibrium dialysis revealed the following: *d*-tubocurarine was bound more strongly than dimethyl-*d*-tubocurarine; this implicates hydrogen bonding in the interaction. Many other quaternary ammonium compounds were bound in what appeared to be a manner parallel to their pharmacological activity on electroplax. The binding of such compounds reached saturation at about 10 molecules/100,000 molecular weight. Since this seemed to correlate with the number of phosphate groups in the protein, it was proposed that these formed the anionic part of the binding sites.

This protein thus appeared to fulfill the first criterion for a receptor (see section III) with respect to the binding of cholinergic drugs, although its affinity for ACh was of a rather low order compared with the potency of this compound on electroplax. Binding alone is obviously an insufficient criterion since many acidic macromolecules were subsequently shown to precipitate with tubocurarine, *e.g.*, chondroitin sulfate, nucleic acid, and casein (76, 81, 87). However, the precipitate formed with the electroplax protein had the characteristic of being very slowly reversible compared with those of other complexes. Many additional aspects of the binding of cholinergic drugs with these macromolecules have been reviewed elsewhere (81).

The deciding factors for this receptor candidate would thus be the quantity isolated as well as its exact cellular location. The first of these appeared to be satisfied in that the amount extracted (76) was of the order predicted on the basis of estimates discussed earlier (section III C).

In order to define the location of the protein, fluorescent antibodies were prepared[7] (78) and applied to sections of electroplax. The distribution of fluorescence indicated that the protein originated from membranes of the cell, none being found in the interior. Contrary to expectations, both the innervated and non-innervated membranes contained the protein. On the basis of current theory, the ACh receptor is located only on the innervated membrane. Hence the original interpretation of the role of this protein was modified (79, 80). The protein is now considered to be an "acceptor" for drugs acting not at ACh receptor sites but within conducting membranes, for example local anesthetics. It has been further proposed (82, 83) that phosphate groups on a similar protein may be sites of binding for such drugs in nerves; subsequently, several investigators have provided additional evidence to support this contention (35, 36, 97).

Nevertheless, there was still an obvious paradox in that the protein does combine strongly with tubocurarine and with other quaternary ammonium compounds that do not ordinarily affect the conducting membrane of electroplax.

From optical density measurement on the peaks that were eluted, it would appear that virtually all of the protein was not eluted under the conditions used. Beychok also reported binding studies with the material removed from the column but these studies obviously were not done on a major fraction of the material applied. Beychok did confirm that the protein was about 85% homogeneous in free electrophoresis (S. Beychok, personal communication).

[7] The antiserum was used to check the homogeneity of the protein by immunodiffusion; this test confirmed its purity as ascertained by electrophoresis.

It is believed that such compounds, unlike lipid-soluble drugs, simply cannot reach the active membrane when applied to the intact tissue. If accessibility can be promoted, then the reaction with such membrane components may be observed pharmacologically. That accessibility is an important factor was shown by the use of cationic detergents and cobra venom; these permitted tubocurarine, chlorisondamine, and even protamine to block action potentials of squid nerves (192, 193), perhaps by reacting with a membrane protein similar to the electroplax protein. Subsequently, it has been shown that a protein or macromolecule that reacts with drugs in a manner similar to that of the eel protein could be isolated from nerve tissue (83).

In another study with electric organ of electric eel as a possible source of receptor material, Trams (220) showed that microsomal proteins bind dimethyl-*d*-tubocurarine at 10^{-8} to 10^{-9} M. The apparent high affinity of this drug was considered to be evidence that the preparation contained receptor substance. In actuality, strength of binding appears to be quite low, particularly since the binding site or sites were not saturated even at the highest concentration of drug used (2.5×10^{-6} M). Nevertheless, the comment was made that this result "substantiated the author's skepticism regarding the isolation of receptors by methods such as proposed by Ehrenpreis." This statement implies that soluble components do not have an important role in the effects of tubocurarine on electroplax. This supposition may well be correct and was pointed out even earlier by Ehrenpreis (78, 80), but the data on which Trams based his conclusions appear to be inadequate. Aside from this, it is rather unlikely that microsomal components could be involved directly in the action of drugs at synaptic sites.

Woolley *et al.* (71, 246–251) attempted to isolate and identify the receptors for ACh and serotonin from various tissues. The "serotonin receptor" substance initially isolated on this basis proved to be a lipid or phospholipid. However, evidence required to meet the criteria for a specific receptor substance was not presented. For example, it was not determined that other compounds known to combine with these receptors *in vivo* actually interact with the isolated material. In examining phosphatidic acid by the method devised by Woolley *et al.*, Vogt (230) found that this could indeed form a complex with atropine and that atropine's affinity was greater than that of ACh. On the other hand, the apparent affinity of calcium for this "receptor" also was greater than that of ACh. Vogt concluded that "phosphatidic acid does not meet the requirement of an ACh receptor as designed by Woolley."

Another point concerns the basic postulate that calcium transport is mediated stoichiometrically by a given hormone, that is, one ACh or serotonin molecule is responsible for the transport of one calcium ion. ACh and other agonists most likely are catalytic since very small amounts promote the transport of very large amounts of calcium (33). Furthermore, smooth or striated muscle may be contracted by direct electrical stimulation or by KCl even when responses to ACh or serotonin are blocked by specific antagonists. Such contractions result from generalized membrane depolarization which, as discussed before, probably does not involve an ACh mechanism. In any event, from the stoichiometry principle,

the amount of "serotonin receptor" extracted from a given tissue may be calculated. This is of the order of 10^{16} molecules/g of intestinal tissue, a value which is 3 to 4 orders of magnitude greater than the estimated number of cholinergic receptor sites in this tissue (179). Similar values have not been presented for the amount of presumed cholinergic receptor isolated in a similar manner. Evidence for the localization of these "receptors" *in situ* was not presented.

Subsequent studies suggested to Woolley *et al.* (252–256) that a more likely candidate for the serotonin receptor is a ganglioside. Binding of serotonin by gangliosides in solution has been demonstrated (226), but these properties do not seem to be sufficiently specific since strychnine and other agents not ordinarily considered as being involved with serotonin effects are also bound strongly (1).

Namba and Grob (165, 166) attempted to isolate the ACh receptor from human skeletal muscle. They used the tubocurarine precipitation procedure devised by Ehrenpreis and developed a sensitive turbidimetric method for measuring drug interactions with the component isolated in this fashion. This was found to be a nucleoprotein containing 20% ribonucleic acid, having a molecular weight between 200,000 and 300,000, and originating primarily from the microsomal fraction. Various compounds known to antagonize the blocking action of curare could reduce the turbidity due to the curare interaction although choline was about as effective as ACh. The amount of material isolated was approximately 400 μg/g of muscle.

It was calculated that the number of isolated "receptor" molecules originating from each endplate of the muscle used was about 10^{10}. Grob and Namba recognized that this value was somewhat high by comparison with Waser's directly determined number, but when the binding capacity of their receptor is taken into consideration, the amount of isolated material becomes even greater. The "nucleoprotein receptor" probably has many binding sites for curare; the number could be as high as 30 since multiple binding to nucleic acid has been demonstrated (81). The number of binding sites at an endplate then would be about 10^{11} if this macromolecule were the ACh receptor. This may be the reason why Namba and Grob (165) more recently suggested that their material might have a more widespread origin than simply from the synaptic region. This conclusion was also based on the localization of antibodies to the material *in vivo*. The authors suggested that the "receptor" might normally exist in an inactive state in the entire muscle fiber but is physiologically active only at the endplate region. The fact that the curare binding component was isolated from the microsomal fraction is interpreted in the following way: "The microsome fraction is believed to be derived from the sarcotubular system, and this system has an important role in excitation-contraction coupling." Of some interest is the finding that immunofluorescent staining by the antibody was inhibited by pretreating the antiserum with their "receptor" or by applying tubocurarine to muscle sections. This is in contrast to the finding that tubocurarine, when applied to sections of electroplax, failed to reduce the amount of binding of electric eel protein antibody (78).

The large amount of material isolated, its multiple binding sites, and the fact

that it originates primarily from the microsomal fraction suggest that this nucleoprotein does not participate as a receptor at the postsynaptic membrane. Nevertheless, it is of interest that some of the fluorescent-labeled antibodies to this component were fixed in synaptic regions. It is conceivable that a small fraction of the material isolated by tubocurarine precipitation does originate from this site and might indeed be the true ACh receptor.

Turpaev *et al.* (141, 173, 222, 223) and later Karlin *et al.* (131, 132) have presented evidence implicating SH groups in cholinergic receptor activity of frog heart and electroplax. The former workers showed that ACh has an effect on SH groups present on macromolecules extracted from frog heart, causing such groups to become more accessible to polarographic titration. They proposed that this effect could be used as an assay for this receptor in solution. They reported that this "receptor" could be isolated as a mercury complex from a fraction precipitated from frog hearts by 45- to 50%-saturated ammonium sulfate.

The conclusion that the ACh receptor is a protein appears valid, although the results obtained do not provide the necessary proof for this contention. First, although such "receptors" were isolated from all ACh sensitive organs, results with tissues that do not contain cholinergic innervation were not reported. This point is raised because of the well known nonspecific protein-precipitating activity of mercury salts. Next, atropine and tubocurarine were shown to act like ACh, *i.e.*, to release SH groups (223). Subsequently it was shown that the ACh polarographic wave of a "receptor" isolated from brain was abolished by nicotine or tubocurarine (174). Lastly, no indication was given of the quantity of material isolated so there is no possibility of comparing this value to those obtained by other procedures designed to estimate receptor content.

De Robertis *et al.* (14, 65) have recently demonstrated that labeled dimethyl-*d*-tubocurarine and other quaternary ammonium compounds are bound to particulate fractions of cat cerebral cortex. Synaptosomes showed the highest capacity to fix these drugs. By extracting the synaptosome fraction with various organic solvents, they concluded that binding of the drugs might be primarily with the proteolipid present in this structure. The suggestion was made that proteolipid might function as the receptor for cholinergic compounds in the brain. It should be indicated that most of the results have been obtained with dimethyl-*d*-tubocurarine, a compound known to bind to many types of macromolecule and thus the specificity of interaction must be checked, possibly by using additional drugs. Nevertheless, it is obvious that this is a most valuable approach, since, in common with microdissected endplates, this preparation has the great advantage of concentrating the receptor population and eliminating large quantities of extraneous material.

V. MODIFICATION *IN SITU*

The preceding sections have dealt mainly with various methods used in the attempt to label, identify, and isolate pharmacological receptors on the basis of the properties of their active sites. However, there are other ways in which additional information can be obtained, that is, by inducing more generalized

changes in receptor macromolecules *in situ*. In this way, the chemical nature of the macromolecules bearing the receptor sites can be deduced, *i.e.*, whether these are proteins, nucleic acids, polysaccharides, or lipids. Alteration in receptor structure is assumed to be reflected by the extent to which the response of an agonist is affected or by the change in affinity of a specific antagonist. The success of this approach obviously depends on the degree of selectivity with which receptor function can be impaired as compared with other elements in the tissue.

Unfortunately, all these studies suffer from a major drawback, namely, uncertainty as to the exact site of action of the modifier. It is conceivable that the alteration might occur either at the receptor level or somewhere along the hypothetical chain of events that links receptor with response mechanism. Thus for these studies, it perhaps is more realistic to attribute changes observed in terms of alteration of either component parts or the total receptor system. In this case, the term "receptor systems" refers not only to the specific macromolecules that directly interact with agonists or antagonists but also to those components, if any, that intervene in the steps linking the receptor to the final cellular component whose change of state directly gives rise to the response (98, 99). In the case of muscles the determination of parameters other than contraction, *e.g.*, membrane permeability or electrical properties, might limit the changes measured to only a part of the total receptor system. Also, if competitive antagonists are used as test drugs, changes in pA values (197, 198) might provide evidence for modification of the receptor itself.

A. Chemical modification

Enzymes, SH reagents, chelating agents, urea, lipid solvents, and changes in pH have been used in studies of this kind. Most agents that could produce even a well defined modification of a receptor molecule undoubtedly could also act on nonreceptor elements and thereby influence the functional integrity of the tissue. Thus, in this kind of study, we cannot overemphasize the importance of using as a control an agonist not dependent on a receptor mechanism for its action, *e.g.*, KCl (88, 98, 99). There is evidence that perhaps even this control is not sufficient, since both smooth and skeletal muscle contain two calcium pools having a role in the contractile process, and Hinke (121) has shown that norepinephrine can release calcium from one such pool while high potassium concentrations release calcium from the other. Caffeine, which appears to release calcium from the same pool as does norepinephrine, might represent a better control (62). Thus Cuthbert (61) found that phospholipase-treated guinea pig taenia coli failed to respond to high concentrations of KCl, whereas, caffeine (1 mg/ml) produced a contractile response in the same preparation.

Another important aspect of the problem is to demonstrate that the agonist under consideration acts directly with its own receptor and not indirectly through the release of another neurohormone, *e.g.*, ACh or norepinephrine. This mechanism has been well documented for a number of very potent agonists, at least on guinea pig ileum (102, 133, 191). Modification of the neural element could be

mistaken for modification of the receptor itself if the agonist were indirectly acting.

1. Enzymes. The specificity of action of several purified enzyme preparations can be used to modify receptors. Alteration of a response by such agents could provide important information concerning component parts of receptors. A narrow specificity is thus a major advantage in the use of enzymes for this type of study. The major disadvantage is the molecular size of most enzymes. If receptors are embedded in membrane crevices, they might not be readily accessible to externally applied substances of high molecular weight; thus a negative result might be of little value. However, there is some evidence that macromolecules of a certain size can reach at least the cholinergic receptors in some tissues. The highly basic protein, protamine (6000 molecular weight) can inhibit responses of electroplax, rabbit atria (85), and ganglia (161) to ACh, presumably by combining with the cholinergic receptor. However, the almost equally basic protein lysozyme (14,000 molecular weight) has a far smaller effect (85), possibly because of its larger size.

Another problem concerns the purity and source of the enzymes in question. Studies of this type may not be reproducible from laboratory to laboratory because of differing enzyme source. In fact, studies in the authors' laboratory showed that various enzymes from the same manufacturer, identical except for control numbers, produced quite variable results.

Just as with any agent used for these purposes, the only successful experiment is one in which the agent has a minimal effect on parts of the tissue other than the receptor substance itself. In the case of enzymes this is a most difficult assignment since nonreceptor elements essential for mediating receptor responses could be attacked. Thus, in these experiments it is extremely important that concentration of enzymes and time of exposure be carefully controlled. These problems were highlighted in a report by Rosenberg and Ehrenpreis (193) on the action of cobra venom applied to squid axon. Without impairing electrical activity of the nerve, this preparation permitted a number of drugs that were ineffective on intact nerves to block the action potential. However, upon prolonged exposure, or when increased concentrations of the venom were used, nerve activity was irreversibly blocked by the venom alone. Probably one of the enzymes present (most likely a phospholipase) hydrolyzed some phospholipid of the conducting membrane, and when this occurred, function was destroyed.

The pioneering work with enzymes used for the purpose of modifying receptors was performed by Lu (149). He showed that trypsin could completely abolish the response of rabbit ileum to ACh. Specificity of action was demonstrated by the fact that there was no alteration in the contractile response to $BaCl_2$. These results strongly suggest that the active site on the ACh receptor is part of a protein molecule and may be subjected to digestion by the enzyme. Lu confirmed and extended this finding in subsequent work (150). These results are in contradistinction to those obtained for the action of trypsin on frog rectus

abdominis muscle wherein sensitivity to ACh and electrical stimulation declined in parallel (109).

One of the most convincing receptor modifications by enzyme treatment involved the use of neuraminidase, an enzyme that splits N-acetylneuraminic acid out of gangliosides (253–255). The isolated rat stomach fundus and rat uterus were used. The enzyme selectively and apparently irreversibly abolished the response of the tissue to serotonin. These experiments seemed to be well controlled in that responses to ACh, bradykinin, and calcium were not at all affected. There was a requirement for EDTA in order for the neuraminidase to be most effective. Inactivation of the serotonin receptor could be reversed by addition of beef brain gangliosides or by lipids extracted from serotonin-susceptible cells (253, 255). On the other hand, only certain ganglioside preparations could restore activity under these conditions (254). These findings implicate gangliosides in serotonin receptor function but Woolley *et al.* did not propose the way in which the action of serotonin or its competitors is mediated by gangliosides. Indeed, gangliosides do bind serotonin in solution (226), but no data have been reported on the binding of specific and potent serotonin antagonists such as brom-LSD.

Further support for Woolley's contention that the serotonin receptor is a ganglioside comes from an experiment of another type (241, 252, 256). The stomachs and uteri of rats made galactosemic by a high galactose diet were tested with serotonin and ACh. The uteri from these animals responded normally to both agonists but the response of the stomach to serotonin was depressed when compared with that of normal control rats. The ACh response was not affected by this diet. As in the neuraminidase experiments, gangliosides restored serotonin receptor activity. Woolley and his colleagues suggested that the galactosemia resulted in the inhibition of an enzyme required for the synthesis of a galactolipid, that is, the serotonin receptor.

Offermeier and Ariëns (175) attempted to repeat the neuraminidase experiments with the enzyme from *Vibrio cholera*. Woolley and Gommi (255) indicated that the *Vibrio cholera* enzyme was inactivated by EDTA and therefore only the enzyme from *Clostridium perfringens* was effective. Offermeier and Ariëns also found some receptor inactivation, but they obtained similar results with EDTA alone (experiments supporting this contention were performed by Fleisch and Ehrenpreis), an effect that was enhanced by the enzyme. The fact that the action of EDTA could be partially reversed by 3×10^{-4} M N-carbobenzoxy-neuriminic acid indicated some degree of nonspecificity in the restoration phenomenon. Wesemann and Zilliken (239, 240) completely restored the response to serotonin of tissues treated with the enzyme by the addition of N-acetyl, N-glycolyl, or N-carbobenzoxy derivatives of neuraminic acid. Carroll and Sereda (45, 46) analyzed purified uterine muscle membrane preparations and concluded that the sialic acid of this tissue is chiefly bound to glycoproteins and not gangliosides. They suggested that the action of neuraminidase is more consistent with the concept that the serotonin receptor is a glycoprotein than that it is a ganglioside. Additional support for a protein nature of the serotonin

receptor in rat stomach has come from studies involving the effects of urea and heat as receptor modifiers (section V A 4 and V B 1). The obvious question that must be raised here is how an enzymatically altered receptor can be reconstituted simply by adding a substance to the bath fluid. Further work is required to clarify this problem.

Albuquerque *et al.* (2, 3, 215) recently initiated studies on the effect of phospholipase C on various tissues in an attempt to modify receptors at the neuromuscular junction. Also included was the effect of the enzyme on extrajunctional elements in the muscle. Their major finding was that the enzyme "interferes with the action potential mechanism leaving cholinergic receptors functionally intact" (215). This evidence appears to be against the proposal that phospholipids constitute at least part of the active site on the ACh receptor (84, 85, 236). However, it is entirely conceivable that lack of effect reflects inaccessibility, *i.e.*, the enzyme simply cannot approach the receptor sites in the postsynaptic membrane. One important aspect of these experiments was the demonstration that the phosphate group of lecithin is part of the structure that controls the passage of sodium ions across cell membranes (82, 83, 107, 108) and thus is involved in generation of the spike. This group may also be essential for maintaining membrane integrity (193, 217) and may be involved with the effects of certain drugs, *e.g.*, local anesthetics (35, 36, 82, 83, 97). Phospholipase A, which does not remove phosphate groups, did not disrupt the conducting membrane (4).

Cuthbert (61) treated the guinea pig taenia coli with *alpha* chymotrypsin and phospholipase C. He examined the subsequent electrical responses to stimulation as well as contractions to ACh. After chymotrypsin, a membrane response to ACh was obtained (an action potential), whereas no mechanical response was evoked. Phospholipolysis, on the other hand, abolished the total response of the taenia coli to ACh at a time when electrical stimulation indicated that the membrane and the contractile mechanism were intact. Cuthbert did not consider that his results provided conclusive evidence on the nature of muscarinic receptors in this muscle because of the many complications involved. The results do actually suggest the involvement of phospholipid in the ACh receptor system, a finding consistent with recent proposals of Watkins (236) and of Ehrenpreis *et al.* (84, 85).

2. Lipid solvents. Dikstein and Sulman (69) found that the frog rectus abdominis muscle treated with a 25% acetone solution for 2 min no longer responded to a supramaximal dose of ACh. Phosphatidylserine and phosphatidylethanolamine were reported to reverse this change. The investigators attributed these findings to alterations in a phospholipid-containing transducer system present in the cell membrane. They obtained essentially the same results on the rat uterus when norepinephrine was the agonist. Ehrenpreis *et al.* (89) repeated these experiments, testing responses to KCl along with ACh and other agonists on the frog rectus abdominis and various other isolated tissues. Exposure to acetone could completely and irreversibly block responses to all agonists, but this effect was not reversed by phosphatidylserine. The fact that KCl contractions were abolished indicated that acetone can have a nonspecific destructive

action and thus was of little value in obtaining information about receptors under the conditions employed in these experiments. Acetone abolished the beating of the isolated rabbit atria within a few seconds, and the beat failed to reappear after exposure to norepinephrine or phosphatidylserine; application of calcium (1 mg/ml) or ouabain (10 μg/ml), however, restored the beat, and then the responses to ACh and norepinephrine returned almost to control values. This result indicates the lack of profound alteration in these atrial receptors by the acetone. It may be that protein denaturation rather than extraction of essential lipids was reponsible for the deleterious action of acetone on the other tissues.

In another report, Dikstein and Sulman (70) stated that acetone could fully extract tissue-bound Dibenamine and on this basis proposed that the "Dibenamine receptor" was a lipid (cephalin). Yong *et al.* (260, 261) stated that only 30% of the labeled Dibenamine could be extracted under these conditions.

3. Sulfhydryl reagents. If drug receptors are proteins, they can be expected to undergo reactions that are known to occur with other proteins. Among the most group-specific reagents are those which cause reduction or oxidation of sulfhydryl groups. Martin and Schild (153) demonstrated the reversible inhibition of responses to vasopressin and oxytocin on rat uterus by various thiol compounds. Responses to bradykinin, a polypeptide that does not contain SH or SS bonds, were unaffected by these reagents. They attributed the antagonism to a reduction of essential disulfide bonds at the active site of their respective receptors and postulated that the sulfur-containing polypeptides interact by a disulfide-sulfide interchange. Thus their data indicate that the receptors for these two polypeptides are sulfur-containing macromolecules, most likely proteins. Their interpretation is consonant with that of Schwartz *et al.* (200), who proposed that the receptor for these polypeptides contains an SH group at the active site. Ishida *et al.* (125, 126) confirmed the involvement of SH groups in the action of oxytocin on rat uterus by demonstrating that mercuric chloride and *p*-chloromercuribenzoic acid acted as competitive antagonists of this polypeptide. The view that the vasopressin receptor contains an SH group has been verified indirectly in experiments by Fleisch and Ehrenpreis (88, 98, 99) (section V B).

SH-reacting compounds block electrical activity of electroplax (131, 132). This has been assumed to reflect the reaction of these compounds with ACh receptors. However, these results must be interpreted with some caution since SH reagents block squid axons (123), and thus it is possible that the block in electroplax could be on the conducting membrane, *i.e.*, extrajunctional. KCl depolarization was not examined after treatment of the electroplax with these reagents.

The studies of Turpaev and coworkers involving the application of SH reagents to the heart have been discussed previously (section IV B).

4. Protein denaturants. One of the main characteristics of proteins is the lability of their secondary and tertiary structures. Various chemical agents are available that induce an unfolding and uncoiling of peptide chains with a concomitant loss in biological activity. Since proteins differ in their susceptibility to such agents, it may be possible to selectively alter those pharmacological receptors which are protein in nature, particularly if their lability is substantially

greater than that of the surrounding macromolecules. This should be manifested as a profound change in the dose-response curve of an agonist. It is assumed that the magnitude of the shift of the dose-response curve and the relationship of the new maximal response to the control reflect the degree of denaturation of a particular receptor. The alteration could be either reversible or irreversible depending on the type or protein involved and denaturing agents employed. It should be pointed out that this type of experiment probably cannot be performed on all types of tissue. Thus, two different tissues may contain muscarinic cholinergic receptors (both blocked by atropine) but if the membrane or the contractile system, or both, in one tissue has an essential constituent that is as readily susceptible to denaturation as the receptor itself, then both the receptor and the essential constituent would be altered and the selectivity would be lost. The importance of using proper controls in this type of approach has already been stressed.

Khairallah *et al.* (134) demonstrated that urea, as well as various amino acids, at concentrations somewhat over 0.2%, reversibly inhibited the response of the rat uterus and guinea pig ileum to angiotensin but not the response to bradykinin, serotonin, or oxytocin. At concentrations of 4% and greater, urea and the amino acids caused a contracture and decreased the response to all of the agonists tested. It was suggested that urea "could block angiotensin by covering the receptor site, by replacing urea for water of hydration around the proteins in the receptor site, or by reversibly denaturing the receptor protein" (134).

Fleisch and Ehrenpreis (88, 99) undertook a more extensive investigation of the effects of urea on the activity of a number of smooth muscle stimulants, extending the urea concentration to the range at which it denatures proteins. Rat stomach strips were exposed to 2 M urea for 19 min. Procedures developed to overcome the marked contracture that ensued enabled the re-establishment of a stable base line. After washout of the urea, essentially 100% of the contraction could be obtained with bradykinin, ACh, and serotonin but the concentration of agonists required was, respectively, 50, 375, and 5000 times greater than in controls. On the other hand, regardless of the concentration used, only about 50% of the maximal contraction to angiotensin could be elicited after treatment with urea. Since responses to KCl were essentially unaffected, the contractile mechanism and the conducting membrane were, as far as could be ascertained, unaltered. These observations, coupled with the effect of heat on the same tissue (section V B 1), give added support to the concept that proteins play a primary role in the activity of the receptors studied. The new dimension added is that such proteins show marked differences in their physicochemical properties.

5. Chelating agents. Evidence has accumulated over the past few years to suggest that at least some pharmacological receptors contain a metal in or near the active site. Chelation is considered to play a prominent role in stabilizing certain types of drug-receptor complex, in particular those involving the catechol nucleus of epinephrine and norepinephrine (23, 26). Direct experimental evidence for this concept has been obtained by Ehrenpreis *et al.* (91, 93) from experimental studies on tissues and through the use of phosvitin, a protein that interacts with

a number of catecholamines and adrenergic antagonists. Our results suggest that the norepinephrine receptor contains iron in the active site, and that the iron is involved in chelation with the catechol groups. This contention is based on the observation that chelating agents with high affinity for $Fe^{(II)}$ (8-hydroxyquinoline, orthophenanthroline, and bipyridyl, all at 10^{-6} M) block the response of the rabbit aortic strip to norepinephrine while having minimal effects on other agonists.

The results obtained by Woolley (section V A 1), using both neuraminidase and EDTA to alter the serotonin receptor, suggest that this receptor might possibly contain a metal. This is based on the finding that EDTA alone can partially inactivate the response to serotonin. Serotonin itself has chelating properties and may complex with a metal at the receptor site.

6. Hydrogen ion concentration. Biological activity of drugs is greatly influenced by the pH of the surrounding medium. The most critical range is around the pK of specific ionizing groups on proteins (amino, carboxyl, imidazole). From the pH dependence of a particular biological activity, *e.g.*, that of an enzyme, much can be deduced about the group or groups present on an active site. Although this might appear to be a relatively simple approach to altering a receptor molecule, many pitfalls exist since tissues contain large numbers of nonreceptor macromolecules, and these undergo similar change with pH and could influence tissue responses. Furthermore, the agonist itself frequently possesses ionizing groups. Thus, interpretations of pH dependence of pharmacological activity is extremely difficult although progress has been made, in particular in the case of the active form of local anesthetics (187) and a few other compounds (9). Rocha e Silva (190) used this approach in his investigation of the molecular nature of the histamine receptor. His results indicated that there is on the receptor an ionizable group, most likely the imidazole group of histidine, with a pK between 6.9 and 7.1. Other receptors investigated in this manner include the inotropic receptors for epinephrine of the turtle heart (112) and the cholinergic receptors of the guinea pig ileum and atrium (150a) and frog stomach (159).

B. Physical modification

1. Heat. It has now been shown that some receptor-associated responses may be selectively altered *in situ* by physical means. Turpaev *et al.* (223, 224), investigated the thermal stability of the cholinergic receptors of the frog heart. Heating the ventricle to 40°C for 1 to 2 min reversibly inactivated the receptors, while repeated heating (3 to 4 times) at 40°C for 3 to 5 min irreversibly abolished the response to ACh. The normal excitability and contractile properties of the myocardium were unaffected. These results were considered in the light of the proposed identity of ChE and the receptor. It is well known that ChE in solution is a relatively heat-stable enzyme (58). In view of the apparent great lability of the ACh receptor, Turpaev *et al.* claimed that their results showed that "the hypothesis of the identity of the ACh receptor and ChE is untenable."

Results of Fleisch and Ehrenpreis on the rat stomach fundal strip are at variance with Turpaev's on the frog heart in that when the preparation was heated to

47°C for 20 min, the highest temperature and duration that it could tolerate, the response to ACh remained unaltered (98). Of course, it is possible that the receptors of the two tissues are different and therefore have very different thermal stabilities. Other agonists whose activities on the rat stomach were unaffected by this treatment included KCl and bradykinin, but responses to serotonin, angiotensin, and vasopressin were greatly depressed. The conclusion reached was that those receptors adversely affected might well be protein in nature. Few tissue macromolecules other than proteins would be expected to be labile under such mild conditions. It may be noted that the order of stability to urea paralleled that of heat (98, 99). These results have a bearing on proposals based on other approaches concerning the molecular nature of certain receptors. As indicated above (section V A 3), it has been postulated that a sulfur-containing group is present at the active site of the vasopressin receptor. The fact that the response to this polypeptide was so sensitive to a comparatively mild rise in temperature supports this claim since sulfhydryl-containing proteins are often denatured by heat (50). Conversely, the lability of the serotonin receptor to heat and urea is more consistent with its being a protein rather than a ganglioside.

2. Cold. Prolonged reduction of tissue temperature also changes apparent receptor activity. Lum *et al.* (151) found that incubation of the rabbit jejunum at 6 to 8°C for 24 to 72 hr produced a loss of response to agonists acting on *alpha* adrenergic receptors. On the other hand, storage of rabbit aortic strips at 4°C for similar periods of time does not significantly change the responsiveness to agonists acting on *alpha* or *beta* receptors (R. F. Furchgott and J. Besse, personal communication). We have shown that cold reduces the response of the rat stomach to serotonin (J. H. Fleisch and S. Ehrenpreis, unpublished data). Responses to other agonists were essentially unaltered under these conditions. Van den Brink (225) recently reported a study on the histamine receptor of guinea pig ileum, the tissue being stored for 6 hr in the refrigerator. He concluded that, although the affinity constant of histamine was unchanged, its intrinsic activity was significantly decreased.

The effects of high temperature on receptor activity may be attributed to protein denaturation. However, it is difficult to understand the mechanism by which a diminished temperature produces its effects on tissue responses. Van den Brink has suggested that in fact the receptor itself is not altered by cold but rather this affects the stimulus-contraction coupling mechanism. This reasonable explanation could be tested by a study of KCl or caffeine response. Another possibility suggested by Ambache (5) is that cold inactivates the neural elements and thus indirectly acting agonists would appear to be depressed. Finally, it is conceivable that certain metabolic processes are required to maintain a particular receptor in its native state; these would be depressed at lowered temperature, resulting in an altered state of the receptor.

VI. MODEL SYSTEMS FOR RECEPTORS

The construction of models of receptors is a highly speculative undertaking fraught with many uncertainties and assumptions. This is especially true for

models that are proposed on an entirely theoretical basis (22, 42, 43) and somewhat less so for models based on drug structure-activity relationships or on enzymes known to be implicated in receptor mechanisms, for example, AChE and adenylcyclase. Nevertheless, such efforts are not without merit as they are attempts to provide a general hypothesis about a central principle of pharmacology.

There are three major aspects of receptor theory that models should attempt to account for. First, the model should provide information about the structure of the receptor site that distinguishes agonists from antagonists on the basis of their structures; second, it should suggest how the interaction is translated into a physiological effect such as a depolarization; and third, it should account for the "effectiveness" with which this translation or its blockade is brought about in relation to the structure of a particular drug molecule. In short, a successful model should provide the theoretical basis for the structure-activity relationships of molecules acting at that site and their mechanism of action.

Receptor models have been proposed that account for some of the above criteria in whole or in part, but in every case there are pharmacological findings for which the model provides no explanation. One of the most serious problems in citing pharmacological evidence for or against a receptor model is that in few cases is it clear that the effect of a drug is due solely to its interaction with the receptor that initiates the response, and not due partially or completely to its interactions with other sites involved in the mediation or modification of the response.

A. The cholinergic receptors

The very elegant and careful work of Beckett, Belleau, and Waser on the dependence of muscarinic activity on stereochemical and conformational properties of agonist molecules is one of the approaches toward a muscarinic receptor model which is least open to criticism since no prior assumptions about the receptor need to be made.

These authors have measured the muscarinic activities of the isomers of acetyl-β-methylcholine, acetyl-α-methylcholine (21), muscarine (233), and 2-methyl-4-dimethylaminoethyl-1,3-dioxolanes (221). The agonistic activities are maximal only in those isomers having a similar and specific steric and electronic configuration irrespective of whether the compound in question has an ester, hydroxyl, ether oxygen, or ketone function. A model of the muscarinic receptor in terms of electron density contours can be constructed from these data (fig. 1). This model is in agreement with the preferred conformation for acetylcholine as deduced from X-ray crystallography (44) and nuclear magnetic resonance (60), and was recently confirmed by Extended Hückel calculations (136). Calculations such as the latter give promise of providing a picture of the shape of the receptor, which is assumed to be complementary to that of the various agonists.

It can also be expected that the receptor site is probably located on a protein and has components similar to those of the active site of acetylcholinesterase. The proposal of Zupancic (263) that the receptor site is composed of the molecular

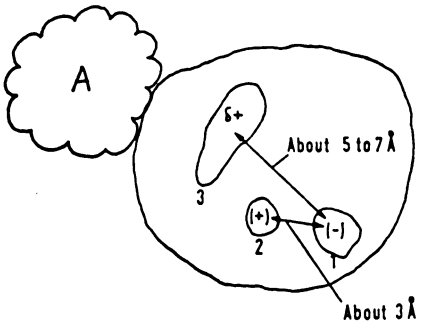

FIG. 1. Diagrammatic representation of muscarinic receptors. 1, Anionic cavity negatively charged to accommodate quaternary nitrogen. 2, Positively charged group or subsite accommodating ether linkage of muscarine or ester linkage of ACh and its analogs. 3, Positively charged area to accommodate hydroxyl of muscarine, carbonyl of ACh and its analogs, or the double bond of furan analogs of muscarine. A is the region of "accessory binding" as suggested by Ellenbroek, Ariëns *et al.* (10, 75) and Moran and Triggle (163). [After Beckett (20). Reproduced by permission.]

components postulated by Krupka for the active site of AChE (144), and that the two are thus identical, is negated by the discrepancy between the enantiomorphs of the acetates of α- and β-methylcholines as substrates for AChE and their relative muscarinic potencies on the guinea pig ileum (20). It must be stressed, however, that these substrate measurements were made on purified enzyme preparations obtained from a different tissue and species, and it is not known whether these results can be extrapolated to the enzyme *in situ* in the guinea pig ileum.

A complementary study of the stereochemical and conformational requirements for potency in antimuscarinic agents of the choline ester type has given results which, contrary to expectation, provided no direct information about the muscarinic receptor site. Among antagonists of the atropine type, the conformation of the choline part of the molecule and even the quaternary nitrogen are relatively unimportant for activity, whereas the stereochemistry and conformation of the acyl moiety is critical (8, 75). This is exactly opposite to what was found with agonists. It has been proposed that compounds of the atropine type interact with adjacent "accessory receptor sites" (fig. 1); nevertheless they have an apparent action which is competitive with ACh. This may even be true in general for muscarinic antagonists. Recent work by Moran and Triggle (163) may provide direct experimental evidence for the concept of adjacent accessory binding sites for antagonists on receptors. By means of a radioactive alkylating atropine-like agent (benzhydryl mustard), they found two different sites of blockade with different recovery rates. Blockade of one site having a slow recovery from the alkylating agent shifted the dose-response curve of complete muscarinic agonists (intrinsic activity of 1) without reducing the maximal contraction height, but it lowered the contraction height of partial agonists, *i.e.*, compounds that are both agonists and antagonists (*e.g.*, hexyltrimethylammonium) (7, 207). Further alkylation of a second, fast-recovering site in the

tissue paralleled a reduction in the maximal contraction height to the complete agonists. On the basis of these results, it was suggested that one of the blocked sites is concerned with the action of agonists and a different site is concerned with the effects of antagonists. Moran *et al.* considered these findings as strong evidence that the muscarinic receptor has subsites (allosteric sites) with stereoselectivity and hydrophobic bonding properties, particularly to Pi electron systems, as in the aromatic moiety of atropine analogs (8). These results also illustrate that, as suggested elsewhere (84, 85), parallel shifts in dose-response curves and apparent kinetic competitive behavior, as is observed with atropine, does not entirely justify the conclusion that such drugs are acting competitively in a mechanistic sense, *i.e.*, directly at the receptor site.

Structure-activity relation studies and correlations with preferred conformations of nicotinic drugs can provide the same kind of general picture for the nicotinic receptor, although the evidence is not quite as clear as in the case of the muscarinic receptor. It has been proposed that the nicotinic receptor has a subsite located about 4 Ångstrom units from the site of binding of the methonium group (120). There is some question as to whether this secondary site is cationic (17) or anionic (59, 201, 202). Recent calculations by the Extended Hückel theory have shown that the ACh molecule has a second preferred conformation, and this has been proposed as its nicotinic conformation (137, 138), and also that the secondary binding site of the nicotinic receptor has a partial negative charge. This confirms earlier suggestions that the nicotinic and muscarinic actions of ACh may be due to at least two different conformations (154).

Observations by Wurzel (257, 258) on the biological activity of esters of choline show that their potency toward nicotinic receptors parallels hydrolysis by pseudo ChE and their potency towards muscarinic receptors parallels hydrolysis by true ChE (AChE). This parallel behavior holds for changes in the acyl moiety and suggests that a site with properties similar to those of the esteratic site of the respective esterase is somehow implicated in biological activity. Wurzel proposed that the esterase produces hydrogen ions and choline cations which accumulate and, by ionic association with membrane polar groups, cause mechanical (conformational) changes (257, 259). He considered the choline cation the effective agent, which by combination with a specific receptor site causes a change in the transmembrane potential. Both choline cations and hydrogen ions would be required simultaneously since either one alone is relatively inactive (259). This hypothesis suffers from a basic flaw as it is unable to account satisfactorily for the activity of poorly hyrolyzed esters such as carbamates, nonester agonists, and antagonists of cholinesterases. The esteratic site of ChE may be an incorporated element of the receptor on an organized membrane; but in this state it has lost its hydrolytic ability while still retaining binding selectivity toward the structure of the acyl moiety of choline esters.

Zupancic (262) also considered that cholinesterase and the cholinergic receptors are closely connected in that the anionic centers are identical, *i.e.*, the receptor is the anionic center of ChE. The role of the esteratic center is indirect—to convert ACh, without dissociation, into choline and thus regenerate the receptor.

This is considered to be a concerted process by which a molecule of high affinity (acetylcholine) associates with the receptor while one of low affinity (choline) dissociates from it. One of the consequences of this hypothesis is that the number of receptors should be about the same as the number of active sites of ChE, *i.e.*, the number of anionic drug-binding sites should be about the same as the number of esteratic sites. This is supported by the results of Waser (235) on the uptake of tubocurarine, presumed to be localized on the receptor sites in mouse diaphragm, in relation to the fixation of radioactive diisopropylfluorophosphate, as determined by Barnard and Rogers in this same tissue (18). The numbers of receptors and AChE sites are close to 1×10^7 per endplate.[8] As a further consequence, the equilibrium constant for the binding of a given agonist and antagonist to the anionic centers of receptor and esterase *in vivo* should be the same. Zupancic (263) has shown with the leech dorsal muscle that this is so for tubocurarine, but it remains to be shown that these values are the same for agonists.

The above-considered receptor models have been discussed first because they have the merit of being based in part on measurements on intact tissues. This point is important as it is entirely likely that a receptor or a receptor system no longer is present after disruption of the organized membrane. Each of the proposals was based on the investigation of a small series of compounds and the major information provided concerns the nature and geometry of the receptor site rather than the mechanism of action of cholinergic drugs. Several other proposals of receptor models will now be considered, representing attempts to define the mechanism whereby combination with an agonist results in a change in ion permeability and how antagonists block this effect.

Some of the earliest proposals for the mechanism whereby large changes in ionic flux may be caused by ACh involved the principle of ion exchange, potassium being displaced by the quaternary nitrogen of the agonist. This idea was later modified, and it is now proposed that the quaternary head displaces a cation (calcium or magnesium) from a phosphate complex (48, 236) and this process leads to the observed general increase in membrane permeability to ions (96 a, b). In the case of the muscle endplate the permeability appears to be relatively nonspecific since tissues subjected to alternate depolarization and repolarization show considerable uptake of a variety of cations including agonists and antagonists (212). This uptake is greatly reduced by preventing depolarization either by the use of drugs (213) or by nerve section (212, 234).

The sites of increased permeability in the muscle endplate are termed "polar pores" by Waser (235). He proposed that in such a pore the flux of Na and K ions is controlled by anionic sites located on its periphery. Quaternary cations interact

[8] Studies from this laboratory on smooth muscle also lend support to this hypothesis in that the number of esteratic sites as determined by enzymatic activity of intact tissue is within one order of magnitude of the number of receptor sites determined by Paton and Rang (179) from atropine uptake, the values being 10^{13} and 10^{12} per g respectively. These calculations indicate that enzyme molecules do not greatly exceed the number of receptors even if they are different entities. This is contrary to the notion that there must be a large excess of enzyme over receptors, in order to effect hydrolysis of ACh.

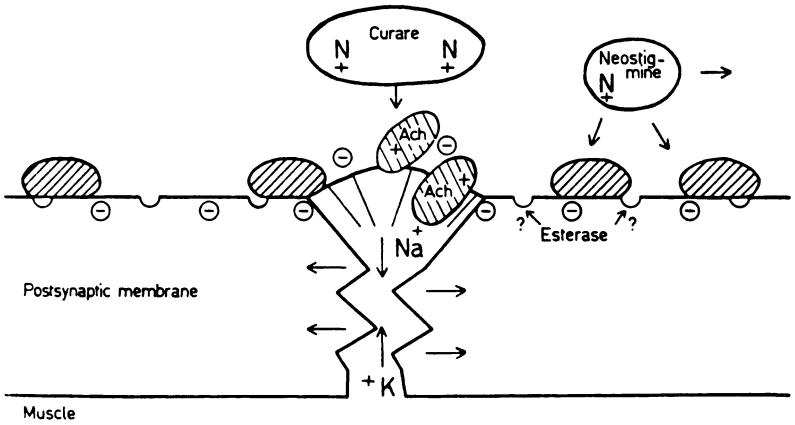

FIG. 2. Hypothetical scheme of receptor area of endplates according to Waser (234). (Reproduced by permission.)

with these anionic sites and modify the ion flux. Large *bis*-quaternary blocking agents, such as tubocurarine, block the pore by combining with anionic sites on opposite sides, and this causes bridging of the pore and thereby interferes with ion flux (see fig. 2). Such speculations have the disadvantage that neither the structural components of the pore nor the mechanism by which the pore controls the passage of specific ions is defined in biophysical terms.

One way out of some of the shortcomings of receptor models is provided by the "induced fit" hypothesis of Koshland (142). These ideas can be profitably applied to receptors if the latter are regarded as analogous in their complexity to enzymes except that no covalent bonds are broken or formed in the substrate (agonist) or inhibitor (antagonist). Different agonists, all equally bound initially, could differ in the efficiency with which they induce a conformational change in the structure of the macromolecular receptor. The kinetic aspects of this approach form the basis of the Clark-Stephenson-Ariëns occupation theory of drug action but the approach can also provide a basis for molecular mechanisms of drug action. Induced conformational changes have been measured by X-ray crystallographic, thermodynamic, and optical rotatory dispersion studies of enzyme-substrate and enzyme-inhibitor complexes, as well as of the binding of small molecules to a variety of macromolecules. Esterases such as trypsin and in particular AChE are no exception and exhibit similar behavior (30, 242, 243).

Belleau has developed this approach for the molecular mechanisms of drug-receptor combinations by a reliance on the thermodynamic parameters of interaction of cholinergic drugs with AChE (24, 27, 28). His basic assumptions are that the enzyme is a valid model for (or "nearly identical with") the receptor in terms of the thermodynamics of its interaction with drug molecules and that results obtained with the enzyme are directly applicable to the receptor. The receptor itself is regarded as a regulatory enzyme, catalyzing an as yet unknown reaction; its activity may be altered by specific interaction of small molecules at a regula-

tory (allosteric) site. The activity is altered by inducing conformational changes when binding occurs at the allosteric site. Such changes may either enhance or depress the activity of the active center from which originates the stimulus leading to a measured response.

A study of the inhibition of AChE by the geometrical isomers of 2-substituted-1,3-dioxolanes (28) showed that in only one isomer was there an appreciable contribution of cohesive (van der Waals) forces to the free energy of binding, and that this was the only compound in the series having appreciable muscarinic activity. This result is cited as evidence in favor of the above mentioned postulate concerning the nature of the active surface of the muscarinic receptor, and, in particular, of the proposal that AChE is a valid model. Free energies of binding were obtained from the values of the inhibitor constants and compared to the values calculated from the sum of the free energy for the unsubstituted dioxolane and the theoretical value for the 2-substituent.

There may be shortcomings to such simplified calculations. The free energy of binding of the substituent to the enzyme surface was assumed to be equivalent to the energy of transfer from water to ethanol and to be a constant for this substituent regardless of its location. In addition, it was assumed that all substituents on the 2-position are equally bound irrespective of their conformation, although it is well known that binding sites on the enzyme are highly stereoselective and thus it is likely that differences in the hydrophobic binding of some stereo-isomers can be expected. For this reason, the correlation of pharmacological activity with van der Waals forces of binding cannot be considered as too significant. The possibility of a substituent having no appreciable hydrophobic bonding has been proposed by Triggle and Belleau in another connection (221), but is not taken into account in this instance.

Belleau *et al.* (30) have measured the free-energy changes associated with a series of AChE inhibitor complexes in which the inhibitor series consisted of alkyltrimethylammonium compounds with linear alkyl chains of from 1 to 12 carbon atoms. The contribution of hydrophobic bonding of these chains to the entropy of binding and the free energy of binding of the antagonists was calculated from the temperature dependence of the inhibitor constants. The free energy of binding as a function of the number of CH_2 groups is not a smooth relationship and has discontinuities at n = 4 and n = 8; the series forms 3 linear groups n = 1 to 4, n = 5 to 8, and n = 9 to 12. The linearity of the points in these three groups was construed as showing that a lengthening alkyl chain from the quaternary head encounters three adjacent regions where the hydrophobic binding of successive CH_2 groups is different but constant in each region. Belleau postulated that in the receptor the quaternary head of the molecule is bound in the first region and molecules binding only in this region enhance the activity of an active center situated in the second adjacent region and should accordingly be agonists. Molecules binding in both the second and third compartment have an increasing inhibitory effect on the active center and should accordingly be antagonists. While it is true that for n greater than 9 the compounds are purely antagonists, the discontinuity at n = 4 is ordinarily not the dividing point between

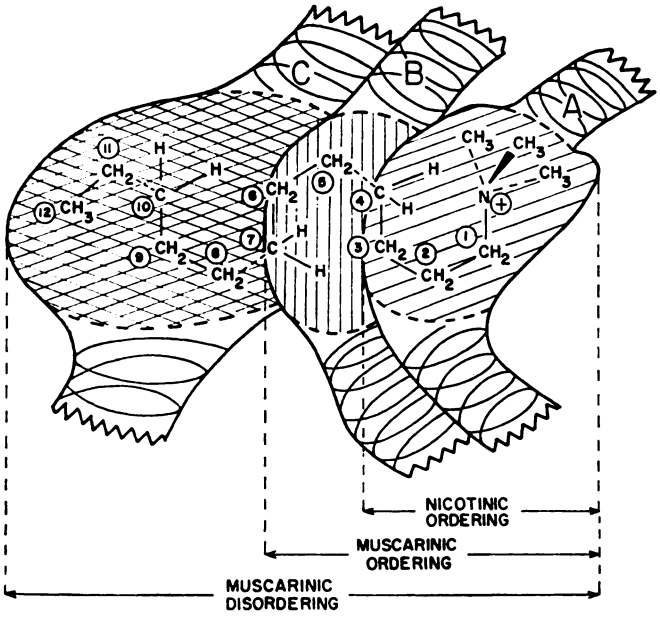

FIG. 3. Adjacent binding compartments for alkyltrimethylammonium drugs on muscarinic and nicotinic receptors and on acetylcholinesterase. [From Belleau (24). Reproduced by permission of Academic Press, Inc.]

agonists and partial agonists. The correlation between discontinuities in free energies of binding and activity determined on rat intestine coincide only in the transition to antagonists. It is possible that too much significance is read into the free-energy discontinuities and that changes in entropy are more meaningful. Agonistic properties appear to correlate with negative entropies of binding, indicating a high degree of order in the complex. Those compounds (C_7 to C_9) showing virtually no net entropy change are partial agonists while those with positive entropies of binding cause disorder in the complex and are antagonists (see fig. 3).

In a later paper Belleau (27) modified this hypothesis by considering only the contribution of entropy and enthalpy of binding of the alkyl chains of increasing length. The binding of the alkyl group, irrespective of length, to AChE was found to be endothermic, and this was attributed to the enthalpy required to displace water envelopes separating the hydrophobic regions of enzyme and the hydrocarbon chain being greater than the gain in enthalpy of binding. From a consideration of the process of binding in detail and estimating partial enthalpies for the steps involved, the conclusion was made that alkyl chains up to C_6 do not form hydrophobic bonds with the binding site whereas those greater than C_6 do form such bonds. The variation of enthalpy with chain length shows breaks at C_3, C_4, C_5, and C_{10}. Since it has been assumed that up to C_6 there is no enthalpy gain due to hydrophobic binding which has the magnitude of an enthalpy of dehydration, the discontinuities must be caused by a change in water structure. Accordingly, to account for the breaks, Belleau has postulated that the 4th CH_2,

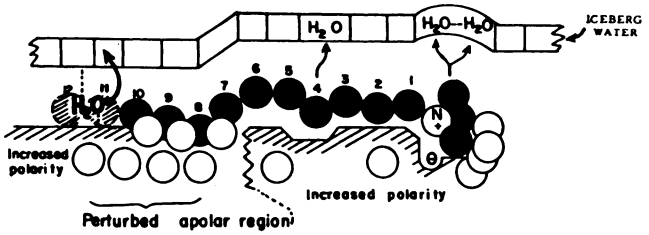

FIG. 4. Schematic representation of the mechanism of physical interaction of long-chain trimethylammonium ions with the AChE and ACh receptor surfaces. The 4th and the 11th carbon atoms are shown to cause the loss of 1 water molecule of hydration. [From Belleau (26). Reproduced by permission.]

the 6th CH_2, and the 11th CH_2 dehydrate the enzyme surface by each displacing one water molecule. The 5th CH_2 is believed to have an opposite effect, causing an ordering or stabilization of the existing enzyme-water structure because there is a gain in enthalpy between C_4 and C_5 (see fig. 4).

The above proposals are ingenious constructions based on somewhat tenuous evidence and on assumptions having slender justification. Nevertheless, the broad picture of the receptor site having adjacent regions of hydrophobic bonding is borne out by the work of Ariëns *et al.* (8, 75) and of Moran and Triggle (163) discussed earlier. There is a need for great caution since the extrapolation of thermodynamic data to such detail tends to outrun the evidence. This entire proposition is founded on the initial assumption that the thermodynamic parameters for binding of the quaternary nitrogen remains constant throughout the entire series of compounds. However, it is equally possible that alkyl chains of different lengths when bound to the enzyme surface exert a modifying effect on the binding of the quaternary group at the end of the chain. Thus, the enthalpy and entropy of this binding may be a variable and partly dependent on the length of the alkyl chain and on its conformation. The postulated activity of the 4th, 5th, 6th, and 11th CH_2 groups on the structured water at the enzyme surface fits in with the enthalpy values rather than the reverse. Since these data are based on AChE, this may be a reasonable physical picture of the binding of inhibitors of this type to the enzyme but is not necessarily so for the receptor. If compounds with C_4, C_6, and C_{11} chain lengths caused dehydration on a receptor surface, this would be expected to produce marked changes in pharmacological activity as compared with other members of the series. However, the activity of these three compounds is not much different from those analogs immediately preceding in chain length, *i.e.*, C_3, C_5, and C_{10}, which apparently do not dehydrate bound surface water. Thus, the mechanism based on this model would appear to have lesser value for an understanding of receptor activity than the earlier and different proposal (30) based on essentially the same thermodynamic data. These concepts have been undergoing development and revision over the past 10 years. Because of their speculative nature they are open to criticism, but this should not obscure their valuable contribution, that is, to stimulate and provoke pharmacologists to come to grips with receptors as real molecular species.

Thus far, the models proposed have dealt with the nature of the receptor site; we will now consider the mode by which combination of a small molecule at this site can induce a change which is the first step in the chain of events leading to a response.

In a recent proposal, Watkins (236) formulated a general relationship between several receptor types and other elements of the organized membrane. There is only circumstantial evidence for this proposal and it encompasses earlier suggestions that phosphate groups within the membrane, complexed to Ca or Mg, control the influex of ions and also interact with quaternary ammonium compounds such as cholinergic agents.

This is also the view of Cavallito (47, 48), who suggested that membrane polyphosphates, being polyanionic regions, are subject to conformational and electrostatic modification under the influence of cations. In this way ACh and quaternary nitrogen analogs interact and upset the existing balance of inorganic ions bound to membrane polyanionic elements. Curarimimetic agents block at this site in the membrane by bridging across two anionic regions.

The related proposal of Watkins differs from Cavallito's in that the receptor (presumably a protein) forms an outer element of the membrane and is coupled to phosphate-calcium (or phosphate-magnesium) complexes within the lipid structure. Cholinergic agents act indirectly on the phosphate-metal complex, perhaps by some conformational change induced by the binding to their receptor protein. More specifically, he proposed that in certain regions of the membrane the complex between lipid and protein occurs in several stages depending on the number of polar groups complexed, and is reversible. Adsorbing substances can displace this equilibrium by competitive complexing with either the lipid or the protein. In the ACh receptor, a lipid containing choline (either lecithin or sphingomelin) forms a complex with a protein (receptor protein) by electrostatic binding of both the ammonium cation and a phosphate-divalent metal cation to anionic groups of the protein (see fig. 5). ACh competes with lecithin and displaces it from the protein complex, changing the conformation of the protein and weakening the electrostatic binding of the divalent cation. Thus ACh "uncouples" a specific protein-lipid complex and thereby changes the selectivity of a cation binding site in such a way that divalent ions are replaced by univalent ions.

One of the chief virtues of this hypothesis is its versatility or generality. It provides a framework for the mechanism of action of a wide variety of drugs, since such a system may be upset or perturbed by compounds that form lipid complexes, even of a relatively nonspecific type (*e.g.*, anesthetic agents) and also by compounds complexing to proteins. Further, such a system would be subject to changes by temperature, pH, pressure, *etc.*, although how such variables could cause a change cannot be predicted from the model.

Ehrenpreis (84, 85) modified this model by considering that the protein of the complex contains the active site of some tissue cholinesterase molecules. The enzyme was thus introduced as part of the actual receptor for ACh and other cholinergic drugs. This proposal was made because the concept that the sole function of tissue ChE is to regulate ACh action by rapidly hydrolyzing the

ester may be invalid. There is no doubt that the hydrolysis of ACh by the enzyme is extremely rapid (164, 244). However, this activity refers to purified electric eel AChE at optimal ACh concentration (about 10^{-3} M). As has been stressed (84, 85, 91), this enzyme most likely is not identical to the one that is bound to tissue membranes and thus could have very different catalytic properties. Furthermore, since the concentration of liberated ACh in the vicinity of the postsynaptic membrane may be in the range of 5×10^{-7} M (129), the turnover of ACh would be very much smaller than at optimum concentration. Barnard and Rogers (18) have determined that the amount of AChE in a single mouse diaphragm endplate (10^7 active sites) is insufficient to hydrolyze the ACh liberated by a nerve impulse within the required time (1–2 msec). Even if the actual concentration of ACh was high, it would appear that diffusion plays a major role in terminating its action as proposed by Eccles and Jaeger (72).

The effects of certain cholinergic drugs are difficult to understand if cholinesterase is considered to act only as the regulator of ACh action. For example, as the alkyl chain length on the nitrogen of alkyltrimethylammonium compounds exceeds 6 or 7 carbon atoms, the compounds become increasingly more potent anatagonists of the action of ACh (7). In parallel with this property, potency as inhibitors of the enzyme increases progressively (30) and thus a potentiating action should be observed. Numerous examples may be cited (49, 56, 130, 237a) in which extremely potent ChE inhibitors exert anti-ACh actions. Such findings suggest a direct relationship between combination with the enzyme and inhibition of a receptor-mediated response. Admittedly, at times there is a discrepancy between affinity for the enzyme and ACh antagonism (100), but some compounds with K_i's in the same range as those for neostigmine and physostigmine antagonize ACh without showing a potentiating effect (237a).

Ehrenpreis attempted to explain some of the above findings on the basis of the modified Watkins model. It was postulated that as the chain length on the quaternary nitrogen is lengthened there is an increasing tendency to form intermolecular hydrophobic bonds through simultaneous interaction with the anionic sites on the protein (ChE) as well as the phosphate group of the phospholipid. This would stabilize the complex against the dissociating action of agonists. Other drugs with a high affinity for the anionic sites on the enzyme generally have many hydrophobic groups. These could act as competitive antagonists by a similar mechanism. Agonists are compounds with a low affinity for the anionic site (affinity constant 10^4 M or less) and dissociate the complex without remaining fixed to the binding sites. Neostigmine, physostigmine, or organophosphates combine with the esteratic site and cause partial protein–lipid dissociation, thereby sensitizing the complex to other agonists.

Although objections may be raised against the proposition that active sites on ChE function as receptor sites for cholinergic drugs, at least two newer aspects should be considered. The first is that a small fraction of the active sites on the enzyme, possibly having low catalytic activity when incorporated into the membrane, represent the cholinergic receptor molecules. Second, allosteric sites of the enzyme may play a more important role than previously considered in the

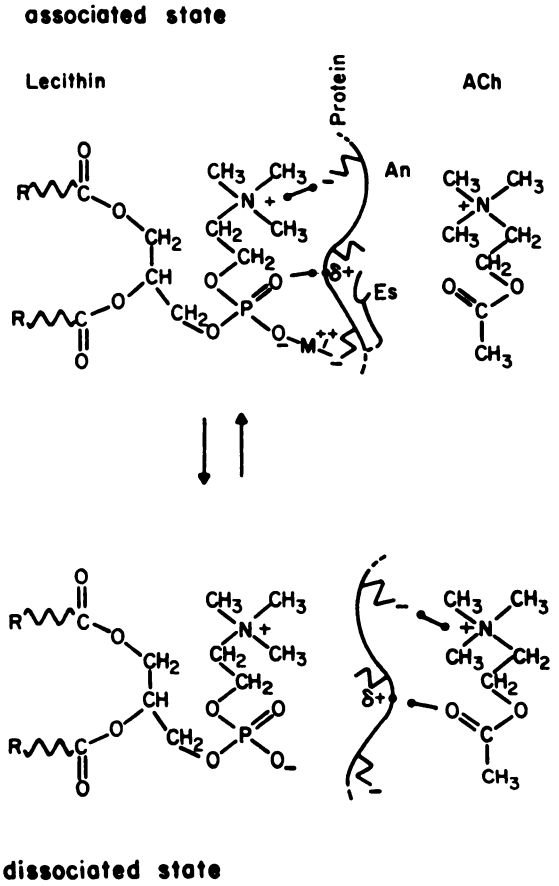

FIG. 5. Watkins' formulation of the ACh receptor site showing lecithin complexed to a protein with an anionic site and an esteratic site (receptor protein). ACh is shown to dissociate the lecithin protein complex (236). (Reproduced by permission of Academic Press, Inc.)

binding of cholinergic agents. One way to test these concepts is to examine the properties of cholinesterases *in situ* using intact tissues, an approach which is being actively pursued in this laboratory (91).

B. The beta adrenergic receptor

Catecholamines as well as indolamines, peptide hormones, certain steroids, and prostaglandins appear to have a regulatory action on the turnover and cellular levels of ATP and cyclic AMP (189). Interactions between these hormones and their receptors must lead to some kind of conformational perturbation and alteration in the catalytic activity of adenylcylase. The resulting changes in cellular levels of cyclic AMP can be regarded as the stimulus that leads to the response of a particular tissue to a hormone.

These ideas have been extensively developed by Belleau (26), Bloom and Gold-

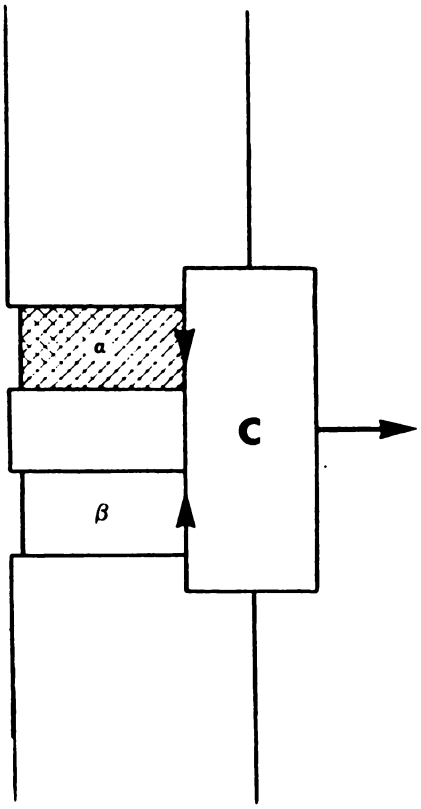

FIG. 6. Possible model of adenylcyclase (C) as related to adrenergic receptors. One type of regulatory subunit is bonded to the catalytic subunit such that it decreases enzyme activity (α), the other such that it increases catalytic activity (β). [From Robison *et al.* (188).]

man (37), and especially the Sutherland group (188) in review articles, and only salient points will be dealt with here. The adenylcyclase enzyme has thus far defied attempts at purification and probably represents a complex of lipids and several proteins in association with the cell membrane. Adenylcyclases of many tissues appear to be highly specific in that they are stimulated by only one or a few hormones. There is a close relationship between the response of a particular tissue to a given hormone and the sensitivity of the adenylcyclase in that tissue. It is the view of the Sutherland group that tissue adenylcyclase consists of two types of subunit: a regulator subunit facing the extracellular fluid and a catalytic subunit with its active center directed toward the interior of the cell (119) (see fig. 6). This proposal has similarities to that which has been made for the enzyme aspartate-transcarbamylase (103, 104). It is thus possible that different tissues contain a variety of catalytic subunits as well as regulatory subunits; alternatively the catalytic subunit may be common to each tissue but the regulatory subunits could have varying specificities for the different hormones that affect

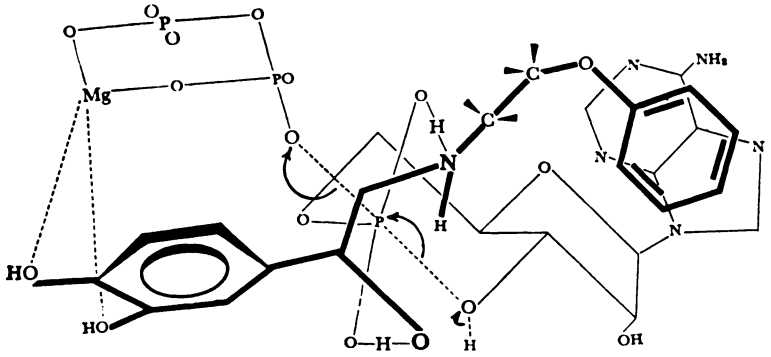

FIG. 7. Mechanism of catalysis by *beta* adrenergic agents of the intramolecular hydrolysis of ATP bound to adenylcyclase. [After Bloom and Goldman (37). Reproduced by permission of Academic Press, Inc.].

activity of adenylcyclase. This extension of the concept of isozymes to receptors, *i.e.*, "isoreceptors" was made (189) to account for tissue-specific differences in the regulation of adenylcyclase.

The *beta* adrenergic stimulants can produce a stimulation of adenylcyclase and a rise in the concentration of cyclic AMP. On this basis Bloom and Goldman (37) and also Belleau (25) considered that the catecholamines interact directly at the active center of adenylcyclase rather than at a regulatory site as proposed by Sutherland and Robison (119). Thus, the former considered the enzyme-substrate complex "adenylcyclase-ATP" to be synonymous with the receptor. The catecholamine binds at the active center of the complex and it participates directly in hydrolysis of the terminal phosphate. The indispensible moiety in the *beta* agonist molecule is the amine group, which has the role of facilitating the intramolecular nucleophilic attack at the innermost phosphorous atom of ATP by the ribose hydroxyl group. This relationship is shown in fig. 7. The catechol group in *beta* agonists as well as the side chain hydroxyl group are considered to be nonessential. These groups, however, do contribute to the potency, particularly the catecholamine ring system, which complexes with magnesium bound at the active site-substrate complex. The view that *beta* agonists interact directly at the active center in this way does not provide an explanation of the lack of stimulation of adenylcyclase by catecholamines in certain tissues that certainly contain the enzyme. To account for this fact, it may be postulated that areas of binding for the catecholamines differ from one tissue-specific adenylcyclase to another. Considered in this way, the views of Bloom and Goldman and of the Sutherland group converge and differ only in degree, *i.e.*, the binding site for catecholamines is either on a separate but regulatory macromolecule or on a site of the same macromolecule adjacent to the adenylcyclase active center.

C. The alpha adrenergic receptor

In view of the stimulating effect on adenycyclase of *beta* agonists, it is tempting to consider that *alpha* receptor stimulation might be related to a depressant effect

on this enzyme system. The *alpha* receptor may be a regulatory subunit of the enzyme which, when it binds to a catecholamine, has a depressing effect on the turnover number of the enzymatic subunit. *Alpha* receptor stimulation has been shown to lower the intracellular level of cyclic AMP in only a few instances (189). This is some support for the attractive hypothesis that *alpha* and *beta* receptors are related to adenylcyclase in a similar way except that the interaction with the catecholamine leading to a change in catalytic activity of adenylcyclase depends on which type of regulatory subunit dominates. This idea can account for the fact that both *alpha* and *beta* receptors are present in a number of tissues and also that one or the other may predominate in a given tissue.

Belleau (26) and Bloom and Goldman (37) have advanced an equally attractive hypothesis that the *alpha* adrenergic receptor is an enzyme-substrate complex, comprising an ATPase and ATP, to which the catecholamine binds. Excitatory *alpha* responses involving smooth muscle cell contraction are mediated by a receptor which consists of ATP directly associated with a specific magnesium-activated ATPase. The nature of the amine substituent on the catecholamine becomes the primary determinant of whether the compound will be an *alpha* or *beta* agonist. In receptor stimulation, the amine group catalyzes a nucleophilic attack at the terminal phosphorous atom of enzyme-bound ATP by an external nucleophile. This activity (*alpha* activity) is dependent on the size of the substituent on the nitrogen atom of the catecholamine. Although the process of muscle contraction is dependent on the initial release of membrane-bound calcium ion, the rate limiting step is postulated to be the hydrolysis of ATP. Thus, according to the hypothesis of Bloom and Goldman (37), the excitatory response to catacholamines in the muscle contraction process involves facilitation of the enzymatic hydrolysis of meromyosin-bound ATP to ADP. This view has some support in the work of Edman and Schild (73) on depolarized smooth muscle, where the catecholamine-induced contractions are independent of changes in cell membrane potential and the contractile effects of calcium and epinephrine were found to be additive.

This proposal suggests that one of the sites of the receptor may be intracellular. However, it is also possible that *alpha* adrenergic agonists are able to release membrane bound calcium in a process which is independent of depolarization of the membrane, as seems to be the case for ACh (74).

As envisaged by Belleau, *alpha* adrenergic receptors mediating membrane depolarization are not intracellular and may be involved in the regulation of membrane ATPase by facilitating ATP hydrolysis (25). In this instance the enzyme is supposed to be the sodium-potassium activated ATPase of membranes which is involved in ion transport (fig. 8). Stimulation of this ATPase by catecholamines could result in an inward release of membrane bound calcium that would initiate muscle contraction.

In the case of both adenylcyclase and membrane ATPase, the degree of purification is only in the initial stage (158). These two enzymes, if more highly purified, could furnish the first examples of isolated receptors, provided that the regulatory subunits survive the purification process.

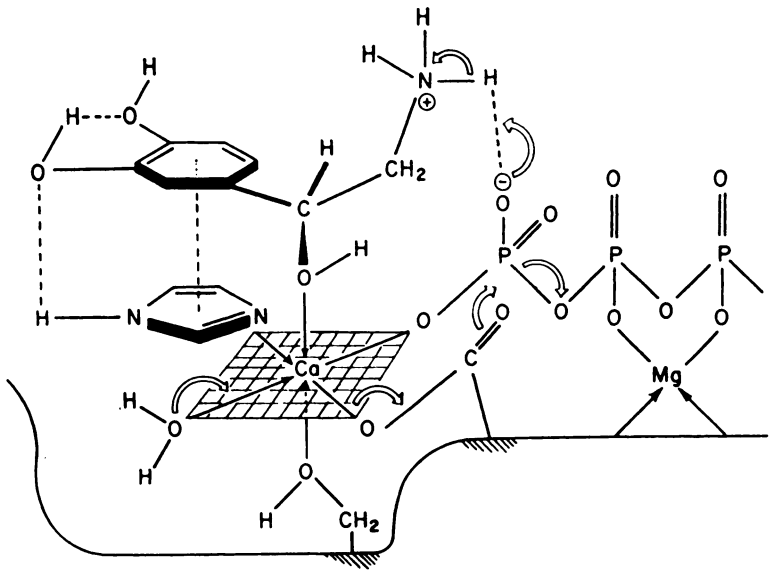

FIG. 8. Norepinephrine co-catalysis of phosphoryl group transfer from ATP bound to a specific sodium-potassium activated membrane ATPase. [From Belleau (26). Reproduced by permission.]

VII. CONCLUDING REMARKS

Until recently, information concerning the properties of pharmacological receptors was obtained almost exclusively from the responses of living tissues to drugs. In recent years, a variety of biochemical and physicochemical approaches have been brought to bear on the problem of the molecular nature of receptors and the fundamental aspects of their interaction with drugs. Several attempts to isolate receptors have been described and, while none has succeeded, they have led to the extraction and characterization of drug-binding macromolecules from a number of tissues. Essential to the identification of a receptor substance in isolated form is a knowledge of the amount and localization in the tissue. Information in both respects has now appeared for the cholinergic receptor in muscle endplates although complications are recognized particularly because of lack of specificity of the methods employed and because of the existence of extra-receptor, drug sensitive sites in nerve terminals, on silent receptors, drug acceptors, *etc.* Tailor made, site-directed reagents for tagging active sites offer perhaps the greatest promise for labeling receptors before their isolation and identification in solution or in subcellular elements.

Despite the fact that true receptor substances have not been isolated, some progress has been made along various lines toward a better understanding of their molecular properties. Models have been devised to visualize how drug-receptor interactions may be translated into an effect, *e.g.*, increased membrane permeability. Suggestions have been made, on the molecular level, to explain the distinction between the actions of antagonists and agonists. Although these pro-

posals are highly speculative, they are of sufficient fundamental importance to stimulate further efforts along these lines. Interactions of drugs with well characterized macromolecules have given insight into certain aspects of drug-receptor interactions *in vivo*.

The selective modification of receptors in various ways (enzymes, heat, urea, group-specific reagents) should provide impetus for more refined work to inquire more deeply into the physicochemical and chemical constitution of receptors and their active sites.

Finally, there is suggestive evidence that receptor-mediated events can be monitored by means other than the contractile response of an organ or tissue. This could perhaps be achieved by measuring the activities of certain key enzymes which undoubtedly are in close proximity to receptors. The enzymes may be part of the same macromolecular complex as the receptors, or, as suggested by some, may even bear the receptive sites themselves. At present, these enzymes in purified or semipurified form serve as useful models for investigating certain drug-receptor interactions. In particular the properties of such enzymes *in situ*, including their reactivity toward drugs ordinarily considered to have an effect by combining only with receptors, could provide the means for a better understanding of receptor-mediated responses on the molecular level.

Acknowledgments. The authors wish to express their sincere appreciation to Dr. Robert F. Furchgott for many suggestions and criticisms which served to improve the manuscript greatly. In addition, we wish to thank Drs. D. Lehr, N. C. Moran, and D. Waud for their critical review of this paper. Miss Ruth Eiss provided skillful and devoted secretarial assistance and Miss Ann Tormay helped considerably with the literature search.

REFERENCES

1. Albers, R. W. and Koval, G. J.: The interaction of gangliosides with cationic molecules. Biochim. Biophys. Acta **60**: 359–365, 1962.
2. Albuquerque, E. X., Sokoll, M.D., Sonesson, B. and Thesleff, S.: Studies on the nature of the cholinergic receptor. Eur. J. Pharmacol. **4**: 40–46, 1968.
3. Albuquerque, E. X. and Thesleff, S.: The effect of calcium on the skeletal muscle membrane after treatment with phospholipase C. Acta Physiol. Scand. **72**: 310–321, 1968.
4. Albuquerque, E. X. and Thesleff, S.: Effects of phospholipase A and lysolecithin on some electrical properties of the muscle membrane. Acta Physiol. Scand. **72**: 248–252, 1968.
5. Ambache, N.: Interaction of drugs and the effect of cooling on the isolated mammalian intestine. J. Physiol. (London) **104**: 266–287, 1948.
6. Ambache, N.: The peripheral action of Cl. botulinum toxin. J. Physiol. (London) **108**: 127–140, 1949.
7. Ariëns, E. J.: Molecular Pharmacology, vol. 1, Academic Press, Inc., New York, 1964.
8. Ariëns, E. J.: Receptor theory and structure-action relationships. Advan. Drug Res. **3**: 235–285, 1966.
9. Ariëns, E. J. and Simonis, A. M.: pH and drug action. Arch. Int. Pharmacodyn. Thér. **141**: 309–330, 1963.
10. Ariëns, E. J. and Simonis, A. M.: Cholinergic and anti-cholinergic drugs, do they act on common receptors? Ann. N.Y. Acad. Sci. **144**: 842–868, 1967.
11. Ariëns, E. J., van Rossum, J. M. and Koopman, P. C.: Receptor reserve and threshold phenomena. I. Theory and experiments with autonomic drugs tested on isolated organs. Arch. Int. Pharmacodyn. Thér. **127**: 459–478, 1960.
12. Arunlakshana, O. and Schild, H. O.: Some quantitative uses of drug antagonists. Brit. J. Pharmacol. Chemother. **14**: 48–58, 1959.
13. Augustinsson, K. B.: Cholinesterases, a study in comparative enzymology. Acta Physiol. Scand. **15**: 1–182, 1948.
14. Azcurra, J. M. and de Robertis, E.: Binding of *d*-tubocurarine-dimethyl-^{14}C, hexamethonium-methyl-^{14}C and alloferin-^{3}H by isolated synaptic membranes of brain cortex. Int. J. Neuropharmacol. **6**: 15–26, 1967.
15. Bachmann, E., Allmann, D. W. and Green, D. E.: The membrane systems of the mitochondrion. I. The S fraction of the outer membrane of beef heart mitochondria. Arch. Biochem. Biophys. **115**: 153–164, 1966.
16. Baker, B. R.: Design of Active Site Directed Irreversible Enzyme Inhibitors; the Organic Chemistry of the Active Site, John Wiley, & Sons, Inc., New York, 1967.

17. Barlow, R. B. and Hamilton, J. T.: Effects of some isomers and analogues of nicotine on junctional transmission. Brit. J. Pharmacol. Chemother. 18: 510–542, 1962.
18. Barnard, E. A. and Rogers, A. W.: Determination of the number, distribution, and some *in situ* properties of cholinesterase molecules in the motor end plate, using labeled inhibitor methods. Ann. N.Y. Acad. Sci. 144: 584–610, 1967.
19. Beani, L., Bianchi, C. and Ledda, F.: The effect of tubocurarine on acetylcholine release from motor nerve terminals. J. Physiol. (London) 174: 172–183, 1964.
20. Beckett, A. H.: Stereospecificity in the reactions of cholinesterase and the cholinergic receptor. Ann. N.Y. Acad. Sci. 144: 675–686, 1967.
21. Beckett, A. H., Harper, M. J. and Clitherow, J. W.: The absolute configuration of the α- and β-methylcholine isomers and their acetyl and succinyl esters. J. Pharm. Pharmacol. 15: 349–361, 1963.
22. Beckett, A. H., Kirk, G. and Vieji, A. S.: Micelles as simple models of drug receptors. J. Pharm. Pharmacol. 19: 827–836, 1967.
23. Belleau, B.: An analysis of drug-receptor interactions. Proc. 1st Int. Pharmacol. Meet. 7: 75–95, 1963.
24. Belleau, B.: Conformational perturbation in relation to the regulation of enzyme and receptor behavior. Advan. Drug Res. 2: 89–125, 1965.
25. Belleau, B.: Steric effects in catecholamine interactions with enzymes and receptors. Pharmacol. Rev. 18: 131–140, 1966.
26. Belleau, B.: Stereochemistry of adrenergic receptors. Newer concepts on the molecular mechanism of action of catecholamines and antiadrenergic drugs at the receptor level. Ann. N.Y. Acad. Sci. 139: 580–605, 1967.
27. Belleau, B.: Water as the determinant of thermodynamic transitions in the interaction of aliphatic chains with acetylcholinesterase and the cholinergic receptors. Ann. N.Y. Acad. Sci. 144: 705–717, 1967.
28. Belleau, B. and Lacasse, B.: Aspects of the chemical mechanism of complex formation between acetylcholinesterase and acetylcholine-related compounds. J. Med. Chem. 7: 768–775, 1964.
29. Belleau, B. and Tani H.: A novel irreversible inhibitor of acetyl-cholinesterase specifically directed at the anionic binding site: structure-activity relationships. Mol. Pharmacol. 2: 411–422, 1966.
30. Belleau, B., Tani H. and Lie, F.: A correlation between the biological activity of alkyltrimethylammonium ions and their mode of interaction with acetylcholinesterase. J. Amer. Chem. Soc. 87: 2283–2285, 1965.
31. Bevan, J. A. Osher, J. V. and Su, C.: Response of vascular smooth muscle to potassium and its antagonism by phenoxybenzamine. J. Pharmacol. Exp. Ther. 139: 216–221, 1963.
32. Beychok, S.: On the problem of isolation of the specific acetylcholine receptor. Biochem. Pharmacol. 14: 1249–1255, 1965.
33. Bianchi, C. P. and Shanes, A. M.: Calcium influx in skeletal muscle at rest, during activity and during potassium contracture. J. Gen. Physiol. 42: 803–815, 1959.
34. Blaber, L. C. and Karczmar, A. G.: Multiple cholinoceptive and related sites at the neuromuscular junction. Ann. N.Y. Acad. Sci. 144: 571–583, 1967.
35. Blaustein, M. P. and Goldman, D. E.: Action of anionic and cationic nerve-blocking agents. Experiment and interpretation. Science 153: 429–432, 1966.
36. Blaustein, M. P. and Goldman, D. E.: Competitive action of calcium and procaine on lobster axon. The mechanism of action of certain local anesthetics. J. Gen. Physiol. 49: 1043–1063, 1966.
37. Bloom, B. M. and Goldman, I. M.: The nature of catecholamine-adenine mononucleotide interactions in adrenergic mechanisms. Advan. Drug Res. 3: 121–169, 1966.
38. Brodie, B. B. and Hogben, C. A. M.: Some physicochemical factors in drug action. J. Pharm. Pharmacol. 9: 345–380, 1957.
39. Brooks, V. B.: The action of botulinum toxin on motor-nerve filaments. J. Physiol. (London) 123: 501–515, 1954.
40. Burgen, A. S. V., Dickens, F. and Zatman, L. J.: The action of botulinum toxin on the neuromuscular junction. J. Physiol. (London) 109: 10–24, 1949.
41. Burn, J. H. and Rand, M. J.: Acetylcholine in adrenergic transmission. Annu. Rev. Pharmacol. 5: 163–182, 1965.
42. Canepa, F. G.: Mechanism of ganglion blocking activity by methonium chains. Nature (London) 195: 573–575, 1962.
43. Canepa, F. G.: The role of ionic environment forces in the chemical transmission of nerve impulses by acetylcholine. Ann. N.Y. Acad. Sci. 144: 918–935, 1967.
44. Canepa, F. G., Pauling, P. and Sörum, H.: Structure of acetylcholine and other substrates of cholinergic systems. Nature (London) 210: 907–909, 1966.
45. Carroll, P. M. and Sereda, D. D.: Cell membrane of uterine smooth muscle. Nature (London) 217: 666–667, 1968.
46. Carroll, P. M. and Sereda, D. D.: Sialic acid of uterine muscle membrane: reappraisal of the serotonin receptor of smooth muscle. Nature (London) 217: 667–668, 1968.
47. Cavallito, C. J.: Bonding characteristics of acetylcholine simulants and antagonists and cholinergic receptors. Ann. N.Y. Acad. Sci. 144: 900–911, 1967.
48. Cavallito, C. J.: Some speculation on the chemical nature of post junctional membrane receptors. Fed. Proc. 26: 1647–1654, 1967.
49. Cavallito, C. J. and Sandy, P.: Anticholinesterase activities of some bisquaternary ammonium salts. Biochem. Pharmacol. 2: 233–242, 1959.
50. Cecil, R.: The role of sulfur in proteins. *In* The Proteins, ed. by H. Neurath, vol. I, pp. 379–476, Academic Press, Inc., New York, 1963.
51. Chagas, C.: Studies on the mechanism of curarization. Ann. N.Y. Acad. Sci. 81: 345–357, 1959.

52. CHAGAS, C., PENNA-FRANCA, E., HASSON A., CROCKER, C., NISHIE, K. AND GARCIA, E. J.: Studies of the mechanisms of curarisation. An. Acad. Brasil Cienc. 29: 53–62, 1957.
53. CHAGAS, C., PENNA-FRANCA, E., NISHIE, K. AND GARCIA, E. J.: Specificity of the complex formed by gallamine triethiodide with a macromolecular constituent of the electric organ. Arch. Biochem. Biophys. 75: 251–259, 1958.
54. CHAGAS, C., PENNA-FRANCA, E., NISHIE, K., CROCKER, C. AND MIRANDA, M.: The fixation of triiodoethyl of radioactive gallamine on electroplates of electrophorus electricus. C. R. Hebd. Seances Acad. Sci. Paris 242: 2671–2674, 1956.
55. CHANGEUX, J. P.: Responses of acetylcholinesterase from torpedo marmorata to salts and curarising drugs. Mol. Pharmacol. 2: 369–392, 1966.
56. CHANGEUX, J. P., PODLESKI, T. AND WOFSY, L.: Affinity labeling of the acetylcholine receptor. Proc. Nat. Acad. Sci. U.S.A. 58: 2063–2070, 1967.
57. COHEN, L. A.: Group-specific reagents in protein chemistry. Annu. Rev. Biochem. 37: 695–726, 1968.
58. COLEMAN, M. H. AND ELEY, D. P.: The thermal inactivation of acetylcholinesterase. Biochim. Biophys. Acta 67: 646–657, 1963.
59. COLEMAN, M. E., HUME, A. H. AND HOLLAND, W. C.: Nicotine-like stimulant actions of several substituted phenylcholine ethers. J. Pharmacol. Exp. Ther. 148: 66–70, 1965.
60. CULVENOR, C. C. J. AND HAM, N. S.: Proton magnetic resonance spectrum and conformation of acetylcholine. Chem. Commun. 15: 537–539, 1966.
61. CUTHBERT, A. W.: Modification of drug responses by hydrolytic enzymes. J. Pharm. Pharmacol. 18: 561–568, 1966.
62. DANIEL, E. E.: Divalent ions in muscle function. *In* Muscle, ed. by W. M. Paul *et al.*, pp. 295–313, Pergamon Press, New York, 1965.
63. DEL CASTILLO, J., RODRIGUEZ, A., ROMERO, C. A. AND SANCHEZ, V.: Lipid films as transducer for detection of antigen-antibody and enzyme-substrate reactions. Science 153: 185–188, 1966.
64. DEL CASTILLO, J., RODRIGUEZ, A. AND ROMERO, C. A.: Pharmacological studies on an artificial transmitter-receptor system. Ann. N.Y. Acad. Sci. 144: 803–815, 1967.
65. DE ROBERTIS, E., FISZER, S. AND SOTO, E. F.: Cholinergic binding capacity of proteolipids from isolated nerve-ending membranes. Science 158: 928–929, 1967.
66. DETTBARN, W. D., ROSENBERG, P. AND NACHMANSOHN, D.: Restoration by a specific chemical reaction of "irreversibly" blocked axonal electrical activity. Life Sci. 3: 55–60, 1964.
67. DETWILER, P., MAENGWYN-DAVIES, G. D. AND BIGO-GULLINO, M.: Kinetics and equilibria in the anti-cholinergic actions of l-hyoscyamine on guinea pig intestinal smooth muscle. Fed. Proc. 27: 661, 1968.
68. DIKSTEIN, S.: Physicochemical models of drug action. *In* Biophysics of Physiological and Pharmacological Actions, ed. by A. M. Shanes, pp. 49–60, American Association for Advancement of Science, Washington, D.C., 1961.
69. DIKSTEIN, S. AND SULMAN, F. G.: Function of phospholipids in receptors. Biochem. Pharmacol. 14: 739–743, 1965.
70. DIKSTEIN, S. AND SULMAN, F. G.: Interaction of dibenamine with the phospholipids of the aorta. Biochem. Pharmacol. 14: 881–885, 1965.
71. DOMBRO, R. S., BRADHAM, L. S., CAMPBELL, N. K. AND WOOLLEY, D. W.: Serotonin receptors. III. Role of salt formation. Biochim. Biophys. Acta 54: 516–524, 1961.
72. ECCLES, J. C. AND JAEGER, J. C.: The relationship between the mode of operation and the dimensions of the junctional regions at synapses and the motor end organs. Proc. Roy. Soc. Ser. B Biol. Sci. 148: 38–56, 1958.
73. EDMAN, K. A. P. AND SCHILD, H. O.: Calcium and the stimulant and inhibitory effects of adrenaline in depolarised smooth muscle. J. Physiol. (London) 169: 404–411, 1963.
74. EDMAN, K. A. P. AND SCHILD, H. O.: The need for calcium in the contractile responses induced by acetylcholine and potassium in the rat uterus. J. Physiol. (London) 161: 424–441, 1962.
75. ELLENBROEK, B. W. J., NIVAARD, R. J. F., VAN ROSSUM, J. M. AND ARIËNS, E. J.: Absolute configuration and parasympathetic action: pharmacodynamics of enantiomorphic and diastereoisomeric esters of β-methylcholine. J. Pharm. Pharmacol. 17: 393–404, 1965.
76. EHRENPREIS, S.: Isolation and identification of an acetylcholine receptor protein from electric tissue. Biochim. Biophys. Acta 44: 561–577, 1960.
77. EHRENPREIS, S.: Isolation and identification of the acetylcholine receptor protein from electric organ of electric eel. *In* Bioelectrogenesis, ed. by C. Chagas and A. P. de Carvalho, pp. 379–396, Elsevier, Publ. Co., Amsterdam, 1961.
78. EHRENPREIS, S.: Immunohistochemical localization of drug-binding protein in tissues of the electric eel. Nature (London) 194: 586–587, 1962.
79. EHRENPREIS, S.: Use of the drug-binding protein from electric tissue to explain the action of neurotropic agents. Int. J. Neuropharmacol. 1: 273–281, 1962.
80. EHRENPREIS, S.: Isolation and properties of a drug binding protein from electric tissue of electric eel. Proc. 1st Int. Pharmacol. Meet. 7: 119–133, 1963.
81. EHRENPREIS, S. The interaction of quaternary ammonium ions with various macromolecules, Georgetown Med. Bull. 16: 148–158, 1963.
82. EHRENPREIS, S.: Acetylcholine and nerve activity. Nature (London) 201: 887–893, 1964.
83. EHRENPREIS, S.: An approach to the molecular basis of nerve activity. *In* Symposium on Active Membranes, Woods Hole, Mass., July, 1965. J. Cell. Comp. Physiol. 66: 159–164, 1965.
84. EHRENPREIS, S.: Possible nature of the cholinergic receptor. Ann. N.Y. Acad. Sci. 144: 720–736, 1967.
85. EHRENPREIS, S.: Molecular aspects of cholinergic mechanisms. *In* Medicinal Chemistry, vol. I, Drugs Affecting the Peripheral Nervous System, ed. by A. Burger, pp. 1–78, M. Dekker, New York, 1967.
86. EHRENPREIS, S., BIGO-GULLINO, M. AND AVERY, M. A.: The effects of cholinesterase inhibitors and lipid soluble

quaternary ammonium compounds on contractility of rabbit aortic strip. Arch. Int. Pharmacodyn. Thér. 156: 1–20, 1965.

87. Ehrenpreis, S. and Fishman, M. M.: Interaction of quaternary ammonium compounds with chondroitin sulfuric acid. Biochim. Biophys. Acta 44: 576–585, 1960.
88. Ehrenpreis, S. and Fleisch, J. H.: Alteration in vasoactive polypeptide receptor activity of the rat fundal strip by heat and urea. Fed. Proc. 27: 70–72, 1968.
89. Ehrenpreis, S., Hazra, J. and Bigo-Gullino, M.: Effects of acetone on responses of various tissues to biogenic amines. Biochem. Pharmacol. 17: 852–853, 1968.
90. Ehrenpreis, S. and Kellock, M. G.: Interaction of quaternary ammonium compounds with hyaluronic acid. Biochim. Biophys. Acta 45: 525–528, 1960.
91. Ehrenpreis, S. and Mittag, T. W.: *In vitro* receptor materials. *In* Symposium on Advances in Screening Methodology, Academy of Pharmaceutical Sciences Meeting, Washington, D.C., Nov. 19–22, 1968, in press.
92. Ehrenpreis, S., Munson, H. R., Jr. and Avery, M. A.: Lack of correlation between cholinesterase activity and responsiveness of nerve (lobster) and smooth muscle (rabbit aorta). Life Sci. 3: 809–819, 1964.
93. Ehrenpreis, S., Munson, H. R., Jr., Kaplan, M. and Platthy-Lang, M.: Spectro-photometric studies on the reaction of noradrenalin with phosvitin. VI Int. Congr. Biochem., New York, July, 1964.
94. Elmquist, D. and Thesleff, S.: Ideas regarding receptor "desensitization" at the motor end-plate. Rev. Can. Biol. 21: 229–234, 1962.
95. Emmelin, N., Nordenfelt, I. and Perec, C.: Rate of fall in choline acetyltransferase activity in denervated diaphragms as dependent on the length of the degenerating nerve. Experientia 22: 725–726, 1966.
96. Fastier, F. N.: Modern concepts in relationship between structure and biological activity. Annu. Rev. Pharmacol. 4: 51–68, 1964.
96a. Fatt, P. and Katz, B.: An analysis of the end-plate potential recorded with an intracellular electrode. J. Physiol. (London) 115: 320–340, 1951.
96b. Fatt, P. and Katz, B.: Chemoreceptor activity at the motor end-plate. Acta Physiol. Scand. 29: 117–125, 1953.
97. Feinstein, M. B.: Reaction of local anesthetics with phospholipids—a possible chemical basis for anesthesia. J. Gen. Physiol. 43: 357–374, 1964.
98. Fleisch, J. H. and Ehrenpreis, S.: Thermal alteration in receptor activity of the rat fundal strip. J. Pharmacol. Exp. Ther. 162: 21–29, 1968.
99. Fleisch, J. H. and Ehrenpreis, S.: A study of the alteration in receptor activity of the rat fundal strip by urea. J. Pharmacol. Exp. Ther. 163: 363–366, 1968.
100. Freiss, S. L., Witkop, B., Durant, R. C., Reber, L. J. and Thommesen, W. C.: Further aspects of stereospecificity in interaction of polyfunctional amine derivatives with biological receptors. Arch. Biochem. Biophys. 96: 158–165, 1962.
101. Furchgott, R. F.: Dibenamine blockade in strips of rabbit aorta and its use in differentiating receptors. J. Pharmacol. Exp. Ther. 111: 265–284, 1954.
101a. Furchgott, R. F. and Bursztyn, P.: Comparison of dissociation constants and of relative efficacies of selected agonists acting on parasympathetic receptors. Ann. N.Y. Acad. Sci. 144: 882–899, 1967.
101b. Furchgott, R. F. and Kirpekar, S. M.: Competition between β-haloalkylamines and norepinephrine for sites in cardiac muscle. Proc. 1st Int. Pharmacol. Meet. 7: 339–350, 1963.
102. Gaddum, J. H. and Picarelli, Z. P.: Two kinds of tryptamine receptors. Brit. J. Pharmacol. 12: 323–328, 1957.
103. Gerhart, J. C. and Pardee, A. B.: The effect of the feedback inhibitor CTP on subunit interactions in aspartate transcarbamylase. Cold Spring Harbor Symp. Quant. Biol. 28: 491–496, 1963.
104. Gerhart, J. C. and Schachman, H. K.: Allosteric interactions in aspartate transcarbamylase. II. Evidence for different conformational states of the protein in the presence and absence of specific ligands. Biochemistry 7: 538–552, 1968.
105. Gill, E. W. and Rang, H. P.: An alkylating derivative of benzilylcholine with specific and long-lasting parasympatholytic activity. Mol. Pharmacol 2: 284–297, 1966.
106. Gissen, A. J. and Nastuk, W. L.: The mechanisms underlying neuromuscular block following prolonged exposure to depolarizing agents. Ann. N.Y. Acad. Sci. 135: 184–194, 1966.
107. Goldman, D. E.: A molecular structural basis for the excitation properties of axons. Biophys. J. 4: 167–188, 1964.
108. Goldman, D. E.: Gate control of ion flux in axons. J. Gen. Physiol. 48: 75–77, 1965.
109. Grossman, M., Kalow, W. and Cooper, P.: Effects of enzymes on the pharmacology of isolated organs. Nature (London) 217: 1151–1152, 1968.
110. Grundfest, H.: General physiology and pharmacology of junctional transmission. *In* Biophysics of Physiological and Pharmacological Actions, ed. by A. M. Shanes, pp. 329–389, American Association for Advancement Science, Washington, D.C., 1961.
111. Günther, W. H. H. and Mautner, H. G.: Analogs of parasympathetic neuroeffectors I. Acetylselenocholine, selenocholine and related compounds. J. Med. Chem. 7: 229–232, 1964.
112. Hardman, H. F. and Reynolds, R. C.: An effect of pH upon epinephrine inotropic receptors in the turtle heart. J. Pharmacol. Exp. Ther. 149: 219–224, 1965.
113. Hasson, A. and Chagas, C.: Purification of macromolecular components of the aqueous extract of the electric organ (*E. electricus* (L)) with binding capacity in vitro, for quaternary ammonium bases. *In* Bioelectrogenesis, ed. by C. Chagas and A. Paes de Carvalho, pp. 362–378, Elsevier, Publ. Co., Amsterdam, 1961.
114. Hasson, A. and Liepin, L.: Reversible inhibition of electric-organ cholinesterase by curare and curare-like agents Biochim. Biophys. Acta 75: 397–401, 1963.
115. Hasson-Voloch, A., Liepin, L. L. and Soares, M. N.: Studies of the inhibitory action of curare and curare-like agents on acetylcholinesterase and its reversion by polyelectrolytes. An. Acad. Bras. Cienc. 36: 441–448, 1964.

116. Hasson-Voloch, A. and Yon, J.: Contribution a l'etude de la fixation du curare sur un récepteur extrait de l'organe electrique de Electrophorus electricus. Biochim. Biophys. Acta 50: 389-391, 1961.
117. Hazra, J.: Effects of botulinum toxin and hemicholinium-3 (HC-3) on injected acetylcholine and its potentiation by diisopropylfluorophosphate. Fed. Proc. 27: 407, 1968.
118. Hebb, C. O.: Acetylcholine content of the rabbit plantaris muscle after denervation. J. Physiol. (London) 163: 294-306, 1962.
119. Hechter, O.: Hormone action at the cell membrane. *In* Mechanisms of Hormone Action, ed., by P. Karlson, pp. 61-82, Academic Press, Inc., New York, 1965.
120. Hey, P.: On relationships between structure and nicotine-like stimulant activity in choline esters and ethers. Brit. J. Pharmacol. Chemother. 7: 117-129, 1952.
121. Hinke, J. A. M.: Calcium requirement in arterial smooth muscle. *In* Muscle, ed. by W. M. Paul *et al.*, pp. 269-285, Pergamon Press, New York, 1965.
122. Hoskin, F. C. G. and Rosenberg, P.: Penetration of an organophosphorous compound into squid axon and its effects on metabolism and function. Science 156: 966-967, 1967.
123. Huneeus, F. C., Fernandez, H. L. and Smith, B. H.: Effects of redox and sulfhydryl reagents on the bioelectric properties of the giant axon of the squid. Biophys. J. 6: 675-689, 1966.
123a. Imaizumi, R., and Yoshida, H.: The involvement of a ferrous protein in natural adrenaline receptor. Jap. J. Pharmacol. 10: 147-156, 1961.
124. Inoue, M.: Mecholyl receptor, especially from the standpoint of the curve analysis of drug antagonism. Kobe Ika Daigaku Kiyo 15: 71-84, 1959.
125. Ishida, Y.: Studies of drug receptors on the plain muscle. III. Effect of hydrogen ion concentration upon ACh, Ba and oxytocin receptors of the isolated rat uterus. Yakugaki Zasshi 81: 1717-1721, 1961.
126. Ishida, Y., Moritoki, H. and Hara, K.: Studies of drug receptors on the plain muscle. II. Influence of heavy metal ions on the oxytocin and barium receptor of isolated rat uterus. Yakugaki Zasshi 81: 1713-1717, 1961.
127. Jenkinson, D. H.: The antagonism between tubocurarine and substances which depolarise the motor end-plate. J. Physiol. (London) 152: 309-324, 1960.
128. Jensen, E. V. and Jacobsen, H. I.: Basic guides to the mechanism of estrogen action. Recent Progr. Hormone Res. 18: 387-414, 1962.
129. Jensen-Holm, J.: The cholinesterase activity, alone and in the presence of inhibitors, at low substrate concentrations. Acta Pharmacol. Toxicol. 18: 379-397, 1961.
130. Karczmar, A. G., Kim, K. C. and Koketsu, K.: Endplate effects and antagonism to *d*-tubocurarine and decamethonium of tetraethylammonium and of methoxyambenonium (Win. 8078). J. Pharmacol. Exp. Ther. 134: 199-205, 1961.
131. Karlin, A. and Bartels, E.: Effects of blocking sulfhydryl groups and of reducing disulfide bonds on the acetylcholine-activated permeability system of the electroplax. Biochim. Biophys. Acta 126: 525-535, 1966.
132. Karlin, A. and Winnik, M.: Reduction and specific alkylation of the receptor for acetylcholine. Proc. Nat. Acad. Sci. U.S.A. 60: 668-674, 1968.
133. Khairallah, P. A. and Page, I. H.: Mechanism of action of angiotensin and bradykinin on smooth muscle *in situ*. Amer. J. Physiol. 200: 51-54, 1961.
134. Khairallah, P. A., Smeby, R. R., Page, I. H. and Bumpus, F. M.: Effect of urea and amino acids on angiotensin interaction with smooth muscle. Biochim. Biophys. Acta 104: 85-91, 1965.
135. Khromov-Borrisov, N. V. and Michelson, M. J.: The natural disposition of cholinoreceptors of locomotor muscles and the changes in their disposition in the course of evolution. Pharmacol. Rev. 18: 1051-1090, 1966.
136. Kier, L. B.: Molecular orbital calculation of preferred conformations of acetylcholine, muscarine and muscarone, Mol. Pharmacol. 3: 487-494, 1967.
137. Kier, L. B.: A molecular orbital calculation of the preferred conformation of nicotine. Mol. Pharmacol. 4: 70-76, 1968.
138. Kier, L. B.: Receptor mapping using molecular orbital theory. *In* Buffalo-Milan Symposium on Drug-Receptor Interactions, Buffalo, August, 1968.
139. Kim, K. C. and Karczmar, A. G.: Adaptation of the neuromuscular junction to constant concentration of ACh. Int. J. Neuropharmacol. 6: 51-61, 1967.
139a. Kitz, R. J. and Kremzner, L. T.: Conformational changes of cholinesterase. Mol. Pharmacol. 4: 104-107, 1968.
140. Koelle, G. B.: A new general concept of the neurohumoral functions of acetylcholine and acetylcholine esterase. J. Pharm. Pharmacol. 14: 65-90, 1962.
141. Koschtojanz, C. S. and Turpaev, T. M.: Role of sulphydril groups in the action of acetylcholine and inhibition of the vagus nerve. Nature (London) 158: 836-837, 1946.
142. Koshland, D. E., Jr.: Mechanisms of transfer ensymes. *In* The Ensymes, ed. by Paul D. Boyer, Henry Lardy and Karl Myrbäck, vol. I, pp. 305-347, Academic Press, Inc., New York, 1959.
143. Krnjevic, K. and Straughan, D. W.: The release of acetylcholine from the denervated rat diaphragm. J. Physiol. (London) 170: 371-378, 1964.
144. Krupka, R. M.: Acetylcholinesterase. Can. J. Biochem. 42: 677-693, 1964.
145. Kruse, R. S.: Effects of salts, temperature, urea, acetylcholine (ACh) and pH on the interaction of *d*-tubocurarine with chondroitin sulfate (CSA). Fed. Proc. 26: 295, 1967.
146. Lewis, J. J., Martin-Smith, M. and Muir, T. C.: Observations upon roles of ether oxygen, alkyl group size, number of onium centers and interonium distance upon neuromuscular block in bis and polyonium compounds. Brit. J. Pharmacol. Chemother. 20: 307-318, 1963.
147. Loomis, T. A.: Neuromuscular function in the absence of cholinesterase. Arch. Int. Pharmacodyn. Thér. 165: 319-327, 1967.

148. Loomis, T. A. and Konker, A. C.: Effects of an organic phosphate on the response of denervated muscle to ACh and some neuromuscular blocking agents. Arch. Int. Pharmacodyn. Thér. 165: 308–318, 1967.
149. Lu, F. C.: The effects of proteolytic enzymes on the isolated rabbit intestine. Brit. J. Pharmacol. Chemother. 7: 637–740, 1952.
150. Lu, F. C.: Some observations on the mode of action of several intestinal muscle stimulants. Rev. Can. Biol. 16: 108–111, 1957.
150a. Lullman, H. and Peters, T.: Influence of pH on the action of parasympathomimetic drugs. Eur. J. Pharmacol. 2: 109–112, 1967.
151. Lum, B. K. B., Kermani, M. H. and Heilman, R. D.: Intestinal relaxation produced by sympathomimetic amines on the isolated rabbit jejunum: selective inhibition by adrenergic blocking agents and by cold storage. J. Pharmacol. Exp. Ther. 154: 463–471, 1966.
152. Maengwyn-Davies, G. D. and Oroszlan, S. I.: The possible implications of complex formation of atropine with amino acids and amines. Ann. N.Y. Acad. Sci. 144: 819–835, 1967.
153. Martin, P. J. and Schild, H. O.: The antagonism of disulphide polypeptides by thiols. Brit. J. Pharmacol. Chemother. 25: 418–431, 1965.
154. Martin-Smith, M., Smail, G. A. and Stenlake, J. B.: The possible role of conformational isomerism in the biological actions of acetylcholine. J. Pharm. Pharmacol. 19: 561–589, 1967.
155. Mautner, H. G.: The molecular basis of drug action. Pharmacol. Rev. 19: 107–144, 1967.
156. May, M., Moran, J. F., Kimelberg, H. and Triggle, D. J.: The noradrenaline α-receptor. II. Analysis of the spare receptor hypothesis and estimation of the concentration of α-receptors in rabbit aorta. Mol. Pharmacol. 3: 28–36, 1967.
157. McCollester, D. L. and Semente, G.: Membrane isolation and cytoskeletal breakdown. I. The effect of endogenous enzymes, ions, pH, temperature, and dissolved gases. Biochim. Biophys. Acta 90: 146–158, 1964.
158. Medzihradsky, F., Kline, M. H. and Hokin, L. E.: Studies on the characterization of the sodium-potassium transport adenosinetriphosphatase. I. Solubilization, stabilization and estimation of apparent molecular weight. Arch. Biochem. Biophys. 121: 311–316, 1967.
159. Mikhel'son, M. Y. and Fruentov, N. K.: Significance of the pH of the medium for cholinolytic and cholinomimetic action of some tertiary amines and quaternary ammonium compounds. Farmakol. Neirotropnykh Sredstv, Akad. Med. Nauk SSSR, Sb. 186–196, 1963.
160. Miledi, R.: Junctional and extra-junctional acetylcholine receptors in skeletal muscle fibres. J. Physiol. (London) 151: 24–30, 1960.
161. Minker, E. and Koltai, M.: Effect of protamine sulphate on the transmission processes, in peripheral sympathetic ganglia. Acta Physiol. Hung. 24: 365–371, 1964.
162. Moran, J. F., May, M., Kimelberg, H. and Triggle, D. J.: The noradrenaline α-receptor. I. Techniques of receptor isolation. The distribution and specificity of action of N-(2-bromoethyl)-N-ethyl-1-naphthylmethylamine, a competitive antagonist of noradrenaline. Mol. Pharmacol. 3: 15–27, 1967.
163. Moran, J. F. and Triggle, D. J.: Approaches to the quantitation and isolation of pharmacological receptors. *In* Buffalo-Milan Symposium on Drug-Receptor Interactions, Buffalo, August, 1968, in press.
164. Nachmansohn, D.: Chemical and Molecular Basis of Nerve Activity, Academic Press, Inc., New York, 1959.
165. Namba, T. and Grob, D.: Cholinergic receptors in skeletal muscle: isolation and properties of muscle ribonucleoprotein with affinity for *d*-tubocurarine and acetylcholine, and binding activity of the subneural apparatus of motor end plates with divalent metal ions. Ann. N.Y. Acad. Sci. 144: 772–800, 1967.
166. Namba, T. and Grob, D.: Ribonucleoprotein in human skeletal muscle with affinity for *d*-tubocurarine and acetylcholine. Biochem. Pharmacol. 16: 1135–1138, 1967.
167. Nastuk, W. L.: Some ionic factors that influence the action of acetylcholine at the muscle end-plate membrane. Ann. N.Y. Acad. Sci. 81: 317–327, 1959.
168. Nastuk, W. L.: Activation and inactivation of muscle postjunctional receptors. Fed. Proc. 26: 1639–1646, 1967.
169. Nastuk, W. L., Manthey, A. A. and Gissen, A. J.: Activation and inactivation of postjunctional membrane receptors. Ann. N.Y. Acad. Sci. 137: 999–1014, 1966.
170. Nedergaard, O. A. and Taylor, D. B.: The uptake by skeletal muscle of tritium-labeled decamethonium and dimethyltubocurarine. Experientia 22: 521–522, 1966.
171. Neet, K. E. and Friess, S. L.: Curare-binding macromolecules from medullated nerve tissue. Arch. Biochem. Biophys. 99: 484–493, 1962.
172. Nickerson, M.: Receptor occupancy and tissue response. Nature (London) 178: 697–698, 1956.
173. Nistratova, S. N. and Turpaev, T. M.: Isolation, of choline-receptor protein from heart muscle. Biokhimiya 26: 952–955, 1961.
174. Nistratova, S. N., Zvezdina, N. D., Mitropolitanskaya, R. L. and Turpaev, T. M.: Isolation and properties of choline-receptive protein from the central nervous system in vertebrates. Probl. Neirokhim., Akad. Nauk SSSR 28–33, 1966.
175. Offermeier, J. and Ariëns E. J.: Serotonin receptors: I. receptors involved in its action. Arch. Int. Pharmacodyn. Thér. 164: 192–215, 1966.
176. Okamoto, M. and Riker, W. F., Jr.: Subacute denervation: A means of disclosing mammalian motor nerve terminals as critical sites of acetylcholine and facilitatory drug actions. J. Pharmacol. Exp. Ther. 166: 217–224, 1969.
177. Oroszlan, S. I. and Maengwyn-Davies, G. D.: The binding of atropine to bovine serum albumin. Biochem. Pharmacol. 11: 1203–1212, 1962.
178. Paton, W. D. M.: A theory of drug action based on the rate of drug-receptor combination. Proc. Roy. Soc. Ser. B Biol. Sci. 154: 21–69, 1961.

179. PATON, W. D. M. AND RANG, H. P.: The uptake of atropine and related drugs by intestinal smooth muscle of the guinea-pig in relation to acetylcholine receptors. Proc. Roy. Soc. Ser. B Biol. Sci. 163: 1–44, 1965.
180. PRESSMAN, D. AND GROSSBERG, A.: The Structural Basis of Antibody Specificity, Benjamin, New York, 1968.
181. RANG, H. P.: The uptake of atropine and related compounds by smooth muscle. Ann. N.Y. Acad. Sci. 144: 756–765, 1967.
182. RIKER, W. F., JR.: Actions of acetylcholine on mammalian motor nerve terminal. J. Pharmacol. Exp. Ther. 152: 397–416, 1966.
183. RIKER, W. F., JR., ROBERTS, J., STANDAERT, F. G. AND FUJIMORI, H.: The motor nerve terminal as the primary focus for drug-induced facilitation of neuromuscular transmission. J. Pharmacol. Exp. Ther. 121: 286–312, 1957.
184. RIKER, W. F., JR. AND STANDAERT, F. G.: The action of facilitatory drugs and acetylcholine on neuromuscular transmission. Ann. N.Y. Acad. Sci. 135: 163–176, 1966.
185. RITCHIE, J. M.: On the role of acetylcholine in conduction in mammalian non-myelinated nerve fibers. Ann. N.Y. Acad. Sci. 144: 505–516, 1967.
186. RITCHIE, J. M. AND ARMETT, C. J.: On the role of acetylcholine in conduction in mammalian non-myelinated nerve fibers. J. Pharmacol. Exp. Ther. 139: 201–205, 1963.
187. RITCHIE, J. M. AND GREENGARD, P.: On the active structure of local anesthetics. J. Pharmacol. Exp. Ther. 133: 241–245, 1961.
188. ROBISON, G. A., BUTCHER, R. W. AND SUTHERLAND, E. W.: Adenylcyclase as an adrenergic receptor. Ann. N.Y. Acad. Sci. 139: 703–723, 1967.
189. ROBISON, G. A., BUTCHER, R. W. AND SUTHERLAND, E. W.: On the relation of hormone receptors to adenylcyclase. *In* Buffalo-Milan Symposium of Drug-Receptor Interactions, August 26–28th, 1968, in press.
190. ROCHA E SILVA, M.: Influence of pH on the interaction of histamine with its receptors in the guinea pig ileum. Arch. Int. Pharmacodyn. Thér. 128: 355–373, 1960.
191. ROCHA E SILVA, M., VALLE, J. R. AND PICARELLI, Z. P.: A pharmacological analysis of the mode of action of serotonin (5-hydroxytryptamine) upon the guinea pig ileum. Brit. J. Pharmacol. Chemother. 8: 378–388, 1953.
192. ROSENBERG, P. AND EHRENPREIS, S.: Reversible block of conduction by curare after treatment with cobra venom and a detergent. Nature (London) 190: 728–729, 1961.
193. ROSENBERG, P. AND EHRENPREIS, S.: Reversible block of conduction by curare after treatment with cobra venom. Biochem. Pharmacol. 8: 192–206, 1961.
194. ROSENBERG, P. AND HIGMAN, H.: An improved isolated single electroplax preparation. II. Compounds acting on the conducting membrane. Biochim. Biophys. Acta 45: 348–354, 1960.
195. ROSENBERG, P., MACKEY, E. A., HIGMAN, H. B. AND DETTBARN, W. D.: Choline acetylase and cholinesterase activity in denervated electroplax. Biochim. Biophys. Acta 82: 266–275, 1964.
195a. ROSENBERG, P. AND MAUTNER, H. G.: Acetylcholine receptor: similarity in axons and junctions. Science 155: 1569–1571, 1967.
196. SCHERAGA, H. A.: Protein Structure, Academic Press, Inc., New York, 1961.
197. SCHILD, H. O.: pA, a new scale for the measurement of drug antagonism. Brit. J. Pharmacol. Chemother. 2: 189–206, 1947.
198. SCHILD, H. O.: pA and competitive drug action. Brit. J. Pharmacol. Chemother. 4: 277–280, 1949.
199. SCHUELER, F. W.: Chemobiodynamics and Drug Design, p. 139. McGraw-Hill, New York, 1960.
200. SCHWARTZ, I. L., RASMUSSEN, H., SCHOESSLER, M. A., SILVER, L. AND FONG, C. T. O.: Relation of chemical attachment to physiological action of vasopressin. Proc. Nat. Acad. Sci. U.S.A. 46: 1288–1298, 1960.
201. SEKUL, A. A. AND HOLLAND, W. C.: Comparative pressor effect of certain unsaturated acid esters of choline. J. Pharmacol. Exp. Ther. 133: 313–318, 1961.
202. SEKUL, A. A., HOLLAND, W. C. AND HOUSE, R.: Relationship between electronic structure and intensity of nicotine-like action of choline esters. Arch. Int. Pharmacodyn. Thér. 141: 404–411, 1963.
203. SINGER, S. J.: Covalent labeling of active sites. Advan. Protein Chem. 22: 1–54, 1967.
204. STANDAERT, F. G.: The action of *d*-tubocurarine on the motor nerve terminal. J. Pharmacol. Exp. Ther. 143: 181–186, 1964.
205. STANDAERT, F. G. AND RIKER, W. F., JR.: The consequences of cholinergic drug actions on motor nerve terminals. Ann. N.Y. Acad. Sci. 144: 517–533, 1967.
206. STEINHART, J. AND BEYCHOK, S.: Interaction of proteins with hydrogen ions and other small ions and molecules. *In* The Proteins, ed. by H. Neurath, vol. II, pp. 139–304, Academic Press, Inc., New York, 1964.
207. STEPHENSON, R. P.: A modification of receptor theory. Brit. J. Pharmacol. Chemother. 11: 379–393, 1956.
208. STEWART, D. M. AND MARTIN, A. W.: Acetylcholine and cholinesterase activity of the denervated hemidiaphragm of the rat. Exp. Neurol. 16: 299–306, 1966.
209. SZÖOR, A., KÖVÉR, A. AND POHANKA, O.: The specificity of muscle cholinesterases. I. The role of active anionic sites. Acta Physiol. Hung. 24: 157–163, 1963.
210. TAKAGI, K., AKO, M. AND TAKAHASHI, A.: Tritium-labeled acetylcholine receptor in the smooth muscle of the small intestine of the dog. Life Sci. 4: 2165–2169, 1965.
211. TAKAGI, K. AND TAKAHASHI, A.: Studies of separation and characterization of acetylcholine receptor labelled with tritiated dibenamine. Biochem. Pharmacol. 17: 1609–1618, 1968.
212. TAYLOR, D. B., CREESE, R., NEDERGAARD, O. A. AND CASE, R.: Labelled depolarising drugs in normal and denervated muscle. Nature (London) 208: 901–902, 1965.
213. TAYLOR, D. B., CREESE, R. AND SCHOLES, N. W.: Effect of curare concentration temperature and potasium ion concentration on the rate of uptake of a neuromuscular blocking agent labeled with radioactive iodine. J. Pharmacol. Exp. Ther. 144: 293–300, 1964.

214. Theleff, S.: Effects of motor innervation on the chemical sensitivity of skeletal muscle. Physiol. Rev. 40: 734–752, 1960.
215. Thesleff, S. and Albuquerque, E. X.: The cholinergic receptor systems of denervated striated muscle and their integration with phospholipid molecules. Ann. N.Y. Acad. Sci. 144: 534–542, 1967.
216. Theron, C. D. and Waud, D. R.: The rate of action of atropine. J. Pharmacol. Exp. Ther. 160: 91–105, 1968.
217. Tobias, J. M.: A chemically specified molecular mechanism underlying excitation in nerve: An hypothesis. Nature (London) 203: 13–17, 1964.
218. Tobias, J. M., Agin, D. P. and Pawlowski, R.: Phospholipid-cholesterol membrane model-control of resistance by ions or current flow. J. Gen. Physiol. 45: 989–1001, 1962.
219. Toft, D. and Gorski, J.: A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. Proc. Nat. Acad. Sci. U.S.A. 55: 1574–1581, 1966.
220. Trams, E. G.: Properties of electroplax protein. II. Biochim. Biophys. Acta 79: 521–530, 1964.
221. Triggle, D. J. and Belleau, B.: Studies on the chemical basis for cholinomimetic and cholinolytic activity. Part I. The synthesis and configuration of quaternary salts in the 1,3 dioxolane and oxazoline series. Can. J. Chem. 40: 1201–1215, 1962.
222. Turpaev, T. M.: On the biochemical mechanism of acetylcholine action. Biokhimiya 23: 71–79, 1958.
223. Turpaev, T. M., Nistratova, S. N. and Putintseva, T. G.: Protein nature of acetylcholine receptor and the role of energy rich compounds in the realization of acetylcholine action. Proc. 1st Int. Pharmacol. Meet. 7: 145–155, 1963.
224. Turpaev, T. M.: Hypothesis on the identity of cholinesterase and the acetylcholine receptor. Fiziol. Zh. (Akad. Nauk Ukr. R.S.R.) 47: 918–922, 1961.
225. Van den Brink, F. G.: Influence of combined cooling and aging on the response of the guinea-pig ileum to histamine. Eur. J. Pharmacol. 2: 224–228, 1967.
226. Van Heyningen, W. E.: The fixation of tetanus toxin, strychnine, serotonin and other substances by ganglioside. J. Gen. Microbiol. 31: 375–387, 1963.
227. van Rossum, J. M.: Pharmacodynamics of parasympathetic drugs. Mechanism of action and structure-action relationship of tertiary ammonium salts. Arch. Int. Pharmacodyn. Thér. 140: 592–605, 1962.
228. van Rossum, J. M. and Ariëns, E. J.: Receptor-reserve and threshold phenomena. II. Theories of drug action and a quantitative approach to spare receptors and threshold values. Arch. Int. Pharmacodyn. Thér. 136: 385–413, 1962.
229. Veldstra, H.: Synergism and potentiation with special reference to the combination of structural analogues. Pharmacol. Rev. 8: 339–387, 1956.
230. Vogt, W.: Formation of lipid-soluble sodium and calcium salts with phosphatidic acid as receptor model. Hoppe-Seyler's Z. Physiol. Chem. 331: 239–244, 1963.
231. Waser, P. G.: Pharmacology of calabash. *In* Curare and Curare-like Agents, ed. by D. Bovet, F. Bovet-Nitti and G. B. Marini-Bettolo, pp. 244–251, Elsevier Publ. Co., Amsterdam, 1959.
232. Waser, P. G.: Investigations of receptors in the endplate with curarizing drugs. *In* Bioelectrogenesis, ed. by C. Chagas and A. Paes de Carvalho, pp. 353–361, Elsevier Publ. Co., Amsterdam, 1961.
233. Waser, P. G.: Chemistry and pharmacology of muscarine, muscarone, and some related compounds. Pharmacol. Rev. 13: 465–515, 1961.
234. Waser, P. G.: Nature of the cholinergic receptor. Proc. 1st Int. Pharmacol. Meet. 7: 101–115, 1963.
235. Waser, P. G.: Receptor localisation by autoradiographic techniques. Ann. N.Y. Acad. Sci. 144: 737–755, 1967.
236. Watkins, J. C.: Pharmacological receptors and general permeability phenomena of cell membranes. J. Theo. Biol. 9: 37–50, 1965.
237. Waud, D. R.: Pharmacological receptors. Pharmacol. Rev. 20: 49–88, 1968.
237a. Webb, G. D.: Affinity of benzoquinonium and ambenonium derivatives for the acetylcholine receptor, tested on the electroplax, and for acetylcholinesterase in solution. Biochim. Biophys. Acta 102: 172–184, 1965.
238. Webb, G. D. and Mautner, H. G.: Sulfur and selenium compounds related to acetylcholine and choline. VI. Effects of homocholine derivatives on the electroplax preparation. Biochem. Pharmacol. 15: 2105–2111, 1966.
239. Wesemann, W. and Zilliken, F.: Adamantanamines and their derivatives as sensitising agents for 5-hydroxytryptamine-induced contraction of smooth muscle. J. Pharm. Pharmacol. 19: 203–207, 1967.
240. Wesemann, W. and Zilliken, F.: Receptors of neurotransmitters. III. Comparison of the 5-hydroxytryptamine receptor of the liver fluke, *fasciola hepatica*, and the rat stomach-fundus. Biochem. Pharmacol. 17: 471–474, 1968.
241. Wild, G., Woolley, D. W. and Gommi, B. W.: Effects of experimental galactosemia on the measured serotonin receptor activity of rat brain. Biochemistry 6: 1671–1675, 1967.
242. Wilson, I. B.: Conformation changes in acetylcholinesterase. Ann. N.Y. Acad. Sci. 144: 664–673, 1967.
243. Wilson, I. B. and Cabib, E.: Acetylcholinesterase: enthalpies and entropies of activation. J. Amer. Chem. Soc. 78: 202–207, 1956.
244. Wilson, I. B. and Harrison, M. A.: Turnover number of acetycholinesterase. J. Biol. Chem. 236: 2292–2295, 1961.
245. Wofsy, L. and Michaeli, D.: Affinity labeling of the anionic site of acetylcholinesterase. Proc. Nat. Acad. Sci. U.S.A. 58: 2296–2298, 1967.
246. Woolley, D. W.: A probable mechanism of action of serotonin. Proc. Nat. Acad. Sci. U.S.A. 44: 197–201, 1958.
247. Woolley, D. W.: Serotonin receptors. I. Extraction and assay of a substance which renders serotonin fat soluble. Proc. Nat. Acad. Sci. U.S.A. 44: 1202–1210, 1958.
248. Woolley, D. W.: Extraction and assay *in vitro* of an "acetylcholine receptor (?)". Fed. Proc. 18: 461, 1959.
249. Woolley, D. W.: Transport of calcium through cell membranes as mediated by certain hormones. *In* Transfer of Calcium and Strontium Across Biological Membranes, pp. 375–382, Academic Press, Inc., New York, 1963.

250. Woolley, D. W. and Campbell, N. K.: Serotonin receptors. II. Calcium transport by crude and purified receptor. Biochim. Biophys. Acta 40: 543–544, 1960.
251. Woolley, D. W. and Campbell, N. K.: Tissue lipids as ion exchangers for cations and the relationship to physiological processes. Biochim. Biophys. Acta 57: 384–385, 1962.
252. Woolley, D. W. and Gommi, B. W.: Serotonin receptors. IV. Specific deficiency of receptors in galactose poisoning and its possible relationship to the idiocy of galactosemia. Proc. Nat. Acad. Sci. U.S.A. 52: 14–19, 1964.
253. Woolley, D. W. and Gommi, B. W.: Serotonin receptors. V. Selective destruction by neuraminidase and EDTA and reactivation with tissue lipids. Nature (London) 202: 1074–1075, 1964.
254. Woolley, D. W. and Gommi, B. W.: Serotonin receptors. VII. Activities of various pure gangliosides as the receptors. Proc. Nat. Acad. Sci. U.S.A. 53: 959–963, 1965.
255. Woolley, D. W. and Gommi, B. W.: Serotonin receptors. VI. Methods for the direct measurement of isolated receptors. Arch. Int. Pharmacodyn. Thér. 159: 8–17, 1966.
256. Woolley, D. W. and Gommi, B. W.: Effects of experimental galactosemia on the measured receptor activity of rat brain. Biochemistry 6: 1671–1675, 1967.
257. Wurzel, M.: A suggested mechanism for the action of choline esters on animal organs inferred from a study of the effect of choline, β-methyl choline, and thio-choline esters. Experientia 15: 430–433, 1959.
258. Wurzel, M.: Hydrolytic activity of cholinesterase at low substrate concentration correlated with the biological effectiveness of choline esters. Bull. Res. Council Israel 9A: 5–13, 1960.
259. Wurzel, M.: The physiological role of cholinesterase at cholinergic receptor sites. Ann. N.Y. Acad. Sci. 144: 694–701, 1967.
260. Yong, M. S. and Marks, G. S.: Studies of the chemical nature of the α-adrenergic receptor II. Investigation of the labeling procedure. Biochem. Pharmacol. 16: 1120–1126, 1967.
261. Yong, M. S., Parulekar, M. R., Wright, J. and Marks, G. S.: Studies of the chemical nature of the α-adrenergic receptor. Biochem. Pharmacol. 15: 1185–1195, 1966.
262. Zupancic, A. O.: Anionic centers of cholinesterases as possible cholinoreceptors. Israel J. Med. Sci. 1: 1396–1399, 1965.
263. Zupancic, A. O.: Evidence for the identity of anionic centers of cholinesterases with cholinoreceptors. Ann. N.Y. Acad. Sci. 144: 689–692, 1967.