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BIOCHEMICAL AND PHARMACOLOGICAL CHARACTERIZATION
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DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Robert Richard Ruffolo, Jr., B.S.

The Ohio State University
1976

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Advisor
College of Pharmacy
DEDICATION

This dissertation is dedicated to my wife, Chris, whose patience, encouragement and love have significantly eased the burden of pursuing graduate studies.
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I wish to express my sincere appreciation and gratitude to:

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for his sound guidance throughout all aspects of my undergraduate and graduate career and whose optimism and encouragement have been directly responsible for completion of this investigation

MY PARENTS
for their unending support and encouragement throughout all facets of my life

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DUANE D. MILLER
for their careful evaluation of this dissertation and their invaluable suggestions
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FIELDS OF STUDY

Major Field: Pharmacology

Structure-Activity Relationships of Adrenergic Drugs
Adrenergic Mechanisms
Biochemistry of the Sympathetic Nervous System
Receptor Identification and Isolation

Professor Popat N. Patil
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CHAPTER I

INTRODUCTION

I.1. Significance of biochemical receptor identification and isolation.

A receptor may be defined as a macromolecule that specifically recognizes and binds a hormone, neurotransmitter or drug and, as a result of this interaction, triggers a series of changes ultimately producing an overt pharmacological response (Cuatrecasas et al., 1975). In recent years, many groups of investigators have attempted to identify and isolate various receptor proteins (reviewed by Cuatrecasas, 1974). Obviously, much has been learned from these experiments about the chemical nature of these receptors and the underlying forces and steric requirements involved in ligand-receptor interactions. Many of these experiments have lead to the development of intriguing new theories as to the mechanisms by which receptors trigger their biological responses (De Robertis, 1975; Cuatrecasas, 1976). While the vast majority of findings from receptor identification and isolation experiments are only of academic interest, there are notable instances where these experiments have answered important clinical and physiological questions related to disease states that would have
otherwise remained a mystery.

The cause of myasthenia gravis, which is characterized by progressive degradation of neuromuscular transmission, is unknown. For years, decreased transmitter release and/or altered nicotinic receptor response to acetylcholine have been proposed (Grob et al., 1956; Churchill-Davidson and Richardson, 1952; Desmedt, 1958; Simpson, 1969). Recently, it was learned that myasthenic muscles bind significantly less alpha-bungarotoxin, a ligand believed to interact specifically and irreversibly with the nicotinic cholinergic receptor, than do normal muscles (Fambrough et al., 1973). The implication is that myasthenic muscles possess fewer, or in some way altered, nicotinic receptors postsynaptically at the neuromuscular junction.

Myasthenia gravis is also believed to be the result of an autoimmune response of the body since patients afflicted with this disease often improve after thymectomy (Comsa and Hook, 1973). Recently, elderly patients with myasthenia gravis were found to have cellular immunity to purified acetylcholine receptors (Richman et al., 1976a,b) which is consistent with the above hypothesis. Furthermore, there is good correlation between T-cell-dependent cellular immunity to the nicotinic receptor and severity of the disease state (Richman et al., 1976b). Injection of
purified acetylcholine receptors into mammals results in the production of antibodies to the receptor and subsequent development of a disease with clinical, pharmacological, electrophysiological and histological manifestations identical to myasthenia gravis (Patrick and Lindstrom, 1973; Lennon et al., 1975).

Although it is not yet possible to answer all the questions about myasthenia gravis, the results detailed above represent an important new approach, possibly leading to an eventual solution. It does appear that the body, for some unknown reason, recognizes the endogenous nicotinic receptor as a foreign entity in some individuals. In response to this, a T-cell-dependent cellular immunity is launched against the receptor which is subsequently destroyed or inactivated. It is apparent that these clinically significant findings had to await the identification and isolation of the nicotinic cholinergic receptor.

Diabetes mellitus is a common disease characterized by increased blood and urine glucose levels secondary to decreased peripheral utilization of glucose (Williams and Porte, 1974). It is believed that insulin, secreted from the pancreas, activates a membrane receptor which facilitates glucose uptake and hence, utilization (Soeldner, 1975; Williams and Porte, 1974). The primary cause of diabetes mellitus has generally been assumed to be inadequate synthesis of insulin by the pancreas (Marble, 1971; Wolf and
Recently, however, it has been learned that many cases of maturity onset diabetes mellitus are not accompanied by low plasma insulin levels. In fact, a good number of these individuals have higher than normal levels of the hormone (Roth et al., 1975). These same individuals are also resistant to injected insulin. What, then, is the cause of diabetes mellitus in these individuals? The answer to this question had to await the advent of techniques used to identify the insulin receptor.

The binding of $^{125}$I-insulin to various tissues, and the characteristics of the insulin receptor, have been reviewed (Cuatrecasas et al., 1975; Roth et al., 1975). Significant advances into the etiology of diabetes mellitus have been made as a result of these investigations. It was learned that those individuals with diabetes mellitus and who simultaneously have normal or greater than normal levels of insulin in their blood, have a decreased capacity to bind $^{125}$I-insulin in peripheral tissues (Roth et al., 1975). The decrease in $^{125}$I-binding sites (presumably insulin receptors) has been attributed to a decrease in the number of receptors since the affinity of insulin for the site was unchanged (Roth et al., 1975). This observation in part explains the numerous cases of "insulin resistance" which occur in many diabetic and obese patients (Roth et al., 1975). It is also believed that the reduction in insulin
receptors is in some way secondary to the increase in circulating insulin levels in this class of individuals. The mechanism whereby insulin can decrease the number of receptors is still a subject of great controversy (see Cuatrecasas et al., 1975; Huang and Cuatrecasas, 1975 and Roth et al., 1975). Much work remains to be done with respect to diabetes mellitus and the insulin receptor, but it is apparent that biochemical studies on the receptor have opened new doors into possible etiologies of the disease and no doubt will continue to be extremely informative.

The term acute leukemia refers to a group of malignancies characterized by uncontrolled and unrestricted proliferation of formed elements in the blood, usually lymphoid or myeloid cells (Lippman, 1976). Many, but not all, patients with an acute leukemia are responsive to glucocorticoid administration. Since administration of glucocorticoids may produce a number of undesirable effects, such as immunosuppression, gastrointestinal bleeding, glucose intolerance and neurologic symptoms (Lippman, 1976), it would be advantageous to be able to predict, in advance, those patients who are more likely to respond favorably to steroid treatment and thereby spare the remainder from further emotional and physical distress due to unnecessary side effects. With the aid of specific binding of labeled glucocorticoids, it has been learned that patients and
animals whose leukemic cells lack the cytoplasmic steroid receptor for glucocorticoids are likely not to be responsive to glucocorticoid therapy whereas those that do possess the receptor most often will respond well to steroid therapy (Baxter et al., 1971; Kirkpatrick et al., 1971; Lippman et al., 1973).

The story for breast cancer is very similar to that described for acute leukemias. That is, many, but not all, cases of breast cancer are responsive to hormonal manipulation such as ovariectomy, adrenalectomy and the administration of estrogen, progestins, androgens and glucocorticoids (Lippman, 1976). It would be most desirable to be able to predict which patients will respond favorably to these treatments so that nonresponders are not subjected to these traumatic procedures unnecessarily. Recently it has been learned that the absence of a cycoplastic estrogen receptor in tumor cells is an excellent method to determine those patients who will not respond to hormonal manipulation (Lippman, 1976). However, the converse was not necessarily true. That is, those patients whose tumor cells possess an estrogen receptor will not always respond favorably to hormonal treatments, although there was a better than half chance that they would (McGuire et al., 1975).

In an attempt to increase the specificity of the screening procedure for hormone responsive candidates with breast cancer, Horwitz et al. (1975) decided to investigate
the likelihood of using the presence or absence of the progesterone receptor as an indicator. It is known that the synthesis of progesterone receptors depends on the action of estrogen on its own receptor (Freifeld et al., 1974). Those tumor cells lacking the estrogen receptor must therefore also be devoid of the progesterone receptor. However, cells containing the estrogen receptor may or may not also possess the progesterone receptor. It was possible that only those cells having both receptors were the ones that would respond favorably to hormonal manipulation. This was determined to be the case (Horwitz et al., 1975). Tumors whose cells possess both estrogen and progesterone receptors responded dramatically to various endocrine therapies. It appears, therefore, that the patients whose tumor cells still retained cytoplasmic estrogen receptors but were never the less resistant to hormonal therapy were so because they lacked the progesterone receptor. The presence of an estrogen receptor alone is not enough to render cells responsive to hormonal manipulation as was originally postulated. As a result of these findings, it becomes apparent that screening for likely candidates to undergo endocrine therapy for breast cancer should involve detection of a cytoplasmic progesterone receptor.

From the preceding discussion, it is apparent that receptor identification and isolation has had an important
impact on clinical medicine and the etiology of certain disease states. No doubt additional advances will be made from investigations on other receptors.

1.2. Criteria for biochemical identification of receptors.

Techniques used in binding studies are relatively simple and require no specific training. However, results from these experiments must satisfy a rigid series of criteria before binding of a labeled ligand may be considered to be to a receptor. These criteria are very general and may be extended to virtually all receptors as well as substrate-recognition sites on enzymes. The investigator should be well aware that satisfying all the criteria to be discussed below does not necessarily prove the existence of a receptor but merely suggests its presence. Ultimate proof of the existence of a receptor must await isolation of the receptor and reconstitution into tissues with reinstatement of biological activity (Dole et al., 1975). This is an ideal but seldomly achieved occurrence.

The pharmacological response of most drugs acting through a receptor-dependent mechanism usually elicits a maximum after which further addition of drug produces no additional effect. This same phenomenon is commonly observed with enzymes suggesting that receptors, as well as enzymes, are present in a limited number. As such, saturation is a necessary criterion to satisfy when studying
binding of a labeled ligand to an alleged receptor protein (Cuatrecasas et al., 1975). Nonspecific, receptor-independent binding sites, on the other hand, are usually of such great capacity that saturation cannot be attained. Thus, as the concentration of a ligand is increased, nonspecific binding to tissue components will also increase, usually linearly, even through extremely high concentrations. If a specific receptor is present, binding will eventually show evidence of saturation, after which, no additional drug may bind.

When a ligand-receptor interaction is observed, the curve resulting when the amount of ligand bound is plotted against the concentration of ligand added takes on the shape of a rectangular hyperbola similar to that observed when plotting the initial rate of an enzymatic reaction against substrate concentration (Christensen and Palmer, 1974). This "saturation curve" follows Michaelis-Menton kinetics and, as such, becomes linear when transposed to double-reciprocal coordinates similar to a Lineweaver-Burke plot for enzymes (Christensen and Palmer, 1974). The linearization technique allows for easy calculation of the dissociation constant for the binding site (analogous to the Michaelis constant, $K_m$, in enzyme kinetics) and the maximum binding capacity (i.e., the maximum amount of ligand that the tissue preparation may bind). The binding capacity is a measure of receptor density if there exists a 1:1
stoichiometry in the interaction between ligand and receptor. The binding capacity is therefore analogous to the $V_{\text{max}}$ one encounters in enzyme kinetics and is obtained in the same manner from the double-reciprocal plot.

Since most biochemical and pharmacological processes are stereoselective (i.e., favors one isomer over its corresponding enantiomer) (reviewed by Patil et al., 1974), binding of a ligand to its pharmacological receptor should also satisfy the criterion of stereoselectivity (Dole et al., 1975). For example, the levo-isomer$^1$ of alprenolol, a beta-adrenoreceptor antagonist, is known to have approximately 50 times higher affinity for the beta-adrenoreceptor than the dextro-isomer (Buckner and Patil, 1971). Consequently, the stereoisomers of alprenolol should show similar degrees of stereoselectivity for adenylate cyclase and binding to a proposed receptor. Lefkowitz (1975) has shown this to be the case.

With respect to the alpha-adrenoreceptor, the (-)-isomers of beta-hydroxylated phenylethylamines are more potent than the corresponding (+)-isomers (Patil et al., 1974). In addition, several competitive antagonists of this receptor display stereoselective effects (personal communication from Dr. W. Nelson). As a result, binding to the alpha-adrenoreceptor should show preference for the biologically active isomer over the weaker or inactive form.

$^1$The levo-isomer [also designated as 1 or (-)] of an optically active compound is defined as that configuration which rotates a plane of polarized light to the left. The dextro-isomer [also designated as d or (+)] of a compound is the mirror image of the levo-isomer and, as such, rotates plane polarized light to the right.
The pharmacological effects of most hormones and neurotransmitters are rapidly reversible. This is an extremely important point since prolonged or irreversible effects would be inconsistent with the cyclical effects of many hormones and the rapidity of onset and cessation by most neurotransmitters. Thus, interaction of these compounds with suspected receptors must satisfy the criterion of reversibility (Dole et al., 1975; Cuatrecasas et al., 1975). Serious problems with the use of $^3$H-norepinephrine as a ligand to identify the beta-adrenoreceptor resulted from irreversible binding which could be demonstrated in some (though not all) systems (Wolfe et al., 1974; Maguire et al., 1974). Consistent with the observation of reversible binding of ligands whose pharmacology suggests that they are truly reversible, is the criterion of equilibrium (Dole et al., 1975). Thus, when a less than maximal concentration of a reversible drug is added to a pharmacological preparation, a certain steady-state response will occur at which time the rate of drug-receptor association equals the rate of dissociation (Furchgott, 1972) and for the latter to occur, the drug-receptor interaction must be of a reversible nature. In line with this, binding of a ligand to a suspected receptor must reach equilibrium and must do so fairly rapidly since most pharmacological responses are quite rapid. An example of where this criterion could not be satisfied has occurred in binding
studies employing $^3$H-norepinephrine as a ligand. Although the stimulatory effects of the ligand on adenylate cyclase, an enzyme believed to be coupled with the beta-adrenoreceptor, reaches equilibrium within minutes, binding of the ligand does not reach equilibrium by 3 hours (Maguire et al., 1974). This inconsistency was interpreted by some to mean that the observed ligand binding was independent of the beta-adrenoreceptor (Wolfe et al., 1974; Maguire et al., 1974).

Most hormones, neurotransmitters and drugs interact potently with their respective receptors. That is to say, pharmacological receptors have high affinity for their substrates. The receptor, therefore, must show equally high affinity for its ligands in binding experiments. It is the criterion of high affinity (i.e., low dissociation constant, $K_D$) that allows a receptor to demonstrate satura-bility at pharmacologically realistic concentrations. If, for example, binding of a ligand was of low affinity, saturation would only occur at extremely high concentrations (i.e., at concentrations greater than the dissociation constant). This problem has arisen when the binding of the extremely potent beta-adrenoreceptor antagonist, propranolol, was investigated (Vatner and Lefkowitz, 1974). The observed affinity for the ligand was approximately 100,000 times lower than that determined pharmacologically. A propranolol
concentration of approximately $10^{-3}$M, which is astronomically high, would have been required to saturate the binding site. As a result, the observed binding of propranolol had to be considered, on the basis of extremely low affinity, to be nonspecific since nonspecific binding sites are characteristically low in affinity.

Receptors are generally responsive to more than one agonist and antagonist. Thus, all drugs known to react with a receptor should likewise interact with a binding site whose identity is believed to be a receptor. Conversely, drugs acting on different receptors should not bind to the site under consideration. This criterion of receptor specificity has been satisfied for many receptors to date. For example, Pert and Snyder (1973) have demonstrated that all opiate receptor agonists and antagonists inhibit the stereoselective binding of $^3$H-naloxone to rat brain homogenate whereas barbiturates, various autonomic drugs and a series of miscellaneous drugs were without effect even at high concentrations. More importantly, however, was the observation that opiate receptor agonists and antagonists could inhibit $^3$H-naloxone binding in concentrations nearly identical to those known to be effective in vivo (Creese and Snyder, 1975). That is, there was excellent (nearly perfect) correlation between known affinity for the receptor and affinity for the opiate binding site. This criterion, which I will call correlation of affinities, for want of a
better name, in addition to receptor specificity, are extremely important characteristics that a binding site must have in order to be called a receptor.

Nonspecific binding sites may satisfy some of the criteria mentioned earlier in this section under unusual circumstances (see section 1.3.). The chances, however, of a nonspecific site having the proper receptor specificity for an entire series of drugs known to interact with a receptor, in addition demonstrating excellent correlation between known receptor affinity and affinity for the binding site, is extremely unlikely making these two latter criteria indispensable.

Finally, binding of a labeled ligand should satisfy the criterion of target organ specificity (Cuatrecasas et al., 1975; Dole et al., 1975). That is, organs known to be responsive to a given drug, and therefore believed to possess a receptor for that drug, should show greater binding capacities (i.e., greater receptor density) than those organs not responding to the drug and therefore believed to be devoid of that particular receptor. This type of analysis has been performed for opiate receptor binding in various regions of the monkey brain (Kuhar et al., 1975). The regions showing the highest level of opiate receptor binding activity were the areas known to be the most important in the perception of pain. An experiment of this
type for the alpha-adrenoreceptor, however, would likely be misleading. The alpha-adrenoreceptor is believed to be the predominant receptor on blood vessels. Thus, any attempt to demonstrate target organ specificity would be complicated by the density of blood supply to that particular organ. Organs having dense blood supplies might show reasonably high levels of receptors even if these tissues were not themselves responsive to alpha-adrenoreceptor agonists and antagonists and therefore presumed to be devoid of the alpha-adrenoreceptor.

This section has dealt with the series of criteria which must be satisfied before a binding site may be called a receptor. Any one criterion in itself has only limited significance. When all are used together, the investigator has a powerful tool with which to try and grasp the receptor. Caution should be exercised, however, even after all criteria have been satisfied since, as mentioned previously, ultimate identification of a receptor requires isolation and reconstitution into a system with subsequent reinstatement of biological activity.

1.3. Problems involved in receptor identification

Although techniques involved in receptor identification are relatively simple, the problems an investigator may encounter can, at times, be insurmountable. As alluded to in the previous section, receptor-specific binding is
always accompanied by a nonspecific component. Nonspecific binding is defined as any binding not directly related to the receptor. One of the goals of the investigator is to reduce the nonspecific component to zero if possible. This, however, is seldom the case. If nonspecific binding is extremely high (greater than 90% of the total binding), it is likely that specific binding will not be observed, or, if observed, will be too small to quantitate. When an experimenter tries to characterize a binding site which represents 10% or less of total binding, the data are subject to enormous variation which may be many times larger than the specific component itself. These same problems occur even if nonspecific binding represents only 30 to 70% of total binding (as is usually the case), but are much less severe and more easily overcome.

The degree of nonspecific binding is characteristic of the ligand used. Lipophilic ligands are more likely to be nonspecifically bound by tissue components than are the hydrophilic ligands (Dole et al., 1975). High lipophilicity favors passive diffusion into membranes and possible entrapment within the membrane or cell. Polycyclic aromatic ligands are generally large flat molecules with delocalized electron clouds on either side available for possible interaction with tissues. The large exposed flat surface of these molecules makes them candidates for hydrophobic interactions with lipids or lipophilic regions of virtually
all nonreceptor protein. Indeed, the long duration of the lipophilic psychotropic drugs is believed to be due to prolonged retention by receptor-independent binding sites (Hollister, 1975). The characteristics of hydrophobic interactions in the nonspecific binding of narcotics has been studied in detail (Höllt and Teschemacher, 1975) and may possibly be extended to nonspecific binding by other lipophilic ligands.

Nonspecific binding may also arise from characteristics of the ligand not related to lipophilicity. For example, if a ligand happens to be a naturally occurring hormone or neurotransmitter, or a close structural analog, it is likely that the ligand will have affinity not only for the receptor of the target organ, but also for uptake and storage sites, metabolic enzymes and other receptors (since most hormones and neurotransmitters are not absolutely specific for one receptor). These additional sites of interaction must all be considered nonspecific and dealt with accordingly. The addition of uptake, storage and metabolic enzyme inhibitors, as well as antagonists of receptors with which the ligand may interact (except the receptor under investigation) might help to increase the specificity of binding. However, one must be aware that no drug is totally specific in its effects and by adding other drugs in an attempt to increase the percentage of specific binding, one might simultaneously decrease specific binding
if these drugs directly affect the receptor.

It is also possible to introduce binding artifacts into an experiment by the type of equipment employed and the methods in which they are used. For instance, Whatman Glass Fiber filters are commonly used to collect membrane fragments containing bound ligand while free ligand passes through. The radioactivity retained by the filters is then determined and used as an estimate of receptor binding. It has been observed (Snyder and Pert, 1975) that these filters bind $^3$H-naloxone in a stereoselective manner. This would have an obvious effect on total binding and could conceivably lead to false results if appropriate corrections were not made. In our laboratory it has been observed that $^3$H-dihydroazepetine, a competitive alpha-adrenoreceptor antagonist, binds to the same filters and also to polyethylene centrifuge tubes. Binding in both cases may be inhibited by phentolamine (unpublished observations). Even the peptide hormone, insulin, has been observed to bind, saturably and with very high affinity, to glass culture tubes (Dole et al., 1975). Obviously, the necessity to correct for all possible artifacts is of the utmost importance and should never be neglected as is often the case.

Nonspecific binding is usually low affinity and not saturable or stereoselective. These characteristics generally make it easy to distinguish nonspecific from specific
binding. In some cases, however, nonspecific binding may have high affinity in which case it may also be possible to saturate with pharmacologically meaningful concentrations (e.g., insulin). Since most body constituents are symmetrically disposed (Snyder and Pert, 1975), it is even possible for nonspecific binding to be stereoselective. For example, D-tryptophan binds stereoselectively to serum albumin (Dole et al., 1975) and \(^3\)H-norepinephrine has been reported to bind stereoselectively to collagen. The stereoselective binding of \(^3\)H-naloxone by glass fiber filters has previously been discussed. It should not be surprising therefore that nonspecific binding can satisfy many of the criteria discussed in section 1.2 and in many ways mimic the receptor that one is trying to locate. In view of these problems, it would be to the investigator's advantage if he would first try to rule out alternate possibilities when a binding site is first found having many characteristics expected for the receptor. An approach of this kind would have avoided much of the confusion that had arisen during early attempts to isolate the beta-adrenoreceptor (see section 1.4).

Chemical instability of the ligand is a problem commonly encountered by investigators. The neurotransmitters, acetylcholine, norepinephrine and dopamine, are chemically unstable. In addition, they are rapidly metabolized in tissue preparations to compounds with much reduced
affinity for the receptor. The irreversible alkylators, such as the nitrogen mustards, 2-haloalkylamines and some of the group selective reagents, usually react with the receptor (and numerous nonspecific sites) through conversion to a reactive intermediate prior to alkylation. These compounds readily undergo transformation to their respective reactive intermediates in buffer and are, as such, stable only for limited periods of time. Once decomposed, these compounds will no longer bind irreversibly to the receptor, yet they may inhibit, by protection, alkylation of receptor sites by molecules that have not already decomposed. This problem may have been responsible, at least in part, for the lack of success in identification of the **alpha**-adreno-receptor by the 2-haloalkylamines (see section 1.5).

One of the criteria discussed in 1.2 concerned the fact that a ligand should bind to the receptor at concentrations known to elicit a pharmacological response. This correlation may be difficult if "spare receptors" exist. To obtain a maximum pharmacological response, it is not always necessary for a drug to occupy all receptors. For example, acetylcholine and carbamylcholine produce maximum contractions of circular muscle from the fundus of the rabbit stomach when only a small percentage of receptors are occupied (Furchgott and Bursztyn, 1967). The excess receptors are referred to as "spare receptors." It is
evident, therefore, that in some pharmacological experiments, we are dealing only with a small fraction of the total receptors and the \( E_D \) \textsubscript{50} is therefore a poor estimate of the true dissociation constant since one-half of all receptors will not be occupied at this concentration (Furchgott, 1972). On the other hand, in a binding experiment using the same ligand, investigators are dealing with all the receptors present and the \( E_D \) \textsubscript{50} is the dissociation constant because half of the total receptor pool is occupied. Since these two \( E_D \) \textsubscript{50} values will disagree, it might be wrongly concluded that the binding site does not represent the receptor since the criterion of correlation of affinities (see section I.2) will not be satisfied. This phenomenon should be kept in mind as a possible explanation when differences between these two values occur. The problem may be avoided if, instead of \( E_D \) \textsubscript{50} values estimated pharmacologically, true dissociation constants are calculated according to the technique described by Furchgott (1972). These dissociation constants should agree well with those determined from binding experiments if the same receptor is involved.


Early attempts to identify the beta-adrenoreceptor through binding studies involved the use of tritiated catecholamines. This class of ligands appeared to be a logical choice in view of the fact that beta-adrenoreceptors
are activated by isoproterenol, epinephrine and norepinephrine. Schramm et al. (1972) first studied the binding of $^3$H-epinephrine to turkey erythrocyte membranes, which had previously been shown by Øye and Sutherland (1966) to possess an epinephrine-responsive adenylate cyclase system. Some of the results of this experiment were consistent with what would be expected if binding were to the receptor. For instance, binding was rapid and saturable as was the known biological response to the catecholamine, and it appeared that the dissociation constant for the epinephrine binding site was nearly the same as that for adenylate cyclase. Furthermore, many catecholamines known to activate the \underline{beta}-adrenoreceptor effectively inhibited $^3$H-epinephrine binding.

In spite of the results detailed above, the majority of the findings of Schramm et al. (1972) were inconsistent with what should occur for the \underline{beta}-adrenoreceptor. The order of potency for activation of adenylate cyclase by catecholamines is: isoproterenol > epinephrine > norepinephrine (Lefkowitz, 1975) and the same order should be reflected in affinities for binding to the receptor. However, Schramm et al. (1972) observed that these three catecholamines were equipotent in inhibiting $^3$H-epinephrine binding as were all other catechol-containing compounds. Even those catechols devoid of agonist or antagonist activity on the receptor appeared to be as potent as isoproterenol on
the binding site. Furthermore, the potent beta-adreno-
receptor antagonist, propranolol, did not inhibit binding
of $^3$H-epinephrine except at concentrations approximately
100,000 times greater than those required to inhibit the
pharmacological response to epinephrine (Furchgott, 1972)
or epinephrine-activated adenylate cyclase (Lefkowitz,
1975). Phentolamine, a potent alpha-adrenoreceptor antag-
onist, also inhibited $^3$H-epinephrine binding when present
in excessively high concentrations suggesting that binding
was not specific. These numerous discrepancies, however,
did not prevent Schramm et al. (1972) from concluding
(incorrectly) that the binding site they were studying was
the beta-adrenoreceptor.

Simultaneously and independently, Lefkowitz and
coworkers (Lefkowitz and Haber, 1971; Lefkowitz et al.,
1973a,b; Lefkowitz, 1973) and Bilezikian and Aurbach
(1973a,b) investigated the binding of $^3$H-norepinephrine and
$^3$H-isoproterenol to membrane fractions isolated from
various tissues believed to contain predominantly beta-
adrenoreceptors. Their results were nearly identical to
those of Schramm et al. (1972) in that binding could be
inhibited by any and all catechol-containing compounds, was
resistant to blockade by beta-adrenoreceptor antagonists
and showed poor correlation between affinities for the
binding site and adenylate cyclase. The series of experi-
ments from these two laboratories also showed a lack of
stereoselectivity for binding of the ligand which is inconsistent with the known pharmacological effects of catecholamines (reviewed by Patil et al., 1974).

Bilezikian and Aurbach (1973a,b) and Lefkowitz et al. (1973a) argued that the lack of stereoselectivity might indicate that they were studying only the "catechol-recognition" site of the receptor and not that region interacting with the ethylamine side chain. Lefkowitz also claimed to observe the proper order of potency for the binding site (i.e., isoproterenol > epinephrine > norepinephrine > dopamine) (Lefkowitz and Haber, 1971; Lefkowitz et al., 1973a). Although the differences obtained were small indeed (all ligands having dissociation constants falling within one-half log unit), Lefkowitz argued that the beta-adrenoreceptor was involved. These results were inconsistent with his belief that the binding site studied was the "catechol-recognition" site since these catecholamines only differed in their side chains. If his findings on the relative potencies were accurate, then the binding site must be recognizing the side chain, and if the side chain is recognized, binding should show stereoselectivity since the side chain is the locus of the point of asymmetry. In spite of these serious inconsistencies, these investigators claimed to have found and characterized the beta-adrenoreceptor.
Additional investigations were published (Dunnick and Marinetti, 1971; Jarett et al., 1974; Lesko and Marinetti, 1975) also claiming to observe binding of $^3$H-catecholamines to beta-adrenoreceptors in tissues known to contain these receptors. The results were identical to those detailed above and offered no additional or convincing findings to support their hypothesis.

The claims that catecholamines bind specifically to the beta-adrenoreceptor were not unanimously accepted. DeSantis et al. (1974) and Tell and Cuatrecasas (1974) could not resolve the problem of nonstereoselectivity in catecholamine binding with the observations of marked stereoselectivity for lipolysis and adenylate cyclase activity, both of which are mediated by the beta-adreno-receptor. These investigators concluded that catecholamine binding was to a receptor-independent site.

Cuatrecasas et al. (1974), in a classical paper, demonstrated that $^3$H-norepinephrine binds not to catecholamine receptors but more likely to a "catechol-binding protein" which he postulated to be catechol-O-methyltransferase (COMT). This hypothesis could account for the ability of all catechol-containing compounds to interfere with the binding of labeled catecholamines as well as the lack of stereoselectivity, since this enzyme is believed to show little or no preference for one isomer over another (Garg et al., 1971). The demonstration that many COMT
substrates and inhibitors were able to displace $^{3}$H-catecholamines from the binding site seemed to strengthen this theory.

The belief that catecholamine binding was largely to COMT was short-lived and subsequently dispelled by Lefkowitz (1974) who was able to demonstrate that tissues with the highest COMT level bound the least amount of $^{3}$H-norepinephrine. Furthermore, the enzyme was largely soluble whereas $^{3}$H-norepinephrine binds almost exclusively to particulate fractions which had no detectable COMT activity. There were also several orders of magnitude difference between the affinities of various substrates and antagonists for the enzyme and the binding site.

The nature of catecholamine binding remained uncertain until the indepth studies on the mechanisms involved by Maguire et al. (1974) and Wolfe et al. (1974). These investigators demonstrated that catecholamine binding was totally oxidation-dependent and that antioxidants or oxygen depletion would therefore inhibit binding. The pharmacological effects of catecholamines, however, are known to be oxidation-independent and thus still occur in the presence of antioxidants. In addition, these investigators, as well as others (Danon and Sapira, 1972; Powis, 1975), were able to demonstrate that $^{3}$H-catecholamines bind to serum albumin in a manner that was identical in every respect to that observed by those advocating that catecholamines bound to
the "catechol-recognition" site of the beta-adrenoreceptor. It was concluded that catecholamine binding was the result of destructive oxidation and subsequent binding to tissue nucleophiles. This process appeared to be independent of the beta-adrenoreceptor.

To avoid the problems encountered with catecholamines, Vatner and Lefkowitz (1974) used $^3$H-propranolol, which does not oxidize, as a ligand to bind to the beta-adrenoreceptor. $^3$H-Propranolol binding had extremely low affinity and very high capacity which is the converse of what one would expect with such a potent antagonist. Binding was poorly inhibited, and then only at very high concentrations, by beta-adrenoreceptor antagonists. No evidence of stereoselectivity could be observed. These investigators concluded that the specificity and specific activity of the ligand were too low to result in detectable interactions with the receptor and that only nonspecific binding could be observed.

It was not until late in 1974 that significant advances were made in beta-adrenoreceptor identification. Simultaneously and independently, Aurbach et al. (1974) and Lefkowitz et al. (1974) were able to demonstrate binding of $^{125}$I-hydroxybenzylpindolol and $^3$H-dihydroalprenolol, respectively, to beta-adrenoreceptors in turkey and frog erythrocyte membranes, respectively. In both cases, binding of the ligand was rapid and reversible with evidence
of stereoselectivity. Binding was inhibited by catecholamines with the following order of relative potencies: isoproterenol > epinephrine > norepinephrine. More detailed studies with $^3$H-dihydroalprenolol (Mukherjee et al. 1975a,b) revealed that binding was saturable with high affinity and low capacity. There was excellent correlation between affinity for the binding site and for adenylate cyclase. All beta-adrenoreceptor agonists and antagonists inhibited binding whereas agents acting on other receptors were without effect. Detailed studies employing $^{125}$I-hydroxybenzylpindolol have produced identical results (Brown et al., 1976a,b; Maguire et al., 1976; Harden et al., 1976).

Recently, Caron and Lefkowitz (1976) have solubilized the beta-adrenoreceptor from frog erythrocytes. The solubilized receptor appears to resemble, both qualitatively and quantitatively, the membrane-bound form. The molecular weight was estimated to be less than 130,000 to 150,000. The solubilized preparation retained adenylate cyclase activity indicating that the beta-adrenoreceptor recognition site is tightly coupled to, or is an integral part of, the enzyme. The solubilization of the receptor has opened new doors to the further purification and understanding of the receptor.
I.5. Identification of the alpha-adrenoreceptor.

Investigations into identification of the alpha-adrenoreceptor were begun over a decade ago. Although attempts to locate the alpha-adrenoreceptor were started long before comparable studies on the beta-adrenoreceptor, the former area of research achieved less success and involved fewer investigations. Unlike the beta-adrenoreceptor, the alpha-adrenoreceptor is not known to be coupled with adenylate cyclase or an equivalent enzyme which could be used as a "marker enzyme." The presence of a "marker enzyme" would aid in the localization of the receptor and serve as a biochemical correlate to binding data. As a result, investigators were forced to perform studies with crude tissue preparations on the assumption that the alpha-adrenoreceptor was present.

The first attempts to identify the alpha-adrenoreceptor were made in 1966 by Lewis and Miller using the irreversible antagonist, $^3$H-phenoxybenzamine. Their procedure involved the technique of receptor protection (Furchgott, 1954) which they felt necessary due to the nonspecificity of irreversible antagonists. The investigators exposed one rat seminal vesicle to $^3$H-phenoxybenzamine and the contralateral tissue to norepinephrine followed by $^3$H-phenoxybenzamine. The difference in radioactivity between the tissues could be interpreted as receptor-dependent binding provided that norepinephrine
interacts specifically with receptor protein. It should be noted, however, that Wolfe et al. (1974) and Maguire et al. (1974) have demonstrated that norepinephrine interacts not only with the receptor, but with many free nucleophiles in the tissue, especially sulfhydryl groups. So the assumption made by Lewis and Miller is incorrect and, as such, their estimate of receptor density in the tissue would be a maximum approximation. Although the investigators did observe a decrease in $^3$H-phenoxylbenzamine binding in tissues pretreated with norepinephrine, this difference was small and subject to extreme variation. The data therefore were not suitable for detailed analysis by the investigators and, as such, the nature of the binding could not be established since most of the criteria discussed in section I.2 were not satisfied.

Shortly after the experiments of Lewis and Miller (1966), Moran et al. (1967) performed a very similar experiment using the irreversible antagonist, $^3$H-N-(2-bromoethyl)-N-ethyl-N-1-naphthylmethylamine ($^3$H-SY.28), as a ligand in the rabbit vas deferens and aorta. Only nonspecific binding could be detected when $^3$H-SY.28 was used alone or in receptor protection experiments which employed an entire series of alpha-adrenoreceptor agonists and antagonists as the protecting species. In a similar experiment by the same group of investigators (May et al. 1967) using $^3$H-N,N-dimethyl-2-bromo-2-phenylethylamine as the ligand, a very
slight decrease in binding was observed in receptor protection studies which the authors suggested might possibly reflect a small receptor-specific component. It should be noted, however, that the specific binding was extremely low compared to nonspecific and did not permit the investigators to characterize the nature of the binding site and thereby confirm their postulation.

Additional experiments with labeled irreversible antagonists employing procedures similar to those detailed above have been performed (Fiszer and DeRobertis, 1968; Yong and Marks, 1969; Graham and Mottram, 1971; Terner et al., 1971 and Mottram, 1974). The results were also similar to those obtained previously in that receptor-specific binding could not be detected or was extremely low. In no case was it possible to characterize specific binding (if indeed it occurred) and thereby achieve a greater understanding of the receptor.

Although gallant efforts were made to identify the alpha-adrenoreceptor with various labeled irreversible antagonists, the results were quite disappointing. The relative lack of success may be attributed to several factors. The most important reason may be the extreme lack of specificity for the alpha-adrenoreceptor by the irreversible antagonists (May et al., 1967). The 2-haloalkylamines appear to block most hormone and neurotransmitter receptors
(with the notable exception of the **beta**-adrenoreceptor) in addition to the nonspecific binding sites which are far greater in number. The result is that specific binding, should it occur, is such a minute part of the total binding that it might not even be detected, let alone subject to the detailed analysis and subsequent characterization required to satisfy the necessary criteria (see section 1.2). In addition, the specific activity of these ligands is low (less than 50 mCi/mmole). This, combined with the low density of receptors (probably in the picomole to femtomole range) complicates experiments. Finally, the drugs used to inhibit ligand binding, and thereby used to estimate specific binding, are relatively nonspecific themselves and are often times substrates for metabolic enzymes, uptake and storage sites and the more prevalent nonspecific sites related to tissue nucleophiles (Maguire et al. 1974). In view of these problems, it is not surprising that early attempts to identify the receptor with labeled irreversible antagonists were unsuccessful.

When it was finally realized that the irreversible **alpha**-adrenoreceptor antagonists were less than ideal for receptor identification, several alternative approaches were explored. DeRobertis began a sophisticated series of experiments involving drug-induced changes in conductance of artificial membranes into which were incorporated proteolipids believed to be **alpha**-adrenoreceptors (Fiszer de
Plazas and DeRobertis, 1972a; Ochoa et al., 1972a,b). These proteolipids were isolated by organic solvent extraction from the bovine spleen, a tissue believed to be rich in alpha-adrenoreceptors. This group of investigators observed that norepinephrine produced stereospecific dose-dependent conductance changes only in those artificial membranes containing proteolipids from the bovine spleen. The effect was antagonized by phentolamine. It should be noted, however, that extremely high concentrations of norepinephrine (greater than \(10^{-4}\)M) were required to produce this effect and high concentrations of phentolamine (greater than \(10^{-5}\)M) were required to block it. The concentrations used were over 1000 times greater than those needed to elicit a pharmacological response. Furthermore, drugs not believed to affect the alpha-adrenoreceptor, such as histamine, serotonin, pilocarpine, propranolol and isoproterenol, also produced conductance changes similar to norepinephrine and in many cases were even more potent. The probability that these proteolipids were ionophores responsive to a broad range of drugs, and were not alpha-adrenoreceptors, seems likely. This system was not subjected to sufficient analysis by the investigators to conclude that a receptor-related proteolipid was involved.

Several investigators have used tritiated norepinephrine as a ligand in an attempt to bind an agonist to the alpha-adrenoreceptor in tissues containing predominantly
these receptors. Although it appears that $^3$H-catecholamines are useless in identification of the beta-adrenoreceptor (Wolfe et al., 1974; Maguire et al., 1974), the same had not been demonstrated conclusively for the alpha-adrenoreceptor. In an early experiment, Fiszer de Plazas and DeRobertis (1972b) studied the binding of $^3$H-norepinephrine to proteolipids isolated from the bovine spleen. There was evidence of saturation and binding could be inhibited by phentolamine. The dissociation constant for norepinephrine was similar to what would be expected based upon pharmacological experiments (approximately $10^{-7}$M). As the authors pointed out, however, high concentrations of phentolamine were necessary to inhibit binding and similar concentrations of propranolol were as effective as phentolamine in this respect. No further analyses were performed by the investigators.

In 1974, Yong et al. investigated the binding of $^{14}$C-norepinephrine to the microsomal fraction of the rabbit aorta. After appropriate treatments, they were able to inhibit binding of the ligand by norepinephrine, epinephrine, phenylephrine and phentolamine. Although isoproterenol also produced significant inhibition of binding, the investigators concluded that binding was to the alpha-adrenoreceptor and that the actions of isoproterenol were due to known alpha-effects of this catecholamine. These results were only published in the form of an abstract. A detailed
investigation of the results has not been published.

In an attempt to resolve the problem as to whether labeled norepinephrine would be useful to study the receptor, Kržan et al. (1974) investigated the binding of $^3$H-norepinephrine to microsomes from the rabbit aorta. Their results resembled those of Wolfe et al. (1974) and Maguire et al. (1974), who used tissues containing mainly beta-adrenoreceptors, in that binding showed no evidence of stereoselectivity and had little in common with the receptor. Again, only an abstract was published and no detailed analysis performed.

From the above discussion it is evident that the utility of labeled norepinephrine in alpha-adrenoreceptor identification has neither been clearly established or refuted. Although this ligand was not useful in studies on the beta-adrenoreceptor, it cannot simply be assumed that the same result would occur for the alpha-adrenoreceptor. In view of the controversy that exists regarding this problem, and considering that only preliminary and inconclusive observations have been published, a further detailed investigation seems indicated to establish whether or not this drug is a reasonable ligand to use to identify the alpha-adrenoreceptor.

Recently, two reports have been published claiming to have identified the alpha-adrenoreceptor. Williams and Lefkowitz (1976) using $^3$H-dihydroergocryptine and Greenberg
et al. (1976) using $^3$H-clonidine and $^3$H-WB-4101, the latter being a competitive antagonist of the alpha-adreno-receptor and structurally related to dibozane, have found binding sites in the rabbit uterus and rat brain, respectively, having characteristics expected for the alpha-adrenoreceptor. The binding in both investigations is saturable and stereoselective. Agonists and antagonists of the alpha-adrenoreceptor appear to be potent inhibitors of binding whereas compounds known to act on different receptors are either much less potent or without effect. Good correlation exists between affinity for the receptor and ability to inhibit binding. The possibility that binding is presynaptic has been eliminated by performing experiments in animals treated with 6-hydroxydopamine. Greenberg et al. (1976) have further identified what they believe to be two "states" in which the receptor may exist. One "state" having higher affinity for agonists and another favoring antagonists. Whether these "states" are inter-convertible or simply different points of attachment for agonists and antagonists on the same macromolecule cannot be determined at the present time.

Although both groups of investigators have promising findings, certain problems exist. Dihydroergocryptine, in addition to blocking alpha-adrenoreceptors, has antagonistic activity, as do other dihydro-ergot alkaloids, on both dopamine and serotonin receptors (Nickerson and
Collier, 1975; Woodruff, 1971), the latter of which might be significant in the rabbit uterus. Clonidine is believed to exert its effects, at least in part, through activation of presynaptic alpha-adrenoreceptors (Dollery and Reid, 1973; Nickerson and Ruedy, 1975) which seems inconsistent with the results of Greenberg et al. (1976) in experiments where denervation was performed by administration of 6-hydroxydopamine. Finally, WB-4101 is not structurally related to the phenylethylamines and might therefore be expected to show different characteristics in binding than for ligands resembling norepinephrine. The latter are postulated to interact with the Easson-Stedman recognition site which is believed to contain complementary structures for interaction with the phenylethylamines but presumably not for structurally unrelated agonists and antagonists (Ariëns and Simonis, 1964).

In spite of the minor problems that exist, the studies of Williams and Lefkowitz (1976) and Greenberg et al. (1976) represent milestones in alpha-adrenoreceptor identification. Further studies, however, are in progress by several groups of investigators in attempts to learn more about this receptor.

At the time this investigation was initiated, identification and isolation of the adrenoreceptors was in its very primitive stages. Many investigators were using the $^3$H-catecholamines in attempts to identify the beta-adrenoreceptor. There were conflicting reports claiming success and failure in these efforts. Since the attempts to find the alpha-adrenoreceptor using the irreversible antagonists had failed several years prior, only a few groups continued to study this receptor. As with the beta-adrenoreceptor, the use of $^3$H-norepinephrine as a ligand to identify the alpha-adrenoreceptor had created considerable conflict as to the utility of labeled catecholamines. Clearly, the value of catecholamines as ligands in adrenoreceptor identification had not been definitely established and some bitter controversies had arisen (e.g., Lefkowitz vs. Cuatrecasas, section I.4.).

The investigators attempting to study the alpha-adrenoreceptor were few in number and generally more conservative than their numerous counterparts investigating the beta-adrenoreceptor. For this reason, we decided to study the alpha-adrenoreceptor since this area was in a considerably less disorganized state.

The investigations employing $^3$H-norepinephrine in tissues containing mainly alpha-adrenoreceptors were either incomplete or only published in abstract form (see section
I.5.). Obviously there was not sufficient data upon which a judgement could be made as to the usefulness of this ligand in identifying the receptor. Indeed an indepth investigation of the binding characteristics of this catecholamine was indicated. This, therefore, was where our first efforts were to be directed aided by $^3$H-norepinephrine with high specific activity which had just been made available.

It was realized that if efforts with $^3$H-norepinephrine should fail, the use of a labeled competitive alpha-adrenoreceptor antagonist was an alternative. The potent antagonist, azapetine, has a double bond in its side chain and could be catalytically reduced with tritium gas thereby incorporating a tritium label into the molecule. If this new compound, $^3$H-dihydroazapetine, still retained alpha-adrenoreceptor antagonistic activity, it might be a possible ligand with which to identify the receptor.

Thus, the specific objectives of the present investigation are: a) to find an appropriate ligand that binds with reasonable specificity to the alpha-adrenoreceptor, b) develop a binding procedure to study the biochemical characteristics of the receptor and c) present a model of the alpha-adrenoreceptor which accounts for all biochemical and pharmacological observations reported on this receptor system.
M.I. A kinetic analysis of a catechol-specific binding site in the microsomal fraction from the rabbit aorta.

M.I.1. Isolation of microsomal fraction from rabbit aorta. Male albino rabbits (New Zealand strain, Kings Wheel Rabbitry) weighing 2.2-3.8 Kg were sacrificed by a sharp blow to the head. The entire length of the thoracic aorta (weighing approximately 400 mg) was isolated and cleaned in sodium phosphate buffer (0.13 M, pH 7.4) at room temperature. The aorta was minced and homogenized at 0°C in sodium phosphate buffer for 1 min in an all-glass tissue homogenizer (Kontes Glass Co.) at the #10 setting on a Polyscience mechanical homogenizer (model RZR64). The homogenizing tube and pestle were washed 3 times with 2 ml portions of ice-cold buffer and the homogenate and washings then sonicated over an ice bath for 2 min at 20% relative output with a Sonic 300 Dismembrator (Artek Systems Corp.). The homogenate was centrifuged at 50,000 X g for 30 min at 4°C in a Beckman Model L5-75 ultracentrifuge using a Model 50Ti titanium rotor. The resulting supernatant was centrifuged at 140,000 X g for 60 min and the sediment...
obtained washed twice with 2 ml portions of ice-cold sodium phosphate buffer. The pellet was resuspended and homogenized in a Teflon-glass tissue homogenizer (0°C for 30 sec at the #6 setting) and a 0.1 ml aliquot assayed for protein according to the method of Lowry et al. (1951). One aorta generally yielded 1.5-2 mg of microsomal protein.

M.1.2. Binding studies. For each experiment, 30 samples were prepared containing 50 µg of microsomal protein each. The volume of each sample was brought up to 1 ml, with sodium phosphate buffer, in a 4 ml plastic incubation cup (Kew Scientific). The protein samples were incubated in a Dubnoff Metabolic Shaking Incubator (Precision Scientific) at a temperature of 27.5°C and a shaking rate of 90 cycles per min. In competition studies, the drug under investigation was incubated with the protein for 15 min followed by addition of (−)^3H-norepinephrine (10^{-8}M; 0.25 µCi/ml) for an additional 15 min. At the end of incubation, protein-bound ^3H-norepinephrine was collected on millipore filters (0.5 µm, cellulose acetate; Millipore Corp.) mounted on a Millipore Sampling Manifold (Millipore Corp. model 3025) maintained under a vacuum of at least 60 cm Hg. The filtering time of each sample was less than 10 sec. The incubation cups were washed once with 2 ml of sodium phosphate buffer which was then filtered and the filters washed twice in rapid succession with 10 ml aliquots of
buffer. The background radioactivity bound by the filter itself was determined for each experiment by preparing 3 samples containing $^{3}$H-norepinephrine ($10^{-8}$M), in buffer, which were filtered and washed as described above. The radioactivity bound to these filters was subtracted from all other samples and was always less than 3% of control binding to protein. Determination of radioactivity adsorbed onto the filters was accomplished by placing the filters in separate 20 ml liquid scintillation counting vials and partially dissolving with 2 ml of 1,4-dioxane. Thirteen ml of 3a40 liquid scintillation cocktail (Research Products International Corp.) were then added to each vial and radioactivity determined by counting each sample for 20 min in a Beckman Liquid Scintillation Spectrometer (Model LS-345). Quench was monitored by automatic external standard and counting efficiency for tritium was consistently between 40 and 45%. Preliminary investigations showed that combined treatment with dioxane and 3a40 could leach all radioactivity from the filters.

In kinetic studies of norepinephrine binding, $^{3}$H-norepinephrine was added to the incubation medium containing protein and buffer and the effects of $^{3}$H-norepinephrine concentration, time, temperature and pH (adjusted with concentrated HCl and 10N NaOH) were determined. The radioactivity bound to microsomal protein was collected and assayed for tritium as described above.
The effects of several concentrations of Mg\(^{+2}\), Ca\(^{+2}\) and ethylenediaminetetraacetic acid (EDTA), in addition to several group selective reagents (dithiothreitol, N-ethylmaleimide and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline) were determined in a manner similar to that described for competition studies. The effect of 1-(1-dimethylamino-propyl)-3-ethylcarbodiimide (EDC), a water soluble carbodiimide used to irreversibly inactivate carboxyl groups on proteins, was determined in the same manner except that incubation of the EDC was allowed to proceed for 1 hr at 25° prior to the addition of 10\(^{-8}\)M \(^{3}\)H-norepinephrine. Bound radioactivity was collected and assayed as described above.

M.1.3. Spectral studies. The effects of drugs on various spectra obtained for microsomal protein were investigated in an attempt to correlate the observed binding with possible conformational changes in the protein. A difference spectrum was obtained for microsomal protein (0.75 mg/ml) and protein plus (-)-norepinephrine (10\(^{-6}\)-10\(^{-5}\)M) in an Aminco-Chance Split-Beam Spectrophotometer (DW-2-UV-VIS, Model 4-9600) scanning from 325-600 nm at a scanning speed of 120 nm/min. For circular dichroism (CD) and optical rotatory dispersion (ORD) spectra, it is essential that the protein solution tested be clear and devoid of particulate matter so as to reduce light scattering (Chignell and Chignell, 1972). Since the microsomal pellet obtained after 140,000 X g centrifugation resulted in a slightly turbid
suspension after re-homogenization in a glass-teflon homogenizer, such a preparation could not be used. For this reason, the supernatant obtained after 50,000 X g centrifugation (containing microsomal protein in a relatively clear solution) was used. The CD and ORD spectra were obtained for the protein in concentrations ranging from 0.7 to 0.2 mg/ml in sodium phosphate buffer over a wavelength range of 220-700 nm and a scanning speed of 12 nm/min in a Jasco CD-ORD Spectropolarimeter (model ORD/UV 5, with modifications by Sproul Scientific, SS 20-2 CD). Wavelengths below 220 nm could not be scanned due to excessive "noise" making interpretation of the spectra impossible. Ambient temperature conditions were employed and a 1 cm sample cell used. The spectra of the protein were then rerun in the presence of $10^{-5}M(-)$-norepinephrine, (+)-norepinephrine or oxymetazoline and the resulting spectra recorded. As a control, the spectrum of each drug was obtained in sodium phosphate buffer in the absence of protein. A similar experimental design is routinely employed by Chignell (1970).

Both the split-beam and CD spectra yielded little information on a possible interaction between microsomal protein and the various amines tested and, as such, will not be discussed further.
M.2. Kinetics of accumulation, efflux and the pharmacological effects of tritiated dihydroazapetine on the rabbit aorta.

M.2.1. Preparation and denervation of the aorta. Male albino rabbits (New Zealand Strain, Kings Wheel Rabbitry) weighing 2.2-3.5 kg were sacrificed by a sharp blow to the head and the thoracic aorta removed. A glass rod (3 mm in diameter) was placed inside the aorta which was then dissected free from fat and connective tissue in physiological salt solution (PSS, pH 7.4) at room temperature. The composition of PSS was (mM): NaCl, 118; KCl, 4.7; MgCl₂·6H₂O, 0.54; CaCl₂·2H₂O, 2.5; NaH₂PO₄, 1; NaHCO₃, 25 and glucose, 11. In experiments utilizing denervated media-intima aortic strips, the adventitia was removed according to the procedure of Maxwell et al. (1968). Briefly, an incision was made with a scalpel blade through the adventitia along the length of the aorta while on the glass rod. Care was maintained so as not to cut the media-intima layers. Fine forceps were then used to gently peel the adventitia free from the media. For pharmacological experiments, the normal or denervated aortae were helically cut into strips approximately 20 mm in length and 2 mm wide according to the technique described by Purchgott (1960).
M.2.2. Pharmacological Experiments. The helically cut intact or denervated aortic strips were tied with thread at one end to a glass tissue hook and placed in a 10 ml glass-jacketed tissue bath containing PSS, at 37.5°C, continuously bubbled with a 19:1 mixture of $O_2:CO_2$. The free end of the tissue was attached, via a thin thread, to a Grass force displacement transducer (model FT-03) which in turn fed into a Grass Ink-writing Polygraph (models 79 and 7) to record tissue contractions. The preparation was allowed to equilibrate for 3 - 3.5 hr at a resting tension of 5 gm (normal strip) or 2 gm (denervated strip) before addition of drugs to the bath. Since the first dose-response curve obtained from a given tissue differed considerably from subsequent curves, each tissue was allowed to respond maximally to $10^{-5}$ M norepinephrine, and the drug washed out, prior to construction of cumulative dose-response curves. The latter were obtained by increasing bath concentrations of agonist (dissolved in 0.9% sodium chloride containing 0.1% sodium metabisulfite) approximately three-fold according to the procedure detailed by Van Rossum (1963). The maximum response of the denervated preparation was approximately one-half that of the normal strip. The partial loss of contractility is likely due to tissue trauma upon removal of the adventitia and is of similar magnitude to that observed by others.
In experiments where pA₂ values were determined (i.e., the negative log of the molar concentration of antagonist that shifts the dose-response curve of an agonist to the right by a factor of two), the technique described by Arunlakshana and Schild (1959) was utilized. The tissue was exposed to the antagonist for 1 hr to insure that equilibrium was reached. In all experiments, a parallel tissue was employed which received no antagonist and was used to make appropriate adjustments for changes in sensitivity to agonist with time (Furchgott, 1972).

M.2.3. Studies with ³H-Dihydroazapetine. To determine the accumulation pattern of ³H-dihydroazapetine, the denervated aorta was cut into rings approximately 2 mm in length and weighing 4.7 ± .17 mg. The rings were placed in 4 ml plastic incubation cups (Kew Scientific) containing 1 ml PSS with 10⁻⁶ M ³H-dihydroazapetine (54 Ci/m mole) and accumulation studied from 0.1 to 60 min with constant shaking (60 cycles/min) at 38° in a Dubnoff metabolic shaker. At the end of the accumulation period, the tissues were washed in 1 ml ice-cold PSS for 5 sec to remove radioactivity associated with the water layer adjacent to the tissue. The aortic rings were then placed
in 20 ml glass scintillation vials containing 1 ml of Soluene-100 (Packard) and allowed to solubilize for 2 hr at 60°. Ten ml of ACS scintillation cocktail (Amersham-Searle) was added to each vial which was then counted for 20 min in a Beckman liquid scintillation spectrometer (model LS-345 or LS-355). Quench was monitored by automatic external standardization and counting efficiencies were routinely between 20 and 30%.

In order to determine the ³H-dihydroazapetine efflux pattern, denervated and spirally cut aortic strips were prepared and mounted in 10 ml glass-jacketed tissue baths maintained at 38° as described previously. The tissues were incubated with ³H-dihydroazapetine (10⁻⁶M) for 60 min to insure equilibrium and then washed with 10 ml PSS at 5 min intervals for 2 hr (except for the first 10 min where washing was at 1-3 min intervals to detect early phases). A 1 ml aliquot of each 10 ml wash was assayed for ³H-dihydroazapetine by adding to 10 ml ACS scintillation cocktail and counting as described above. At the end of the 2 hr washing period, the radioactivity still retained by the tissue was determined by solubilizing each strip and counting as described above.

In one experiment, ³H-dihydroazapetine (10⁻⁶M) was incubated with aortic rings for 1 hr. Radioactivity in the tissue was extracted with 0.4N perchloric acid, 1 N
sodium hydroxide, 0.13 M phosphate buffer, chloroform, ethylacetate and butanol and the extracts checked for metabolites with a chloroform: methanol (7:1) thin layer chromatographic system on silica gel plates. The only radioactivity observed in the tissue or medium was that for authentic dihydroazapetine.

M.2.4. Preparation of Dihydroazapetine and $^3$H-Dihydroazapetine. Unlabeled Dihydroazapetine was prepared in our medicinal chemistry laboratory from the parent compound, azapetine phosphate, by the following procedure; 400 mg of azapetine phosphate (1.25 mmole) was suspended in 10 ml H$_2$O, made alkaline with 10% NaOH and extracted 3 times with 30 ml portions of diethylether. The combined extracts (containing azapetine as the free base) were washed once with 20 ml H$_2$O, dried (MgSO$_4$), and then evaporated in vac to give an off-white oil which was then taken up in 15 ml ethanol (95%). At the same time, 200 mg of 10% palladium-on-charcoal (Pd/C) in 60 ml ethanol plus 10 ml of 10% NaOH (aq) were shaken under 30 psi H$_2$ until uptake of H$_2$ was complete (approximately 1 hr). Layered upon this was the azapetine (as the free base in ethanol) and the H$_2$ pressure raised to 25 psi while shaking was

The reduction of azapetine was kindly performed by John W. Fowble.
begun at room temperature. Shaking was allowed to proceed until one equivalent of $\text{H}_2$ uptake was reached (approximately 5 min). The resulting solution was filtered by suction through a celite pad and the filtrate evaporated in vac. The residue was taken up in diethylether and washed and dried with $\text{MgSO}_4$. Dry HCl gas was bubbled into the solution to give a gummy solid. The ether was evaporated off and the residue taken up in a small volume of chloroform (with gentle heat) and ether added until clouding occurred, and the product slowly crystallized as the HCl salt. The yield of the reaction was 70%.

The melting point of the final preparation was 188-190°C (decomp.) and the structure verified by proton magnetic resonance spectroscopy. The chemical purity of the preparation was determined by thin layer chromatography (TLC) in a solvent system of chloroform: methanol (7:1), with 4 drops of acetic acid per 100 ml, on silica gel plates with fluorescent indicator. The resulting spot (visualized both by ultraviolet light and iodine vapor) consisting of dihydroazapetine had an $R_f$ of $0.69 \pm 0.03$ ($n=6$) compared to the parent compound, azapetine, whose $R_f$ was $0.86 \pm 0.03$ ($n=4$). These values differ significantly ($p < 0.05$) indicating that no significant overlap between the two compounds occurred. No contaminant, as determined
by TLC, could be detected in the final preparation of dihydroazapetine.

The tritium labeled dihydroazapetine was prepared for us by The Amersham-Searle Corporation using the technique described above except that tritium gas was substituted for H₂ gas. The ³H-dihydroazapetine supplied had a specific activity of 49-54 Ci/m mole. Radiochemical purity was determined as described in section M.6.

M.3. Binding of ³H-dihydroazapetine to alpha-adrenoreceptor-related proteins from the rat vas deferens.

M.3.1. Isolation of membrane fragments. Male albino rats (Sprague Dawley, Lab Supply Co.), weighing 200-300 gm, were sacrificed by a sharp blow to the head. The vasa deferentia from one to three rats were removed and cleaned of surrounding connective tissue in 0.13 M sodium phosphate buffer (pH 7.4) at room temperature. The pooled tissues were cut into segments approximately 3 mm in length and washed 3 times in ice-cold sodium phosphate buffer to remove blood and semen. The cut segments were homogenized in 1 ml of sodium phosphate buffer (0°C) by 12 strokes with a glass-teflon tissue homogenizer at the maximum setting on a Polyscience mechanical homogenizer (Model RZR64). The homogenizing apparatus was washed 3 times with 2 ml aliquots of cold sodium phosphate buffer which
were combined with the original homogenate. The homogenate was filtered, by suction, through muslin and then centrifuged at 17,000 X g for 30 min at 4°C. The resulting pellet was resuspended by gentle homogenization in distilled water and centrifuged at 50,000 X g for 20 min. The supernatant was discarded and the pellet resuspended in distilled water as described above and used in binding studies (except for the pH profile where the pellet was resuspended in 50 mM TRIS-HCl buffers of varying pH). The protein content of the final suspension was determined by the method of Lowry et al. (1951).

M.3.2. Binding studies. In all binding experiments, 500 µl aliquots of the crude membrane preparation were used. Incubation of protein, \(^3\)H-dihydroazapetine and specific drugs (where indicated) was allowed to proceed, with constant shaking (60 cycles per min) supplied by a Dubnoff Metabolic Shaking Incubator, for at least 20 min at 37°C in 4 ml plastic incubation cups (Kew Scientific). This time was chosen to insure that equilibrium of specific binding was reached. In the present experiment, specific binding refers to that binding of \(^3\)H-dihydroazapetine which may be decreased by a 10\(^{-5}\)M concentration of phentolamine. Except where indicated, the concentration
of $^3$H-dihydroazapetine was $10^{-8}$M. When catecholamines were included in the incubation mixture, EDTA (10 μg/ml) and sodium metabisulfite (10 μg/ml) were added to all samples to retard spontaneous oxidation. These antioxidants produced only a negligible increase in $^3$H-dihydroazapetine binding. At the end of incubation, 450 μl aliquots of each sample were filtered through glass fiber filters (Whatman GF/C, 2.4 cm diameter) with the aid of suction supplied from a sink aspirator. The filters were washed once with 5 ml distilled water (0°C) and then placed in 20 ml scintillation vials containing 1 ml of 5% triton X-100. The vials were mechanically shaken for at least 60 min to remove protein-bound and free radioactivity from the filters and thereby minimize self-adsorption of tritium. Ten ml of Thrift Solve liquid scintillation cocktail (Kew Scientific) were added to each vial and tritium assayed by counting each sample for 10 min in a Beckman Liquid Scintillation Spectrometer (LS-345). Appropriate corrections were made for quench which was monitored by an automatic external standard. Counting efficiencies were routinely between 40 and 45%. Since many of the drugs used in this experiment decreased binding of $^3$H-dihydroazapetine to the glass fiber filters, an equal number of samples were prepared which were
identical to those described above and thus served as background standards. The amount of $^3$H-dihydroazapetine retained by the filters under these conditions was subtracted from the corresponding sample containing membrane protein.

M.3.3. **Effect of surgical denervation of the rat vas deferens on the binding of $^3$H-dihydroazapetine.** Rat vasa deferentia were unilaterally denervated *in vivo* according to the procedure of Kasuya *et al.* (1969). Briefly, male rats were lightly anesthetized with ether and restrained on an animal board. A 2 cm incision was made approximately 0.5 cm to the right of midline and the right seminal vesical pulled through the opening while simultaneously retracting the urinary bladder. The hypogastric plexus is located at the junction of the bladder, prostate, seminal vesical and the origin of the vas deferens. Since the hypogastric plexus is not readily visible, all the connective tissue at this junction (presumably containing the nerve plexus) was removed (with the exception of blood vessels). This procedure guaranteed the excision of the hypogastric plexus resulting in postganglionic sympathetic denervation. In addition, the blood vessel running along the surface of the vas deferens was separated,
intact, from the organ at the proximal end. This procedure sections nerves that course with the vessel and subsequently enter the vas deferens while leaving the circulation to the organ unaltered.

The rats were allowed to recover for 5 to 7 days prior to sacrifice. Before any tissue was used, a simple experiment was performed in an isolated organ bath to establish the extent of denervation (see section M.4 for experimental details). Only those tissues not responding to the indirectly-acting sympathomimetic amine, tyramine ($3 \times 10^{-4}\text{M}$), but still contracting to the directly-acting amine, phenylephrine ($10^{-4}\text{M}$), were considered denervated and used in binding experiments. The contralateral tissue, which was left intact to serve as a control, responded both to tyramine and phenylephrine as expected.

Membrane fragments from the control and denervated tissues were prepared as described in section M.3.1 and binding of $^3\text{H}$-dihydroazapetine performed according to the procedure detailed in section M.3.2.

**M.4. Densensitization experiments on the rat vas deferens.**

Male albino rats weighing 150 to 250 gm were sacrificed by a sharp blow to the head. The vasa deferentia were removed and placed in physiological salt solution (PSS) at room temperature for removal of extraneous connective tissue. The composition of PSS has been
previously described (section M.2.1). One end of the tissue was fastened to a glass tissue hook and placed in a 10 ml organ bath containing PSS maintained at 37.5°C and continuously bubbled with a 19:1 mixture of O₂:CO₂. The free end of the tissue was fastened, via a thin thread, to a lever which was attached to an isotonic myograph transducer (Narco Bio-Systems). Drug-induced contractions were recorded on a Desk Model Physiograph (DMP-4B). The tension on the tissue was adjusted to 250mg.

Before addition of drugs to the bath, the tissue was allowed to equilibrate for a period of at least 30 min. Desensitization was produced by successive exposures to $10^{-5}$ M oxymetazoline (for no longer than 2 min) at 20 min intervals with 4 to 5 washings with PSS between additions. Approximately 6 to 7 exposures to oxymetazoline were required to completely desensitize the vas deferens. Various drugs were tested on the desensitized tissue at 20 min intervals usually at a point where spontaneous activity was low. At the end of each experiment, the vas deferens was contracted maximally with 50 mM KCl to demonstrate that the tissue was not fatigued.

Some experiments were performed in reserpinized or denervated tissues. Reserpine was administered (5 mg/kg) intraperitoneally 17 to 21 hr prior to sacrifice. Unilateral denervation was performed as described in the previous section (M.3.3).
M.5. **Statistical analysis.** All determinations were repeated 3 to 18 times (except for part of the data presented in figure 7) permitting the calculation of a mean ± standard error of the mean (S.E.M.) (Sokal and Rohlf, 1969). Difference between two means was tested for statistical significance (\( P < .05 \)) by Student's t-test for unpaired observations or by testing for overlap of 95% confidence limits (Goldstein, 1964). When any relation appeared to conform to the linear model upon visual inspection, the data were subjected to an analysis of variance in regression and then tested for linearity using an F-test according to the method described by Woolf (1968). When the data were shown **not** to differ significantly from linearity (\( P > .05 \)), a regression line was drawn, using model I regression analysis in all cases, according to the least square method described in detail by Sokal and Rohlf (1969). In all cases, the significance of the regression coefficient (slope of regression line) was tested using an F-test as described by Woolf (1968) and when two slopes were tested for significant differences, overlap of 95% confidence limits was used (Woolf, 1968).

Cumulative log dose-response curves (expressed as percent of maximum) as well as some binding curves, were transposed to log concentration-probit coordinates which serve to linearize the symmetrical sigmoidal type curves.
typically obtained on log concentration-response coor-
dinates (Goldstein, 1964). This procedure permits the use of linear regression analysis on the data.

In kinetic studies, the data were subjected to first-order analyses using standard feathering procedures described in detail by Notari (1975). The first-order rate constants, obtained by feathering, for accumulation and efflux of $^3$H-dihydroazapetine in media-intima sections, were tested by comparing them to those obtained from a computer-generated non-linear regression analysis (see Section II of Appendix). In addition, theoretical accumulation and efflux curves, using the constants obtained from feathering, were shown to accurately describe the experimental data points.

M.6. Radiochemicals and radiochemical purity.

(-)-Norepinephrine [7,8-$^3$H(N)] (specific activity = 25-27.7 Ci/mmole) was obtained from New England Nuclear shipped in dry ice, and stored under nitrogen at -15°C. $^3$H-Dihydroazapetine was prepared for us by the Amersham Searle Corporation according to our specifications (see section M.2.4). The drug was shipped in benzene:ethanol (9:1) under nitrogen and stored at 2 - 4°C until used.

Radiochemical purity was tested periodically by thin-layer chromatography (TLC) on silica gel plates using a solvent system of ethyl acetate:formic acid:water
(70:20:10) for norepinephrine and chloroform:methanol (7:1), with 4 drops of acetic acid per 100 ml, for dihydroaza-
petine. The plates were photographed with a Baird-Atomic Beta Camera (model 6000). The vast majority of radio-
activity for each compound was found as a single spot with R_f values identical to authentic standards. The radio-
chemical purity, as judged by regional scanning with the Beta Camera, was never below 96% for \(^3\)H-norepinephrine or 98% for \(^3\)H-dihydroazapetine.

No detectable decomposition of \(^3\)H-dihydroazapetine could be seen after 4 months when stored as described above. Radiochemical purity tests conducted by Amersham-
Searle using TLC with three solvent systems (methanol:
chloroform, 4:1; methanol:ammonium hydroxide, 100:1.5; and ethanol:acetic acid:water, 6:3:1) yielded results identical to those detailed above.

Stocks of (-)-\(^3\)H-norepinephrine were used quite rapidly and were not kept for longer than one month.

**M.7. Materials**

Drugs and chemicals used in this study are listed, with their molecular weights and suppliers, in Table 1.
**TABLE 1**
MOLECULAR WEIGITS AND MANUFACTURERS OF DRUGS AND CHEMICALS
USED IN THE PRESENT INVESTIGATION

<table>
<thead>
<tr>
<th>Drug or Chemical</th>
<th>Molecular Weight</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-alprenolol tartrate</td>
<td>415</td>
<td>AB Hässle</td>
</tr>
<tr>
<td>atropine sulfate</td>
<td>695</td>
<td>Mallinckrodt</td>
</tr>
<tr>
<td>azapetine phosphate</td>
<td>332</td>
<td>Hoffman-LaRoche</td>
</tr>
<tr>
<td>carbamylcholine chloride</td>
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</tr>
<tr>
<td>(+)-chlorpheniramine maleate</td>
<td>392</td>
<td>Schering Corp.</td>
</tr>
<tr>
<td>(+)-cobefrin hydrochloride</td>
<td>220</td>
<td>Sterling-Winthrop</td>
</tr>
<tr>
<td>1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide</td>
<td>247</td>
<td>Aldrich Chemical Co.</td>
</tr>
<tr>
<td>dithiothreitol</td>
<td>154</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>dopamine hydrochloride</td>
<td>190</td>
<td>Regis</td>
</tr>
<tr>
<td>(-)-epinephrine hydrochloride</td>
<td>202</td>
<td>Mallinckrodt</td>
</tr>
<tr>
<td>(-)-epinephrine bitartrate</td>
<td>333</td>
<td>Sigma</td>
</tr>
<tr>
<td>(+)-epinephrine bitartrate</td>
<td>333</td>
<td>Sigma</td>
</tr>
<tr>
<td>epinine hydrochloride</td>
<td>204</td>
<td>Burroughs Wellcome and Co.</td>
</tr>
<tr>
<td>N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline</td>
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<td>Aldrich Chemical Co.</td>
</tr>
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<tr>
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<td>Ciba-Geigy</td>
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<td>Mead Johnson &amp; Co.</td>
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<tr>
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<td>Regis</td>
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<td>Ciba-Geigy</td>
</tr>
<tr>
<td>tyramine hydrochloride</td>
<td>174</td>
<td>Cyclo Chemical Co.</td>
</tr>
<tr>
<td>xylometazoline hydrochloride</td>
<td>280</td>
<td>Ciba-Geigy</td>
</tr>
<tr>
<td>yohimbine hydrochloride</td>
<td>390</td>
<td>K &amp; K Laboratories</td>
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</table>
CHAPTER III

RESULTS

R.1. Binding of $^3$H-norepinephrine to the microsomal fraction of the rabbit aorta.

The microsomal fraction obtained from the rabbit aorta is capable of binding relatively high amounts of $^3$H-norepinephrine. Binding of $^3$H-norepinephrine ($10^{-8}$M) in control samples (without inhibitor) generally resulted in about 3,000 - 6,000 cpm bound per sample which corresponds roughly to about 2-4 pmoles bound/mg protein. Figure 1 shows the time course of $^3$H-norepinephrine binding after the addition of $10^{-8}$M. As may be seen, there is a linear increase in binding with time for at least 30 min. Since no maximum in binding is evident, 15 min was arbitrarily chosen as a standard incubation time for $^3$H-norepinephrine in all subsequent experiments.

The data presented in figure 2 indicate the relative specificity of the binding site. All catechols tested were able to inhibit the binding of $^3$H-norepinephrine ($10^{-8}$M) in approximately equal concentrations. The concentrations of pyrogallol, (-)-norepinephrine, (-)-isoproterenol, and (-)-epinephrine needed to inhibit
FIGURE 1

Time course of $^3$H-norepinephrine ($^3$H-NE; $10^{-8}$M; 0.25 µCi/ml) binding to microsomal protein of the rabbit aorta. The larger points are the means of 4-5 observations (smaller points). The data do not deviate significantly from linearity ($P > .05$) enabling the line to be drawn by linear regression.
FIGURE 2

Reduction in $^3$H-norepinephrine ($^3$H-NE; $10^{-8}$M; 0.25 μCi/ml; 15 min) binding to microsomes of the rabbit aorta by various agents possessing the catechol or 3-methoxy-4-hydroxyphenyl- moieties. All compounds tested not containing either of these groups (see Results) failed to produce significant blockade at $10^{-5}$M. Means and S.E.M. bars (less than 5% of control binding) have been omitted for simplicity, n = 5-7.
FIGURE 2
binding of $^3$H-norepinephrine by half (apparent dissociation constant) ranged between $7-9 \times 10^{-8}$ M. The binding showed no stereoselectivity since the curves for (+)-norepinephrine, (+)-isoproterenol and (+)-epinephrine were superimposable on those of their respective enantiomers (data not shown). Adrenergic agonists or antagonists not possessing a catechol moiety, such as (-)-phenylephrine, (-)-ephedrine, (+)- and (-)-propranolol, phentolamine, naphazoline, phenoxybenzamine, azapetine, oxymetazoline and (±)-synephrine, were ineffective in blocking the binding of $^3$H-NE when tested in concentrations ranging from $10^{-9} - 10^{-5}$ M ($n = 3-6$, data not shown). Concentrations of these agents higher than those needed to elicit a pharmacological response in vivo or in vitro were not employed since high concentrations of most drugs usually lead to nonspecific effects which are of little significance in binding studies. The only noncatechol compound tested which possessed the ability to block the binding site was the O-methylated metabolite of norepinephrine, (±)-normetanephrine. It should be noted, however, that the blocking effect of this agent is about 100-fold less than that of the catechol-containing compounds and has an apparent dissociation constant of approximately $10^{-5}$ M.

In the absence of a blocker, the binding of $^3$H-norepinephrine increases with concentration up to a finite amount. Figure 3a is a saturation curve obtained for
FIGURE 3

a. Saturation curve for the binding of increasing concentrations of $^3$H-norepinephrine ($^3$H-NE; $3 \times 10^{-9}$ - $10^{-6}$M; 15 min) to microsomal protein of the rabbit aorta. The points represent the mean of 3 observations and the vertical bars are S.E.M.

b. Double reciprocal plot of $^3$H-NE binding to microsomal protein of the rabbit aorta. The larger points are the means of 3 observations (smaller points). The data do not deviate significantly from linearity ($P > .05$) enabling the line to be drawn by linear regression.
**Figure 3**

\[ \text{[H-NE CONCENTRATION] [M]} \]

- \( K_m = 8.5 \times 10^{-8} \text{ M} \)
- \( V_{max} = 28.33 \text{ pmoles/mg protein} \)

![Graph 1](image1)

![Graph 2](image2)
$^3$H-norepinephrine and is the characteristic rectangular hyperbola (since the double reciprocal plot is linear) expected for interaction of a substrate with one site (Christensen and Palmer, 1974). As may be seen binding increases rapidly with concentration up to about $10^{-7}$M, after which evidence of saturation becomes apparent. The binding sites are fully saturated at a concentration of $10^{-6}$M which agrees well with data obtained from figure 2 which shows that a $10^{-6}$M concentration of (-)-norepinephrine (or any catechol-containing compound for that matter) completely inhibits the binding of $^3$H-norepinephrine presumably by occupying all binding sites normally exposed to the labeled catecholamine. When the data from figure 3a are transposed to double reciprocal coordinates, figure 3b is obtained. The $V_{\text{max}}$, corresponding to the maximum number of binding sites present, is obtained from the slope and is equal to 28.33 pmoles/mg protein.

Figures 4 and 5 represent Hill and Scatchard plots (Van Holde, 1971; Scatchard, 1949), respectively, constructed for $^3$H-norepinephrine binding. The slope of the Hill plot does not differ significantly from unity ($P > .05$) indicating that binding is noncooperative. The Scatchard plot results in a curve (data deviates significantly from linearity, $P < .05$) which indicates that more than one type of binding site is present. The curve may be resolved into
Hill plot for the binding of $^3$H-norepinephrine ($^3$H-NE) to microsomal protein of the rabbit aorta. The larger points represent the means of 3 observations (smaller points). The data do not deviate significantly from linearity ($P > .05$) enabling the line to be drawn by linear regression. The slope does not deviate significantly from 1 ($P > .05$).
FIGURE 5

Scatchard plot for the binding of $^3$H-norepinephrine ($^3$H-NE) to microsomal protein of the rabbit aorta. The curved line represents the actual data whereas the straight lines are the linear components of the curve obtained as described in Results. Each point is the mean of 3 observations and the vertical bars are S.E.M. The data, in their entirety, do deviate significantly from linearity (P < .05). The slopes of the two straight lines differ significantly from one another (P < .05). The dissociation constant for the higher affinity site is $3.4 \times 10^{-8} M$ (association constant = $3.0 \times 10^7 M^{-1}$) and for the lower affinity site is $2.4 \times 10^{-7} M$ (association constant = $4.2 \times 10^6 M^{-1}$), $n_1$ and $n_2$ represent the concentration of binding sites in the higher and lower affinity sites, respectively.
\[ K_1 = 3.4 \times 10^{-8} \text{ M} \]
\[ n_1 = 10 \text{ pMOLES/MG} \]

\[ K_2 = 2.4 \times 10^{-7} \text{ M} \]
\[ n_2 = 25 \text{ pMOLES/MG} \]

**Figure 5**
two linear components according to the methods of Rosenthal (1967) and Klotz and Hunston (1971) which yield dissociation constants of $3.4 \times 10^{-8}$ M and $2.4 \times 10^{-7}$ M for the higher and lower affinity sites, respectively. The higher affinity site has 10 pmoles of binding sites/mg protein whereas the lower affinity site has 25 pmoles/mg.

As a check on the analysis performed on the Scatchard plot in figure 5, the constants generated were substituted into the general binding equation (Van Holde, 1971) permitting calculation of theoretical binding curves. The equation is:

$$\bar{v} = \frac{n_1[A]}{K_1 + [A]} + \frac{n_2[A]}{K_2 + [A]} + \cdots$$

where $\bar{v}$ is the quantity of drug A bound, $n$ is the binding capacity and $K$ is the dissociation constant for the interaction of A with the binding site. The subscripts denote different and distinct binding sites. When the values for the higher affinity site are substituted into the equation (i.e., $K_1 = 3.4 \times 10^{-8}$ M; $n_1 = 10$ pmoles/mg; and the second term on the right side of the equation is dropped as if only one site existed), the theoretical curve labeled "site 1" in figure 6 is obtained. A similar analysis for the lower affinity site (i.e., $K_2 = 2.4 \times 10^{-7}$ M; $n_2 = 25$ pmoles/mg; and the first term on the right side of
FIGURE 6

Theoretical saturation curves determined from the kinetic constants obtained in figure 5. The curve for "site 1" was calculated from the equation for binding to one site using $K_1$ and $n_1$ as the constants. Similarly, the curve labeled "site 2" was obtained from the same equation except that $K_2$ and $n_2$ were used as the constants. The solid line was calculated using the equation for two simultaneously occurring binding sites whose constants are $K_1$, $K_2$, $n_1$ and $n_2$ (see text). The points are actual experimental data points (see figure 3a) which are in good agreement with the theoretical curve. The location of the dissociation constants ($K_{m1}$ and $K_{m2}$ which are identical to $K_1$ and $K_2$, respectively) along the abscissa are for reference.
FIGURE 6
the equation is dropped) yields the curve labeled "site 2". If all these constants are substituted into the equation so that two distinct sites are considered simultaneously (i.e., both terms on the right side of the equation are used), or if the curves labeled "site 1" and "site 2" are simply added, the solid curve labeled "total" in figure 6 is obtained. The theoretical curve is in excellent agreement with the experimental data points (compare the points in figure 6 with the solid line) indicating that resolution of the Scatchard plot (figure 5) into two linear components is correct and accurately describes the observed binding.

The free energy of binding ($\Delta G_b$) of norepinephrine to the binding site(s) is calculated from the following equation:

$$\Delta G_b = -RT \ln K$$

where $R$ is the gas constant, $T$ the absolute temperature and $K$ the association constant (Van Holde, 1971). For the two sites observed in the Scatchard plot, free energies of $-10.3$ and $-9.1$ Kcal/mole (for the higher and lower affinity sites, respectively) are obtained.

Figure 7 shows the effects of pH on $^3$H-norepinephrine binding to the microsomal protein. At pH values greater than 7 and less than 4, the binding was greatly enhanced. Since maximum binding occurred at pH 9, an
FIGURE 7

pH profile for binding of $^3$H-norepinephrine ($^3$H-NE; $10^{-8}$M; 0.25 µCi/ml; 15 min) to microsomal protein of the rabbit aorta. The solid curve represents binding in the absence of sodium metabisulfite and each point is the mean of 4 determinations. The vertical bars are S.E.M. The dashed line is binding in the presence of 0.1% sodium metabisulfite and each point is determined from 1-2 observations.
alkaline pH known to cause a rapid oxidation of norepinephrine, the pH profile was repeated in the presence of 0.1% sodium metabisulfite, an antioxidant used as a preservative for catecholamines in solution. As may be seen in the figure, the increases in binding formerly observed at high and low pH values are no longer evident. In fact, the binding that had occurred at all pH values in the first case had been inhibited almost completely by sodium metabisulfite.

Calcium has been shown to be an absolute requirement for the contraction of the aorta in response to norepinephrine (Somlyo and Somlyo, 1968). For this reason, it was of interest to determine what effects Ca$^{+2}$, Mg$^{+2}$ and EDTA, an agent capable of chelating divalent metals, had on $^3$H-norepinephrine binding. The results are presented in figure 8. As may be seen, 1.1 and 2.2 mM Ca$^{+2}$, in sodium phosphate buffer, caused a significant decrease in binding of the labeled amine. Conversely, Mg$^{+2}$ at 2.5 and 5 mM concentrations had no effect or slightly enhanced binding. EDTA (0.1 mM), an agent known to retard the spontaneous oxidation of catecholamines presumably by chelation of trace metals, markedly decreased binding. When Ca$^{+2}$ or Mg$^{+2}$ were added to sodium phosphate buffer, a precipitate formed. This precipitate, most likely the
FIGURE 8

Effect of Ca$^{+2}$, Mg$^{+2}$ and EDTA on binding of $^3$H-norepinephrine ($^3$H-NE; $10^{-8}$M; 0.25 μCi/ml; 15 min) to microsomal protein of the rabbit aorta, in two buffer systems, expressed as percent of control. Each bar is the mean of 3-6 observations and the vertical bracketed lines represent S.E.M. * represents a significant difference from control (P < .05).
phosphate salts of Ca$^{+2}$ and Mg$^{+2}$, is not biochemically inert (Lishajko, 1970; Euler and Lishajko, 1973). Since the effect of the precipitate on $^3$H-norepinephrine binding is unknown, the results presented in figure 8 when sodium phosphate buffer was employed cannot be definitely attributed to the free metals or their corresponding phosphate salts. Indeed, the concentration of free metal is not known due to precipitation of a fraction of the metal ions added. For this reason, the same experiment was repeated in phosphate-free tris-HCl buffer (pH 7.4) where no precipitate forms upon addition of Ca$^{+2}$ or Mg$^{+2}$. The results were qualitatively similar. That is, Ca$^{+2}$ significantly lowered the amount of $^3$H-norepinephrine bound while Mg$^{+2}$ had little effect. Although not evident from the figure, control binding of $^3$H-norepinephrine was significantly lower when tris-HCl buffer was used.

Figure 9 illustrates the results of an experiment intended to study the effect of various group specific reagents on $^3$H-norepinephrine binding in an attempt to determine what type of active groups exist at the binding site(s). Dithiothreitol (DTT, $10^{-3}$M) drastically reduces binding of the catecholamine. This agent is used to reduce disulfide bridges in protein (Karlin, 1973) and so it might be assumed that an S-S linkage must remain intact for binding to be observed. Several complicating factors
FIGURE 9

The influence of various group selective reagents on the binding of $^3$H-norepinephrine ($^3$H-NE; $10^{-8}$M; 0.25 µCi/ml) to microsomal protein of the rabbit aorta expressed as percent of control. Each bar is the mean of 3-6 observations and the vertical bracketed lines are S.E.M. * represents a significant difference from control (P < .05).
result from the use of DTT when one considers its possible reactions with catecholamines. Not only is it a good antioxidant, but it is also a nucleophilic primary mercaptan. It has been reported elsewhere (Tse et al., 1976) that primary mercaptans such as glutathione act as nucleophiles toward oxidized catecholamines, and react to form the sulfhydryl adducts. In addition, unpublished results (personal communication from Dr. R. L. McCreery) indicate that such an adduct does occur between DTT and 4-methylcatechol. Therefore the nucleophilicity or the antioxidant property of DTT could result in decreased binding to proteins. N-ethylmaleimide (NEM, $10^{-3} \text{M}$), which irreversibly alkylates free sulfhydryl groups (Gregory, 1955), reduces the binding of $^{3}$H-norepinephrine by over 90% suggesting that binding requires at least one free sulfhydryl group. The combination of DTT with NEM ($10^{-3} \text{M}$) causes an even greater reduction in binding.

N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ, $5 \times 10^{-4} \text{M}$), an irreversible alkylator of free carboxyl groups on proteins (Belleau et al., 1968), was without effect on $^{3}$H-norepinephrine binding. 1-(3-Dimethylaminoethyl)-3-ethylcarbodiimide (EDC), also known to irreversibly inactivate free carboxyl groups (Hoare and Koshland, 1967), when present in a concentration of $2 \times 10^{-4} \text{M}$ for 1 hr, produced a small but statistically significant
(P < .05) decrease in binding. This concentration of EDC is sufficient to inhibit the contractile response of the aorta to norepinephrine and histamine by about 80\% (unpublished observation). When EDC is incubated in the presence of a nucleophile (histidine, 2 X 10^{-4} M), blockade of carboxyl groups should also occur (Hoare and Koshland, 1967). In the present experiment, the combination of EDC with a nucleophile markedly increased binding of ^3_H-norepinephrine to microsomal protein.

The marked increase in ^3_H-norepinephrine binding observed in the presence of EDC plus histidine was of considerable interest. It was possible that this treatment simply increased the availability of the binding site(s) under investigation or unveiled yet another type of binding site for the ligand. In an attempt to determine which case had occurred, the stereoisomers of norepinephrine were employed to compete for available binding sites with ^3_H-norepinephrine to microsomal protein after 1 hr exposure to EDC plus histidine (2 X 10^{-4} M each). Both isomers proved to be equally effective in inhibiting catecholamine binding (figure 10). The apparent dissociation constants were 5 X 10^{-8} and 7 X 10^{-8} M for the (+)- and (-)-isomers, respectively. In addition, the binding of the labeled amine was unaffected by 10^{-6} M concentrations of phentolamine and propranolol (n = 3). Since the binding
Effects of (-)- and (+)-norepinephrine (NE) on the binding of $^3$H-norepinephrine to rabbit aortic microsomes in the presence of EDC plus histidine. There is no evidence of stereoselectivity or change in dissociation constants indicating that EDC plus histidine simply increase the number of the same binding sites and do not expose a new type of site. Each point is the mean of 4 observations and the vertical bars are S.E.M.
of \(^3\text{H}\)-norepinephrine, in the presence of EDC plus histidine, was not stereoselective and exhibited the same affinities for catecholamines as in figure 2, coupled with the fact that \textit{alpha-} and \textit{beta-} adrenergic antagonists were ineffective in blocking the binding, it is concluded that the increase in binding observed in the presence of EDC plus histidine is simply due to a greater availability of the catechol-specific binding site.

The rate of \(^3\text{H}\)-norepinephrine binding is extremely sensitive to changes in temperature, showing an increase in binding with temperature. The Arrhenius equation relates the rate constant of a chemical reaction to temperature as follows:

$$\log k = \frac{-E_\text{a}}{2.303 R \frac{1}{T}} + \log A$$

where \(k\) is the rate constant, \(E_\text{a}\) the energy of activation, \(A\) the preexponential factor and \(R\) and \(T\) the same as described previously (Daniels and Alberty, 1966). As is evident from the equation, if one plots the log of the rate constant against the reciprocal of the absolute temperature measured in °K, a straight line should result whose slope is \(-E_\text{a}/2.303R\) from which the \(E_\text{a}\) is readily calculated. In the present situation, the \(E_\text{a}\) is the energy that the system must absorb from the environment.
in order for $^3$H-norepinephrine to bind to the microsomal protein. Since binding is linear with time (figure 1), it is a relatively simple task to determine the rate of binding at various temperatures. The reaction,

$$^3\text{H-norepinephrine} + \text{Protein} \rightarrow ^3\text{H-norepinephrine} - \text{Protein}$$

is a second-order process described by the following rate equation:

$$v = k_2 [^3\text{H-norepinephrine}] [\text{Protein}]$$

where $k_2$ is the second order rate constant. If the concentration of protein is in excess, then the $[\text{Protein}]$ term may be considered as a constant and the reaction treated as a pseudo-first-order process described as:

$$v = k_1 [^3\text{H-norepinephrine}]$$

where $k_1$ is the pseudo-first-order rate constant which equals $k_2$ [$\text{Protein}$]. If the rate of $^3$H-norepinephrine binding is determined at very early times, when the concentration of free $^3$H-norepinephrine has not decreased appreciably (i.e. < 1-2% of the total reaction completed), the $[^3\text{H-norepinephrine}]$ term is approximately constant and the very initial rates of the binding process therefore proceed as pseudo-zero-order (Frost and Pearson, 1961) described as:

$$v = k_0$$
where \( k_0 = k_2 [^3\text{H}-\text{norepinephrine}] \text{[Protein]} \). As a result, at very early times in the reaction, the rate of binding is proportional to the true second order rate constant. Therefore a plot of \( \log \) (initial rate of binding) vs \( 1/T \) should give the expected straight line allowing calculation of the \( E_a \) from the slope. Such an Arrhenius plot is constructed in figure 11. The \( E_a \) calculated for the binding process is 14.8 Kcal/mole.

The \( E_a \) calculated above appears to be rather high for the simple adsorption of a small molecule to a protein. To explain this high value, it was theorized that the protein had undergone some kind of conformational change (perhaps a change in secondary, tertiary or quaternary structure) which might be at least partly responsible for the calculated \( E_a \). In an attempt to detect such a conformational change, several types of spectroscopy were employed. The difference-spectrum and CD spectrum yielded little information about a possible interaction between norepinephrine and microsomal protein. The ORD spectrum was then obtained for protein which showed no major peaks or troughs in the region scanned. However, after the addition of \( 10^{-5} \text{M} \) (-)-norepinephrine to the protein, a drastic change in the spectrum was observed with a peak at 220 nm and a trough at 231 nm (figure 12). When (-)-norepinephrine was scanned by itself, neither the peak nor
Arrhenius plot for the initial rate of $^3$H-norepinephrine ($^3$H-NE) binding ($10^{-8}$M; 0.25 μCi/ml; less than 5 min) to microsomal protein of the rabbit aorta. The larger points represent the means of 4 observations (smaller points). The data do not deviate significantly from linearity ($P > .05$) enabling the line to be drawn by linear regression. The $E_a$ calculated from the slope is 14.8 Kcal/mole.
FIGURE 12

ORD spectra for the binding of (-)- and (+)-norepinephrine and oxymetazoline (10^{-5}M) to microsomal protein of the rabbit aorta (0.2 mg/ml). The dashed line is the spectrum of the protein alone while the dotted line is the spectrum of the adrenergic agonist alone. The solid curve is the spectrum obtained when the protein and agonist are combined.
trough was present. Similarly, addition of (+)-norepinephrine (10\(^{-5}\) M) to the protein caused a peak at 220 nm and a trough at 230 nm which were of similar magnitude and direction as that observed for the (-)-isomer though slightly larger. Again, neither the protein nor the (+)-norepinephrine alone induced such a spectral change. Finally, oxymetazoline (10\(^{-5}\) M) was added to the system and no significant spectral change was observed either alone or when in combination with protein.

R.2. Studies with the reversible alpha-adrenoreceptor antagonist, dihydroazapetine.

R.2.1. Pharmacological activity of dihydroazapetine on the rabbit aorta. Azapetine possesses a double bond in its side chain which makes the compound subject to catalytic reduction and the formation of dihydroazapetine (figure 13). Like the parent compound, dihydroazapetine has the ability to produce competitive blockade of alpha-adrenoreceptors. Figure 14 shows the shifts to the right in the response of the aorta to norepinephrine after addition of various concentrations of azapetine or dihydroazapetine with all lines being parallel (P > 0.05). Dose ratios were calculated (i.e., ED\(_{50}\) of norepinephrine in the presence of antagonist divided by ED\(_{50}\) in the absence of antagonist)
Reaction scheme illustrating the reduction of azapetine to dihydroazapetine with hydrogen or tritium gas and the catalyst, palladium-on-charcoal (Pd/C). The asterisks indicate the carbon atoms to which the newly introduced hydrogen or tritium atoms are attached.
FIGURE 14

Log concentration-probit curves for norepinephrine in the presence of various concentrations of dihydroazapetine (A) or azapetine (B) in the intact aorta. Each point represents the mean of 5 observations and the vertical bars are S.E.M. The data for each line do not differ significantly from linearity (p > 0.05) and all lines are parallel (p > 0.05).
for each concentration of antagonist and expressed in figure 15 as a plot of log(dose ratio - 1) against the negative log of the molar concentration of antagonist. The intercept at log(dose ratio - 1) = 0 is the pA₂ (Arunlakshana and Schild, 1959). As may be seen, reducing the double bond of azapetine decreases the pA₂ from 7.9 to 6.6 which corresponds to approximately a 20-fold decrease in potency. The pA₂ value of 7.9 determined for azapetine is identical to that obtained by Sheys and Green (1972). The parallel shifts in log concentration-probit curves, along with the slopes in figure 15 that do not differ significantly (p > 0.05) from unity, together indicate that blockade is of a competitive nature (Furchgott, 1972). The above experiments were conducted in normal aortic strips. The dissociation constant (Kₐ) was also determined in denervated aortic strips (n = 3) and found to be identical to the Kₐ calculated from the above pA₂ value. Dihydroazapetine (5 × 10⁻⁶ M) does not block the effects of histamine on the normal aortic strip or carbamylcholine (carbachol) on the denervated strip (figure 16). Denervated aortic strips were used when carbamylcholine was the agonist to prevent a presynaptic effect that consistently occurred at higher concentrations of the drug in the normal tissue (unpublished data).
Plot of log(dose ratio - 1) against the negative log of the antagonist concentration (M) for azapetine and dihydroazapetine in the intact aorta. The intercept on the abscissa represents the pA$_2$. The data for each line do not differ significantly from linearity (p > 0.05) and the slopes do not differ significantly from unity (p > 0.05). Each point is the mean of 5 observations and the vertical bars are S.E.M.
FIGURE 16

Log concentration-probit curves on the rabbit aorta for histamine and carbachol (carbamylcholine) in the presence of 5 x 10^{-6}M dihydroazapetine (DHA). Each point is the mean of 3 observations and the vertical bars are S.E.M. The effects of carbachol were studied in denervated tissues to eliminate a presynaptic effect that complicated the analysis.
R.2.2. **Kinetics of uptake and efflux of $^3$H-dihydroazapetine in the rabbit aorta.** Figure 17 shows the pattern of accumulation, expressed as percent of the equilibrium value, for $^3$H-dihydroazapetine in the denervated aorta. Equilibrium is reached after approximately 50 min. When the amount of $^3$H-dihydroazapetine accumulated at various times is subtracted from the equilibrium value and expressed as percent of the latter, it is possible, after transposing to semilog coordinates, to conduct a first-order kinetic analysis on $^3$H-dihydroazapetine accumulation (see Section I of Appendix). Such is the case in figure 18. The data, in their entirety, are plotted in figure 18a from which may be seen a large (70% of total accumulation) but slowly filling (rate constant, $k_1$, of 0.042 min$^{-1}$) compartment. Figure 18b is simply an expanded time scale for the accumulation that occurs earlier than 3 min. As may be seen, two additional compartments of approximately the same size each (i.e., 16% of total accumulation) exist with rate constants of 0.38 and 3.86 min$^{-1}$. When the three rate constants for the compartments are substituted into rate equations (see Sections I and II of Appendix), the theoretical line in figure 17 is obtained. The close agreement between this line and experimental data indicates the reliability of the feathering technique used to obtain the constants.
Accumulation of $^3$H-dihydroazapetine ($^3$H-DHA), expressed as percent of the equilibrium value, in denervated aortic rings. Each point is the mean of 4 to 6 observations and the vertical bars are S.E.M. The solid line is the theoretical accumulation curve expected from the rate constants obtained in figure 18.
FIGURE 18

Data for the accumulation of $^3$H-dihydroazapetine ($^3$H-DHA) in denervated aortic rings after transformation to facilitate first-order kinetic analysis. Accumulation of $^3$H-dihydroazapetine at various times ($A_t$) was subtracted from the accumulation at equilibrium ($A_{eq}$) and divided by the latter giving the amount of accumulation that may still occur, as a fraction of the equilibrium value. The percent was obtained by multiplying by 100. 18A shows the entire accumulation with a regression line marking the slowest phase whose rate constant ($k_1$) is the slope. 18B is an expanded time scale for accumulation before 3 min subsequent to feathering out the slower phase in A. Each point is the mean of 4 to 6 observations. The intercepts along the ordinate represent the relative sizes of the compartments in terms of percent of total $^3$H-DHA accumulated.
$k_1 = 0.42 \text{ min}^{-1}$
$t_{1/2} = 18.5 \text{ min}$

$ki = 3.68 \text{ min}$
$t_{1/2} = 0.16 \text{ min}$

FIGURE 18
The efflux pattern of $^3$H-dihydroazapetine from the denervated aorta, previously loaded with the drug, is shown in figure 19. When transposed to semilog coordinates (figure 20), it is apparent that $^3$H-dihydroazapetine is also released from three compartments, the sizes of which are 42, 30 and 28% (of total uptake), and whose rate constants are 0.01, 0.075 and 0.51 min$^{-1}$, respectively, for the slowest through fastest compartments. As before, the rate constants, obtained by standard feathering techniques employed linear regression analysis, have been substituted into standard rate equations to yield a theoretical curve (figure 19) which agrees well with experimental data.

R.2.3. Kinetics of onset and offset of alpha-adrenoreceptor blockade by dihydroazapetine. In figure 21a may be seen the time course of receptor blockade by $10^{-6}$M dihydroazapetine, expressed as percent of maximum blockade of a $10^{-7}$M concentration of norepinephrine, in denervated aortic strips. As evident from the figure, blockade increases rapidly, for approximately 1 min, after which equilibrium is reached. When the percent blockade at various times (less than 1 min) is subtracted from the equilibrium value in percent and plotted on semilog coordinates (figure 21b), it becomes
**FIGURE 19**

Efflux of $^3$H-dihydroazapetine ($^3$H-DHA) from denervated aortic strips previously incubated with the labeled antagonist for 1 hr. The data represent $^3$H-dihydroazapetine remaining in the tissue as a percent of that value at $t_0$. Each point is the mean of 5 observations and the vertical bars are the S.E.M. The solid line is the theoretical efflux pattern that is described by the three first-order efflux constants obtained from figure 20.
FIGURE 20

Efflux data from figure 19 transposed to semilog coordinates to facilitate first-order kinetic analysis. The slope of each line represents the first-order rate constant (k₁) for that particular phase of efflux and the intercepts on the ordinate are the relative sizes of the compartments in terms of percent of total ³H-dihydroazapetine (³H-DHA) in the tissue at t₀. Each point is the mean of 5 observations.
FIGURE 21

A. Blockade of response, in denervated aortic strips, to $10^{-7}$M norepinephrine by a $10^{-6}$M concentration of dihydroazapetine (added at $t_0$). The data are expressed as percent of the maximum blockade that occurs to the given dose of antagonist. Each point is the mean of 5 observations and the vertical bars are the S.E.M.

B. Obtained by subtracting the degree of blockade by $10^{-6}$M dihydroazapetine, expressed as percent of maximum blockade, from 100 (i.e., maximum blockade). This value, which represents the response of the tissue to $10^{-7}$M norepinephrine (expressed as a percent of the response blocked by $10^{-6}$M dihydroazapetine) is plotted against $t$. The rate constant ($k_1$) obtained from the slope is the rate constant for onset of blockade by dihydroazapetine. Each point is the mean of 5 observations and the vertical bars are S.E.M.
FIGURE 21

A

B

BLOCKADE OF RESPONSE (% of blockage at t=0)

RESPONSE TO $10^{-7} M$ NOREPINEPHRINE (% of Control)

TIME (min)

TIME (min)

$100$

$75$

$50$

$25$

$10$

$60$

$105$

$0.50$

$0.25$

$0.18$

$3.88$

$0.18$

$0.50$

$0.50$

$0.25$

$0.18$

$3.88$

$10^{-7} M$
apparent that the rate of blockade of alpha-adrenoreceptors by dihydroazapetine proceeds as a first-order process with a rate constant of $3.88 \text{ min}^{-1}$. Similarly, the rate of loss of alpha-receptor blockade to a $10^{-6}$ M concentration of dihydroazapetine, as determined by measuring initial rates of contraction to $10^{-7}$ M norepinephrine following washout of dihydroazapetine from denervated aortic strips, proceeds as a first-order process whose rate constant is $0.096 \text{ min}^{-1}$ (figure 22). After washout of dihydroazapetine and addition of $10^{-7}$ M norepinephrine, blockade could not be observed, even at early times, if the tissue was allowed to reach equilibrium. This, presumably, was due to continuous displacement, or inhibition of re-occupation after spontaneous dissociation, of receptor-bound antagonist by the agonist until blockade was overcome. For this reason, the initial rates of contraction to $10^{-7}$ M norepinephrine were determined at various time intervals after washout of dihydroazapetine and used to estimate the degree of receptor blockade. The blockade at $t_0$ was considered as 100%. It has been determined, independently, that the initial rate of response to an agonist is inversely proportional to the degree of receptor blockade (unpublished observations).
The blockade of response to $10^{-7}$M norepinephrine, expressed as percent of blockade at $t_0$, at various times after removal of $10^{-6}$M dihydroazapetine from the tissue bath containing denervated aortic strips. The degree of blockade was determined from the initial rates of contraction to $10^{-7}$M norepinephrine. The bathing medium was replaced every 5 min with fresh PSS and the rate constant for offset of blockade ($k_1$) is the slope. Each point is the mean of 6 observations and the vertical bars are S.E.M.
R.3. Binding of $^3$H-dihydroazapetine to a membrane fraction from the rat vas deferens.

Membrane protein from the rat vas deferens binds a considerable amount of $^3$H-dihydroazapetine. A portion of the total binding (approximately 30%) was inhibited by a $10^{-5}$M concentration of phentolamine and was termed "specific" binding to distinguish it from ligand that was "nonspecifically" bound. Specific binding of $^3$H-dihydroazapetine was directly proportional to the quantity of membrane protein filtered up to at least 90 μg (figure 23). In addition, the binding of $^3$H-dihydroazapetine ($10^{-8}$M) rapidly reaches equilibrium (figure 24). It is evident from the figure that equilibrium is established after approximately 10 min of incubation at 37°C. Dilution of the incubation mixture by addition of 49 volumes of distilled water completely dissociates bound $^3$H-dihydroazapetine from the membrane fragments in less than 5 min, demonstrating that binding is of a reversible nature. Similar results were obtained after addition of $10^{-5}$M phentolamine to the incubation mixture after equilibrium is attained. In all subsequent experiments, $^3$H-dihydroazapetine was incubated with membrane protein for at least 20 min to insure that equilibrium was reached.

Figure 25a is a Scatchard plot (Scatchard, 1949) for the total binding of $^3$H-dihydroazapetine. The curve
FIGURE 23

Dependence of $^3$H-dihydroazapetine ($^3$H-DHA) binding on the quantity of protein added. Binding is linear with protein concentration up to at least 90 μg. Each point is the mean of 6 observations and the line was drawn by linear regression.
FIGURE 24

Time course of $^3$H-dihydroazapetine ($^3$H-DHA) binding to membranes from the rat vas deferens. Binding rapidly approaches equilibrium and once equilibrium is attained, bound ligand may be completely dissociated by either 50-fold dilution with distilled water or addition of $10^{-5}$M phentolamine. Each point is the mean of 6 to 12 observations.
a. Scatchard plot for total binding of $^3$H-dihydroazapetine ($^3$H-DHA) to membrane fragments from the rat vas deferens. The curve asymptotically approaches a limiting bound/free ratio (lim B/F) indicating the presence of a nonsaturable, nonspecific binding site.

b. Scatchard plot for $^3$H-dihydroazapetine after correction for nonspecific binding. The curve has been resolved into two linear components which represent higher and lower affinity sites whose dissociation constants are $K_1$ and $K_2$, respectively, with binding capacities of $B_{max1}$ and $B_{max2}$, respectively.
asymptotically approaches a limiting bound/free ratio which typically occurs in the presence of a nonsaturable, nonspecific binding site. The limiting bound/free ratio may be used to correct for the nonspecific binding according to the procedure detailed by Chamness and McGuire (1975). Figure 25b is the resulting Scatchard plot following appropriate corrections. The curve has been resolved into two linear components according to the procedure of Rosenthal (1967). As is evident, $^3$H-dihydroazapetine binds to two distinct binding sites in the membrane fraction. The higher affinity site has a dissociation constant ($K_1$) of $5 \times 10^{-9}$M and binds a maximum of 5 pmoles of $^3$H-dihydroazapetine per mg protein ($B_{max1}$) whereas the lower affinity site has a dissociation constant ($K_2$) of $3.7 \times 10^{-7}$M and binds 40 pmoles/mg protein ($B_{max2}$). When the corrected $^3$H-dihydroazapetine binding is plotted against the concentration of $^3$H-dihydroazapetine in the incubation medium, saturation of binding sites becomes evident (figure 26a). Transformation of the data to double reciprocal coordinates (figure 26b) and analysis of the resulting curve according to the technique described by Segel (1975) indicates the presence, again, of two binding sites with dissociation constants and binding capacities similar to those obtained from the Scatchard plot (figure 25b).
FIGURE 26

a. Saturation curve for corrected $^3$H-dihydroazapetine ($^3$H-DHA) binding to membrane fragments from the rat vas deferens. Each point is the mean of 5 observations.

b. Double reciprocal plot of the data from "a". $K_1$, $B_{\text{max}1}$, $K_2$ and $B_{\text{max}2}$ are the same as in figure 25b.
A Hill plot for the corrected binding (figure 27) yields a straight line with a slope whose absolute value is 0.42 suggesting that binding is negatively cooperative (i.e., slope less than 1.0). It is possible that negative cooperativity in binding could explain the nonlinearity of the Scatchard plot and that two distinct sites do not actually exist. Furthermore, it is possible to exhibit binding to more than one site in addition to observing negative cooperativity since both need not be mutually exclusive. From the data obtained, it is not possible to determine which case, if either, occurs for the binding of $^3$H-dihydroazapetine. It should be noted, however, that the simultaneous occurrence of a nonlinear Scatchard plot and a negative Hill coefficient are consistent with the mathematically sound mobile-receptor hypothesis presented by Jacobs and Cuatrecasas (1976).

To determine the specificity of the binding site, various receptor antagonists (figure 28) and agonists (figure 29) were tested for their ability to inhibit $^3$H-dihydroazapetine binding. As may be seen, the potent competitive alpha-adrenoreceptor blocking agent, azapetine, and the irreversible alpha-blocker, phenoxybenzamine, significantly inhibited the binding of $^3$H-dihydroazapetine. (-)-Alprenolol, atropine and chlorpheniramine were without effect. The concentrations of these antagonists were in a 100 to 1000-fold excess relative to the concentrations
Hill plot for the binding of $^3$H-dihydroazapetine ($^3$H-DHA) to membrane fragments from the rat vas deferens. Each point is the mean of 5 observations. The slope of -0.42 (i.e., absolute value less than 1) indicates that binding is negatively cooperative.
FIGURE 28

Ability of several specific receptor antagonists to inhibit the binding of $^3$H-dihydroazapetine ($^3$H-DHA) to membrane fragments from the rat vas deferens. Each bar is the mean of 6-9 observations and the asterisk represents significant differences from control ($p < 0.05$).
FIGURE 28

SPECIFIC $^3$H-DHA BOUND (% OF CONTROL)

Control
Azapetine $10^{-6}$ M
Phenoxybenzamine $10^{-6}$ M
Alprenolol $10^{-6}$ M
Atropine $10^{-6}$ M
Chlorpheniramine $10^{-6}$ M
FIGURE 29

Ability of several selective receptor agonists to inhibit the binding of $^3$H-dihydroazapetine ($^3$H-DHA) to membrane fragments from the rat vas deferens. Each bar is the mean of 6 observations and the asterisk represents a significant difference from control ($p < 0.05$).
necessary to block their respective receptors. Consistent with the above findings are the observations that the alpha-adrenoreceptor agonist, oxymetazoline, significantly inhibited the binding of $^3$H-dihydroazapetine whereas high concentrations of soterenol and carbamylcholine were without effect. Quite unexpectedly, however, tubocurarine ($10^{-5}$M) produced a marked decrease in binding (data not shown). To exclude the possibility that this compound interacted with the specific binding site for $^3$H-dihydroazapetine, the ability of tubocurarine to inhibit binding in the presence of a large excess of phentolamine was studied. Under these conditions, tubocurarine still produced significant inhibition of binding indicating that the site of interaction of the drug is located in what we have classified as the non-specific (i.e., phentolamine-resistant) $^3$H-dihydroazapetine binding.

Figure 30 shows dose-dependent decreases in $^3$H-dihydroazapetine binding by several alpha-adrenoreceptor antagonists. The relative potencies of these antagonists in inhibiting ligand binding roughly parallels their ability to block the alpha-adrenoreceptor in isolated tissues (Table 2). Two pairs of stereoisomers of alpha-adrenoreceptor blockers were also tested (figure 30b). Although the curves for the stereoisomers converge at higher concentrations, the isomeric-activity ratios observed at lower concentrations approach one log unit which is similar to the values observed in isolated tissues (personal communication from Dr. Wendel Nelson and unpublished observations).
FIGURE 30

a. Dose-dependent decreases in $^3$H-dihydroazapetine ($^3$H-DHA) binding to membrane fragments from the rat vas deferens by several reversible competitive antagonists of the alpha-adrenoreceptor. Each point is the mean of 6 to 12 observations. The data were transformed to probits, to facilitate analysis, and the lines drawn by linear regression.

b. Same as in "a" except that two pairs of stereoisomers were used. Each point is the mean of 6-18 observations. The notations, S and R, refer to the absolute configuration about the asymmetric carbon atoms.
Table 2  Affinity of alpha blockers for the presynaptic and the T325hik handshake at sites

<table>
<thead>
<tr>
<th>Structure</th>
<th>1-Log KJ</th>
<th>1-Log K8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azaeprine</td>
<td>8.26</td>
<td>7.94</td>
</tr>
<tr>
<td>Phenidolene</td>
<td>7.02</td>
<td>6.10</td>
</tr>
<tr>
<td>Dihydropine</td>
<td>6.92</td>
<td>6.60</td>
</tr>
<tr>
<td>Dihyroxy</td>
<td>5.65</td>
<td>5.05</td>
</tr>
<tr>
<td>Hydroxy</td>
<td>5.25</td>
<td>6.05</td>
</tr>
<tr>
<td>Yohimbine</td>
<td>6.57</td>
<td>6.12</td>
</tr>
<tr>
<td>Piroxizine</td>
<td>5.51</td>
<td>7.12</td>
</tr>
<tr>
<td>Proximal</td>
<td>6.21</td>
<td>5.60</td>
</tr>
<tr>
<td>Tolazolene</td>
<td>6.01</td>
<td>5.50</td>
</tr>
</tbody>
</table>

a. Data from Ruffolo et al. (1976, submitted for publication).
b. Data from Sanders et al. (1975).
c. Personal communication from Dr. Wendel Nelson.
d. Unpublished observation.
Dose-dependent decrease in $^3$H-dihydroazapetine ($^3$H-DHA) binding to membrane fragments from the rat vas deferens by several alpha-adrenoreceptor agonists of the imidazoline class. Each point is the mean of 6-9 observations. The lines were drawn by linear regression after transformation of the data to probits.
### Table 3. Affinity of alpha-agonists for the receptor and the [\(^{3}H\)]dihydroazapetine binding site

<table>
<thead>
<tr>
<th>Structure</th>
<th>-log $K_D$</th>
<th>-log $ED_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxymetazoline</td>
<td>8.02</td>
<td>8.40(^{b})</td>
</tr>
<tr>
<td>Naphazoline</td>
<td>7.19</td>
<td>7.95(^{b})</td>
</tr>
<tr>
<td>Xylometazoline</td>
<td>6.58</td>
<td>7.40(^{c})</td>
</tr>
<tr>
<td>Tetrahydrazoline</td>
<td>6.33</td>
<td>6.50(^{b})</td>
</tr>
<tr>
<td>Methyltetrahydrazoline</td>
<td>4.33</td>
<td>4.60(^{c})</td>
</tr>
</tbody>
</table>

---

a. $-\log ED_{50} = pD_2$

b. Data from Sanders et al. (1975).

c. Unpublished observations. Methyltetrahydrazoline was synthesized in our laboratory. Synthesis and pharmacology to be published.
Correlation between ability to inhibit the binding of $^3$H-dihydroazapetine ($-\log K_D$) and affinity of the various antagonists ($pA_2 = -\log K_B$) or agonists ($pD_2 = -\log ED_{50}$) for the receptor in intact tissues. The line was drawn by linear regression and the correlation coefficient ($r$) is 0.84.
(Furchgott, 1972). Furthermore, since the rabbit aorta is only sparsely innervated by sympathetic nerves (Patil et al., 1972), the pD₂ values for agonists on this tissue should more closely reflect the true affinities for the \textit{alpha}-adrenoreceptor.

In another series of experiments, agonists of the phenylethylamine class (i.e., structural analogs of norepinephrine) were tested over a 1000-fold concentration range for their ability to inhibit the binding of $^{3}\text{H}$-dihydroazapetine. It was noted that these agonists did not inhibit binding except at very high concentrations. However, at low concentrations, these compounds produced a stereoselective increase in $^{3}\text{H}$-dihydroazapetine binding in favor of the (-)-isomer. This biphasic effect is shown for the stereoisomers of methoxamine in figure 33. The effects of low concentrations of three pairs of stereoisomers are shown in figure 34 at concentrations that produced the maximum difference between each pair of isomers.

Total binding of $^{3}\text{H}$-dihydroazapetine is inhibited by Ca$^{++}$, Mg$^{++}$, Na$^{+}$ and K$^{+}$ (figure 35). It is apparent that both divalent cations are equipotent in this effect as are the univalent ions. The potency difference between the divalent and univalent cations is approximately 150. The binding of $^{3}\text{H}$-dihydroazapetine is very low, though
FIGURE 33

Curves showing the biphasic effects of the stereoisomers of methoxamine on the binding of $^3$H-dihydroazapetine ($^3$H-DHA) to membrane fragments from the rat vas deferens. At low concentrations there is a stereoselective increase in binding while higher concentrations produce an inhibitory effect. Each point is the mean of 5 to 8 observations.
Effects of stereoisomers of the phenethylamine class of agonists on $^3$H-dihydroazapetine ($^3$H-DHA) binding to membrane fragments from the rat vas deferens. Each bar represents the mean of 6-15 observations and the vertical lines are S.E.M. The large asterisks represent significant differences ($p<0.05$) between the stereoisomers and the smaller asterisks on the accompanying chemical structures indicate the points of asymmetry.
FIGURE 35

Effects of univalent and divalent cations on the total binding of $^3$H-dihydroazapetine ($^3$H-DHA) to membrane fragments from the rat vas deferens. Individual data points have been omitted for clarity but each curve was determined from at least 15 individual observations.
still detectable, at physiological concentrations of the ions.

Specific binding of $^3$H-dihydroazapetine varies considerably with pH over approximately 3 pH units (figure 36). There is a minimum in the binding at pH 7.35 whereas binding increases rapidly with pH above this point, suggesting that perhaps the unionized (i.e., more lipophilic) form of dihydroazapetine has higher affinity for the site. The slight increase in binding near pH 6 might possibly reflect a change in ionization of a function at or near the binding site since dihydroazapetine is nearly completely ionized throughout this range ($pK_a$ approximately 9).

Specific binding of the labeled ligand is extremely sensitive to changes in incubation temperature. There is a progressive decrease in $^3$H-dihydroazapetine binding as the temperature is raised from 25 to 60°C (figure 37). This effect is probably not related to denaturation since it also occurs throughout the physiological temperature range.

The effects of various group selective reagents is presented in Table 4. As may be seen, dithiothreitol and N-ethylmaleimide, agents that reduce disulfide bridges and irreversibly alkylate free sulfhydryl groups, respectively, both cause significant reductions in $^3$H-dihydroazapetine binding. A combination of these two compounds
FIGURE 36

The effects of pH on specific $^3$H-dihydroazapetine ($^3$H-DHA) binding to membrane fragments from the rat vas deferens. Each point is the mean of 4 to 5 observations. Tris-HCl buffer (50 mM) was used and the pH was varied by changing the relative proportions of Tris and hydrochloric acid.
FIGURE 37

Effects of temperature on specific $^3$H-dihydroazapetine ($^3$H-DHA) binding to membrane fragments from the rat vas deferens. Each point is the mean of 8 observations.
### TABLE 4

**EFFECT OF VARIOUS GROUP SELECTIVE REAGENTS ON BINDING OF [3H]DIHYDROAZAPETINE**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Total [3H]dihydroazapetine Bound (% of Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ( \pm ) 3.6</td>
</tr>
<tr>
<td>Phentolamine, 3 ( \times ) 10^{-5} M</td>
<td>39 ( \pm ) 2.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dithiothreitol, 10^-3 M</td>
<td>84 ( \pm ) 3.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>N-ethylmaleimide, 10^-3 M</td>
<td>73 ( \pm ) 4.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dithiothreitol, 10^-3 M plus N-ethylmaleimide, 10^-3 M</td>
<td>77 ( \pm ) 2.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>N-ethoxycarbonyl-2-ethoxy-1,2- dihydroquinoline (EEDQ), 10^-3 M</td>
<td>73 ( \pm ) 1.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Each mean was obtained from 5 observations.

<sup>b</sup>Significant difference from control (\( p < 0.05 \)).
produced no greater effect than either of the compounds alone.  N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoiline, which irreversibly alkylates free carboxyl groups, also produces a significant reduction in binding.

All protein denaturants tested produced significant decreases in \(^3\)H-dihydroazapetine binding (Table 5). This effect was dose-dependent. Specific binding, as well as nonspecific, was reduced by all denaturants except glycerol (10 and 20%) which did not inhibit binding by more than 40%.

Phospholipase A and C significantly inhibited total binding of \(^3\)H-dihydroazapetine as did higher concentrations of the proteolytic enzyme, trypsin (Table 6). Low concentrations of trypsin, however, produced a marked enhancement in binding (Table 6 and figure 38a). In an attempt to determine whether this action was on specific or nonspecific binding, the effect of a low concentration of trypsin (1 \(\mu\)g/ml) was investigated in the presence and absence of a high concentration of phentolamine (figure 38b). Trypsin alone produced a 6058 dpm increase in \(^3\)H-dihydroazapetine binding when compared to the control samples without the enzyme. However, after all specific binding was inhibited by excess phentolamine, the same increase in binding also occurred after treatment with trypsin. It is concluded, therefore, that the effect of low concentrations of the proteolytic enzyme represents actions on the nonspecific component of \(^3\)H-dihydroazapetine binding.
TABLE 5

EFFECT OF VARIOUS PROTEIN DENATURED REAGENTS ON BINDING OF $[^3H]$DIHYDROAZAPETINE $^a$

<table>
<thead>
<tr>
<th>Denaturant</th>
<th>Total $[^3H]$dihydroazapetine Bound (% of Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 5.4</td>
</tr>
<tr>
<td>Phentolamine, 3 X 10$^{-5}$M</td>
<td>33 ± 1.0$^b$</td>
</tr>
<tr>
<td>Urea, 0.9M</td>
<td>71 ± 5.1$^b$</td>
</tr>
<tr>
<td>Urea, 2M</td>
<td>44 ± 3.8$^b$</td>
</tr>
<tr>
<td>Urea, 4M</td>
<td>21 ± 1.2$^b$</td>
</tr>
<tr>
<td>Urea, 6M</td>
<td>16 ± 1.7$^b$</td>
</tr>
<tr>
<td>Guanidine-HCl, 0.9M</td>
<td>2 ± 0.2$^b$</td>
</tr>
<tr>
<td>Guanidine-HCl, 2M</td>
<td>1 ± 0.2$^b$</td>
</tr>
<tr>
<td>Guanidine-HCl, 3M</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Guanidine-HCl, 4M</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Glycerol, 10% (v/v)</td>
<td>76 ± 5.2$^b$</td>
</tr>
<tr>
<td>Glycerol, 20% (v/v)</td>
<td>60 ± 4.1$^b$</td>
</tr>
<tr>
<td>Trichloroacetic acid, 5% (w/v)</td>
<td>2 ± 0.3$^b$</td>
</tr>
</tbody>
</table>

$^a$ Each mean was obtained from 4 to 5 observations.

$^b$ Significant difference from control (p < 0.05).
### TABLE 6

**EFFECTS OF VARIOUS ENZYMES ON BINDING OF \(^3\text{H}\)Dihydroazapetine**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Total [^3\text{H}]Dihydroazapetine Bound (% of Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 7.9</td>
</tr>
<tr>
<td>Phentolamine, 3 X 10(^{-5})M</td>
<td>40 ± 1.6(^\text{b})</td>
</tr>
<tr>
<td>Trypsin, 0.002 mg/ml</td>
<td>150 ± 6.9(^\text{b})</td>
</tr>
<tr>
<td>Trypsin, 0.015 mg/ml</td>
<td>46 ± 0.8(^\text{b})</td>
</tr>
<tr>
<td>Phospholipase A, 0.05 mg/ml</td>
<td>25 ± 4.1(^\text{b})</td>
</tr>
<tr>
<td>Phospholipase A, 0.2 mg/ml</td>
<td>6 ± 1.6(^\text{b})</td>
</tr>
<tr>
<td>Phospholipase C, 0.5 mg/ml</td>
<td>5 ± 1.1(^\text{b})</td>
</tr>
<tr>
<td>Phospholipase C, 1.0 mg/ml</td>
<td>&lt; 1</td>
</tr>
</tbody>
</table>

\(^\text{a}\)Each mean was obtained from 4-6 observations.

\(^\text{b}\)Represents a significant difference (p < 0.05) from control.
FIGURE 38

a. Effects of various concentrations of trypsin on the specific binding of $^3$H-dihydroazapetine ($^3$H-DHA) to membrane fragments from the rat vas deferens. Each point is the mean of 3 to 6 observations.

b. Effects of a low concentration of trypsin that increases $^3$H-DHA binding to membrane fragments. Binding is still enhanced by trypsin in the presence of a large excess of phentolamine which completely blocks specific binding of the ligand. Each bar is the mean of 3 observations.
Finally, to demonstrate that specific binding of $^3$H-dihydroazapetine is to postsynaptic elements, and not to structures located presynaptically, such as the "amine-pump" or presynaptic alpha-adrenoreceptors, specific binding was measured in vasa deferentia from rats that were unilaterally denervated. The contralateral vas deferens was untouched and served as a control. As evident in figure 39, surgical denervation did not decrease specific binding but slightly enhanced it. The increase, however, was not statistically significant ($p > 0.05$).

R.4. Desensitization experiments on the rat vas deferens.

It is evident from figure 40a that repeated administration of oxymetazoline ($10^{-5}$M) at 20 minute intervals results in desensitization of the vas deferens by the sixth to seventh administration. On the desensitized tissue (figure 40b), the imidazolines, oxymetazoline and tetrahydrozoline, produce only small responses. The three phenylethylamine agonists, (-)-phenylephrine, (-)-norepinephrine and (±)-methoxamine, however, are able to produce significant responses after desensitization by oxymetazoline. The contraction produced by depolarization with 50 mM potassium chloride indicates that tissue fatigue cannot explain the lack of effect of the imidazolines in the desensitized tissue.
FIGURE 39

Effects of unilateral surgical denervation of the rat vas deferens on the specific binding of $^3$H-dihydroazapetine ($^3$H-DHA) to membrane fragments. The contralateral vas deferens was not denervated and serves as a control. Each bar is the mean of 5 observations. The binding in denervated tissues does not differ significantly from control ($p > 0.05$).
FIGURE 40

a. Contraction of the rat vas deferens (expressed as percent of the oxymetazoline maximum) to successive administrations of $10^{-5}$M oxymetazoline at 20 min intervals. The number below each bar refers to the number of exposures to oxymetazoline. Each bar is the mean of 8 observations and the brackets are S.E.M.

b. Ability of various alpha-adrenoreceptor agonists to contract the rat vas deferens after desensitization by oxymetazoline. Each bar is the mean of 6 to 8 observations and the brackets are S.E.M. OXY = oxymetazoline, PE = phenylephrine, NE = norepinephrine, MXA = methoxamine, THZ = tetrahydrozoline. The number in parentheses under each bar refers to the concentration of agonist used (mM).
FIGURE 40
The ability of a series of 12 phenylethylamines and 4 imidazolines to produce a response in the oxymetazoline-desensitized rat vas deferens is presented in Table 7. As may be seen, all the phenylethylamines, except dopamine, were resistant to desensitization by oxymetazoline whereas the imidazolines were not. The possibility that phenylethylamines and imidazolines interact at different sites on the receptor seems likely.

The susceptibility of dopamine to desensitization by oxymetazoline is of considerable interest. Since phenylephrine, norepinephrine and methoxamine paradoxically increased the binding of \(^{3}\text{H}\)-dihydroazepetine (figure 34), and were also resistant to oxymetazoline desensitization, it was decided to investigate the effects of dopamine on \(^{3}\text{H}\)-dihydroazepetine binding. The procedures used were the same as those described in sections M.3.1 and M.3.2 and the results are presented in figure 41. Unlike phenylephrine, norepinephrine and methoxamine, dopamine, which showed cross-desensitization to the imidazolines, potently \textit{inhibited} specific binding of \(^{3}\text{H}\)-dihydroazepetine. It is apparent that dopamine, for some unknown reason, seems to mimic the imidazolines in both binding and pharmacological studies.
TABLE 7
EFFECTS OF VARIOUS SYMPATHOMIMETIC AMINES ON OXYMETAZOLINE-DESENSITIZED VAS DEFERENS

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration (mM)</th>
<th>n</th>
<th>Contraction (% of Maximum) c</th>
<th>Mean ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylethylamines</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(-)-Phenylephrine</td>
<td>0.1</td>
<td>8</td>
<td>114 ± 5</td>
<td></td>
</tr>
<tr>
<td>(-)-Norepinephrine</td>
<td>0.01</td>
<td>6</td>
<td>90 ± 4</td>
<td></td>
</tr>
<tr>
<td>(+)-Norepinephrine</td>
<td>3.0</td>
<td>5</td>
<td>61 ± 9</td>
<td></td>
</tr>
<tr>
<td>Methoxamine</td>
<td>0.1</td>
<td>6</td>
<td>115 ± 4</td>
<td></td>
</tr>
<tr>
<td>(+)-Cobefrin</td>
<td>1.0</td>
<td>8</td>
<td>82 ± 7</td>
<td></td>
</tr>
<tr>
<td>Epinephrine</td>
<td>3.0</td>
<td>5</td>
<td>90 ± 3</td>
<td></td>
</tr>
<tr>
<td>(+)-Synephrine</td>
<td>3.0</td>
<td>5</td>
<td>65 ± 5</td>
<td></td>
</tr>
<tr>
<td>(-)-Epinephrine</td>
<td>0.1</td>
<td>5</td>
<td>75 ± 3</td>
<td></td>
</tr>
<tr>
<td>(+)-Epinephrine</td>
<td>1.0</td>
<td>5</td>
<td>74 ± 11</td>
<td></td>
</tr>
<tr>
<td>Metaraminol</td>
<td>3.0</td>
<td>5</td>
<td>79 ± 3</td>
<td></td>
</tr>
<tr>
<td>(+)-Isoproterenol</td>
<td>100.0</td>
<td>1</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>Dopamine</td>
<td>3.0</td>
<td>8</td>
<td>7 ± 5 d</td>
<td></td>
</tr>
<tr>
<td>Imidazolines</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxymetazoline</td>
<td>0.01</td>
<td>7</td>
<td>3 ± 3</td>
<td></td>
</tr>
<tr>
<td>Tetrahydrozoline</td>
<td>0.1</td>
<td>8</td>
<td>12 ± 5</td>
<td></td>
</tr>
<tr>
<td>Xylometazoline</td>
<td>0.1</td>
<td>4</td>
<td>1 ± 1</td>
<td></td>
</tr>
<tr>
<td>Naphazoline</td>
<td>0.1</td>
<td>3</td>
<td>2 ± 2</td>
<td></td>
</tr>
</tbody>
</table>

aVasa deferentia were desensitized by 6 to 7 administrations of 10^-5 M oxymetazoline at 20 minute intervals with 4 washings between each administration.

bNumber of individual observations.

cData are expressed as percent of the oxymetazoline maximum (10^-5 M).

dDopamine showed significant cross-desensitization with the imidazolines.
FIGURE 41

Effects of various concentrations of dopamine on the specific binding of $^3$H-dihydroazapetine ($^3$H-DHA) to membrane fragments from the rat vas deferens. Each point is the mean of 5 observations.
CHAPTER IV

DISCUSSION

D.1. Binding of $^{3}\text{H}$-norepinephrine to a microsomal fraction of the rabbit aorta.

The binding of $^{3}\text{H}$-norepinephrine to microsomes obtained from the rabbit aorta is in many ways similar to the binding of catecholamines observed by other investigators in tissues containing mainly beta-adrenoreceptors. $^{3}\text{H}$-Norepinephrine binding to aortic microsomes increased linearly with time showing no evidence of a maximum during 30 min. In a similar experiment performed on the particulate fractions of cultured rat glioma cells (C6TG1A) and canine ventricle, Maguire et al. (1974) observed $^{3}\text{H}$-norepinephrine binding that was nearly linear with time for at least 30 min which subsequently reached a maximum after 2 hr. Likewise, the binding of $^{3}\text{H}$-epinephrine to proteins obtained from the rat ventricle appears to increase linearly with short periods of time but shows no evidence of a maximum after about 6 hr (Wolfe et al., 1974).

From the studies aimed at determining the specificity of binding, it was shown that the catechol
nucleus is an absolute requirement, and that all compounds containing this moiety are approximately equipotent in inhibiting $^3$H-norepinephrine binding. Compounds lacking a catechol were shown to be ineffective in blocking the binding. Similar results have been obtained by others (Schramm et al., 1972; Bilezikian and Aurbach, 1973a; Cuatrecasas et al., 1974; Maguire et al., 1974; and Wolfe et al., 1974) for catechol-specific sites obtained from various tissues using several different labeled catecholamines as ligands.

The only noncatechol to inhibit binding of $^3$H-norepinephrine in aortic microsomes was (±)-normetanephrine. This effect, however, was 100-fold less than for compounds containing a catechol moiety. In other systems studied, this O-methylated metabolite of norepinephrine was ineffective in inhibiting $^3$H-norepinephrine binding (Schramm et al., 1972; Maguire et al., 1974; Bilezikian et al., 1973a; Lefkowitz, 1973).

The lack of stereoselectivity in the binding of catecholamines to a catechol-specific site has been noted by many investigators (Cuatrecasas et al., 1974; DeSantis and Patil, 1974; Maguire et al., 1974; Kržan et al., 1974). This nonstereoselectivity was also observed in the present investigation where it was shown that the blockade of $^3$H-norepinephrine binding by one isomer of a catechol-containing sympathomimetic amine was identical to its
corresponding enantiomer. Since the pharmacologic activity-ratio for norepinephrine isomers in the rabbit aorta is 300, the nonstereospecific binding must be a receptor-independent phenomenon (Patil et al., 1974).

The catechol-specific binding site in the rabbit aorta is clearly saturable (figure 3a) and has a $K_m$ and $V_{max}$ for $(-)^3H$-norepinephrine of $8.5 \times 10^{-8} M$ and 28.33 pmoles/mg protein, respectively (figure 3b). The $K_m$ is roughly equivalent to the apparent dissociation constant of $8.3 \times 10^{-8} M$ obtained for $(-)$-norepinephrine in figure 2 (as it should be in an idealized situation). These values ($K_m$ and apparent dissociation constant) compare well with those calculated from association constants or from competition studies presented by Lefkowitz and coworkers on the canine ventricle (Lefkowitz et al., 1973a) or cultured myocardial cells (Lefkowitz et al., 1973b), but are generally one-half to one and one-half log units less than those determined in other studies on other systems (Lefkowitz and Haber, 1971; Cuatrecasas et al., 1974; Maguire et al., 1974).

The total number of binding sites present is equal to the $V_{max}$ in figure 3b. The value of approximately 30 pmoles/mg protein agrees favorably with that reported for a similar site on the turkey erythrocyte plasma membrane (Cuatrecasas et al., 1974) but is considerably
lower than those values reported by Lefkowitz et al. (1973a) on cardiac muscle and Jarett et al. (1974) on the plasma membrane of the rat adipocyte.

On the basis of a Hill plot (figure 4) it was felt that binding was to only one class of sites and was noncooperative. However, a Scatchard plot (figure 5) reveals that two classes of sites are present which, by virtue of the closeness in their dissociation constants, behave in binding studies as one site. A similar Scatchard plot has been presented for binding of $^3$H-norepinephrine to canine ventricle protein by Lefkowitz et al. (1973a). Krzan et al. (1974) found both high and low affinity binding sites for $^3$H-norepinephrine in the rabbit aorta. The higher affinity site had a similar binding capacity (approximately 10 pmoles/mg protein) as did the high affinity site observed in the present study; however, their dissociation constant for this site was some 30-fold higher. Using association constants obtained from the slopes of a Scatchard plot, Lefkowitz et al. (1973a) have calculated free energy changes for combination of norepinephrine with each binding site. Their values of -9.96 and -8.70 Kcal/mole for the higher and lower affinity sites, respectively, compare favorably with the values of -10.3 and -9.1 Kcal/mole calculated in the present study. These free energy changes indicate that binding of norepinephrine to catechol-specific binding sites in microsomal fractions
of dog heart and rabbit aorta are energetically favorable.

While studying the dependence of $^3$H-norepinephrine binding on pH, it was noted that at pH extremes, the binding was markedly enhanced (figure 7). The potentiation of catecholamine binding to a catechol-specific site by elevated pH has been previously documented (Lefkowitz et al., 1973a). Since the increase in $^3$H-norepinephrine binding at high and low pH values was abolished by 0.1% sodium metabisulfite (as was also the binding in the more physiological pH range), it was concluded that, under the conditions employed in the present study, the binding of an oxidized form of the catecholamine was involved. A similar hypothesis has previously been set forth by Maguire et al. (1974) for binding to particulate fractions of rat glioma cells in culture or canine ventricle and Wolfe et al. (1974) for binding to proteins of the rat ventricle.

$^3$H-Norepinephrine binding was inhibited significantly by 1.1 and 2.2 mM Ca$^{+2}$ in two different buffer systems. Lefkowitz et al. (1973a) determined that both Ca$^{+2}$ and Mg$^{+2}$ reduced $^3$H-norepinephrine binding to cardiac microsomes, however the effect of Ca$^{+2}$ was considerably greater than that of Mg$^{+2}$, the latter only causing a 16% inhibition at a 5 mM concentration. EDTA markedly inhibited the binding of $^3$H-norepinephrine in the present study. This action, presumably due to inhibition of
catecholamine oxidation by chelation of trace metals, has been observed in other systems and is reversed by addition of Cu$^{+2}$, Fe$^{+2}$ and Mn$^{+2}$ but not Ca$^{+2}$, Mg$^{+2}$ or Ba$^{+2}$ (Maguire et al., 1974).

The binding of $^3$H-norepinephrine to microsomes of the aorta is also similar to binding in other organs (Cuatrecasas et al., 1974; Lefkowitz et al., 1973a) in that it is extremely labile to dithiothreitol and N-ethylmaleimide, agents that reduce disulfide bonds and irreversibly alkylate free thiol groups, respectively. The significance of the effect of dithiothreitol is uncertain since this compound also possesses antioxidant and nucleophilic properties which may be responsible for the observed decrease in binding. The influence of N-ethylmaleimide on catecholamine binding may, on the other hand, indicate that a free thiol group(s) is required to maintain the integrity of the binding site.

It appears, based on the data presented in figure 9, that a free carboxyl group is not required for $^3$H-norepinephrine binding to microsomes of the aorta. N-Ethoxy-carbonyl-2-ethoxy-1,2-dihydroquinoline, which irreversibly alkylates free carboxyl functions on protein, is without effect on binding. Although 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) does produce a small but statistically significant decrease in binding, the combination of
EDC plus a nucleophile (histidine), which has also been reported to irreversibly alkylate free carboxyl groups (Hoare and Koshland, 1967), does not decrease \(^3\)H-norepinephrine binding. On the contrary, EDC plus histidine significantly enhance binding presumably by exposing or making available more binding sites of the same type.

The influence of temperature on binding of catecholamines to microsomal protein from various tissues has previously been reported (Maguire et al., 1974; Lefkowitz et al., 1973a; Bilezikian and Aurbach, 1973b; Lesko and Marinetti, 1975). In general, increasing the temperature in the range of 0-50°C increases binding. Similar results have been observed in the present study. The dependence of a rate constant on temperature is described by the Arrhenius equation (see section R.1). When the rate of binding is determined at various temperatures, and the appropriate assumptions satisfied, an Arrhenius plot, similar to figure 11, may be constructed. The energy of activation (\(E_a\)) for the binding process may be easily calculated from the slope of such a plot. In the present experiment, the \(E_a\) is 14.8 Kcal/mole which is the amount of energy that the environment must supply to allow the binding of \(^3\)H-norepinephrine to proceed.

It was felt that 14.8 Kcal/mole was fairly high for the simple process of drug adsorption onto a macromolecule.
To explain such a high $E_a$, it was postulated that the process may involve some type of conformational change in protein structure, induced by $^3$H-norepinephrine, which enables the protein to accept the oxidized catecholamine. This is a similar concept as that presented in the macro-molecular perturbation theory for interaction of a drug with its pharmacological receptor (Belleau, 1964). Optical rotatory dispersion yielded valuable information on a presumable interaction between protein and norepinephrine (figure 12). In the region scanned, neither the microsomal protein (0.2 mg/ml) nor (-)-norepinephrine ($10^{-5}$M) alone produced any marked peaks or troughs in the spectrum. However, when (-)-norepinephrine was combined with protein, a drastic spectral change, possibly reflecting a conformational change in protein structure, resulted. Similarly, (+)-norepinephrine, when in combination with microsomal protein, produced a spectral change that was similar in shape and magnitude to that obtained for the combination of (-)-norepinephrine plus protein. Neither the protein nor (+)-norepinephrine alone produced such a spectrum. These results are significant since both (-)- and (+)-norepinephrine also have equal affinities for the microsomal proteins as determined by their ability to inhibit the binding of $^3$H-norepinephrine. Oxymetazoline ($10^{-5}$M), a noncatechol alpha-adrenergic agonist which does not
inhibit the binding of $^3H$-norepinephrine, likewise does not produce a spectral change when added to microsomal protein. These results indicate that the ability of catechol-containing compounds to inhibit the binding of $^3H$-norepinephrine is dependent upon interaction with a catechol-specific site on microsomal protein which induces a conformational change in the protein. Those agents that do not block the binding of $^3H$-norepinephrine presumably cannot interact with the catechol-specific site and therefore do not induce such a change in protein structure.

These conclusions may be summarized through reference to an oxidation mechanism (figure 42) reported by several other investigators (Maguire et al., 1974; Saner and Thoenen, 1971; Sternson et al., 1973). Reaction $a$ in figure 42 is carried out by oxygen in these experiments, and may be catalyzed by trace metal ions such as Fe$^{+2}$, Mn$^{+2}$, Cu$^{+2}$. Addition of EDTA will reduce the catalysis by metal ions already present in the preparations. An increase in pH lowers the oxidation potential of the catecholamine, thus promoting its oxidation (Sternson et al., 1973). The fate of the ortho-quinone has been examined in some detail (Tse et al., 1976), and it was found that sulfhydryl groups add very rapidly to the electron deficient ring. Only if the oxidation is inhibited by strong antioxidants may this addition reaction be
FIGURE 42

Possible oxidation and binding mechanisms, for catechol containing compounds, to free or protein bound nucleophilic groups. Reaction a is catalyzed by oxygen enabling binding to proceed through the ortho-quinone.
suppressed. Dithiothreitol may act either as an anti-
oxidant or as a substitute for protein sulfhydryl groups
in a nucleophilic addition reaction. The inhibition of
binding by N-ethylmaleimide supports the conclusion that
binding occurs to a protein-bound sulfhydryl group.

(±) Normetanephrine may also be oxidized to an ortho-
quinone but at a potential significantly higher than the
oxidation potential of norepinephrine (Sternson et al.,
1973). This higher potential results in less oxidation,
and a higher concentration of normetanephrine is therefore
required to observe interaction with the protein.

Although we propose that covalent binding occurs
between catechols and free thiol groups on protein, many
investigators have observed that binding is reversible
(Lefkowitz et al., 1973a; Jarett et al., 1974; Vallières
et al., 1975). It is unknown at the present time whether
there exists a mechanism present in the microsomal fraction
whereby catecholamines, covalently bound to thiol functions
of protein, are cleaved, thereby allowing the system to
behave in an apparent reversible fashion.

Based on the preceding findings, and those of
others (Wolfe et al., 1974; Maguire et al., 1974; Kržan
et al., 1974) it must be concluded that 3H-norepinephrine,
and probably all labeled catecholamines for that matter,
are not of significant value in identification of the
adrenoreceptors. The existence of a high affinity and relatively high capacity binding site, which appears to be oxidation-dependent and receptor-independent, serves to mask the small amount of specific binding that may occur. Possibly the use of a different ligand not possessing the catechol moiety or being subject to rapid oxidation and destruction would be more fruitful.


In view of the problems associated with the binding of $^3$H-norepinephrine, it was decided to study a derivative of a chemically stable antagonist. Reduction of the alpha-adrenoreceptor blocking agent, azapetine, results in the formation of dihydroazapetine which retains qualitatively the same pharmacological actions. The potency, however, of dihydroazapetine is some twenty-fold lower than that of the parent compound although still high enough to be used in a kinetic analysis of alpha-adrenoreceptor blockade.

Since dihydroazapetine did not affect the response of histamine or carbamylcholine, it is concluded that this blocker is relatively specific for the alpha-adrenergic receptor. Thus, before using dihydroazapetine in binding experiments, it was thought that some important information concerning receptor mechanisms could be gained by investigating, in detail, the kinetics of alpha-adrenoreceptor
blockade by the antagonist.

The radiolabeled antagonist is accumulated in the denervated aorta into three compartments, the anatomical nature of which is unknown. Similarly, the labeled blocker is also released from three compartments during washout of a preloaded denervated preparation. Ideally, the sizes (in percent of total accumulation) of the three individual compartments that accumulate the drug should be equal to the sizes of the three compartments from which the antagonist washes out. Inspection of the data in figures 18 and 20 show this not to be the case. An explanation for the discrepancy may lie in the different manner in which the experiments were performed. For example, the first and most rapid phase of accumulation (representing 16% of total radioactivity accumulated) of $^3$H-dihydroazapetine has a rate constant of 3.86 min$^{-1}$ with a half-life of 0.18 min. Unless binding or some other type of retention occurs, the rate constant for diffusion out of a compartment should be equal to that for accumulation, and so it is possible that this phase has not been directly observed in efflux studies where the first observation was made at 2 min. Thus, any extremely rapid efflux phase could have long ended before the first sampling was made. The possibility exists, therefore, that the first two compartments (approximately 16% each) to accumulate
$^3$H-dihydroazapetine appear in the efflux experiments as the first rapid phase whose size is 28%. The lack of discrimination between the two phases might be due to the fact that the more rapidly releasing compartment has emptied prior to 2 min and is therefore part of the first sample collected.

Since the rate constant for filling of a compartment does not have to equal the rate constant for emptying if binding or some other form of retention is occurring, the remaining slowly accumulating compartment (70%) might consist of the two more slowly emptying compartments whose sizes are 30 and 43% of total $^3$H-dihydroazapetine in the tissue at $t_0$. The two compartments may accumulate at identical rates or rates so close that resolution is impossible and thus appear as one compartment on accumulation, whereas the labeled antagonist may exit at different rates permitting resolution into two phases.

Although it is not possible in most kinetic studies to attribute any particular phase or compartment to a specific anatomical structure, it is not unreasonable to assume, in the present case, that the most rapid phase of accumulation ($k_1 = 3.86 \text{ min}^{-1}$, 16% of total accumulation) represents $^3$H-dihydroazapetine in either the extracellular space, the "biophase", a term used by Furchgott (1955) to describe the location of receptors, or both, since these areas would be expected to be among the first to accumulate the drug. The rate constant of accumulation in this
rapidly filling compartment is identical to the rate constant of onset of alpha-receptor blockade by dihydroazapetine ($k_1 = 3.88 \text{ min}^{-1}$). The implication is that the factor limiting the onset of the pharmacological effect of dihydroazapetine is diffusion to the site of action (extracellular spaces or "biophase") (Furchgott, 1964; delCastillo and Katz, 1957; Goldsmith, 1963; Bevan, 1960) and not the interaction of the drug with the receptor (Paton, 1967a,b). It is believed, therefore, that dihydroazapetine must first penetrate a "barrier" [(i.e., the central compartment which may be the extracellular fluid and/or "biophase" (see Section II of Appendix)], to come into close proximity to the receptor and that once the drug reaches the receptor, it binds instantaneously due to the large second-order forward rate constant (a value greater than $3.84 \times 10^5 \text{ M}^{-1}\cdot\text{min}^{-1}$) for the drug-receptor interaction.

If the rate constant, $3.88 \text{ min}^{-1}$, indeed represents diffusion of the drug into the "biophase," then the same rate constant should describe passive diffusion out of the "biophase" (Furchgott, 1955). It has been shown that, in certain cases, the offset of drug action is accounted for by rate limiting passive diffusion out of the "biophase," and not dissociation of drug from the receptor, since the rate constant for onset (and thus partition into
the "biophase") equals the rate constant for offset of drug response (Furchgott, 1955). If, however, dissociation of the antagonist-receptor complex occurs at a slower rate than diffusion out of the "biophase," then the former process would be rate limiting and thus describe offset of blockade. In the present situation, the rate constant for loss of blockade to dihydroazapetine is 0.096 min$^{-1}$, a value approximately 40-fold slower than the rate constant for onset of drug response, which, as stated above, possibly represents rate limiting diffusion into the "biophase." The possibility exists, therefore, that the rate constant of 0.096 min$^{-1}$ represents a minimum estimation for the dissociation of dihydroazapetine from the receptor since dissociation could not possibly be at a rate slower than loss of pharmacological blockade, whereas it is possible for dissociation to be faster than the loss of blockade observed pharmacologically (Furchgott et al., 1973).

The dissociation constant for an antagonist ($K_D$) is defined as the ratio of the first-order rate constant for dissociation of the drug from the receptor divided by the second-order rate constant for association with the receptor (Furchgott, 1972). If, indeed, the rate constant for loss of receptor blockade to dihydroazapetine is the lowest possible estimation of the rate constant for
dissociation (figure 22), then, knowing the $K_B$ for dihydroazapetine ($2.5 \times 10^{-7}$M), one may calculate the smallest value that the second-order forward rate constant could have (see Section III of Appendix). Thus, dihydroazapetine reacts with the receptor with a second-order forward rate constant of at least $3.84 \times 10^{5} \text{M}^{-1} \text{min}^{-1}$.

As noted above, the rate constant for the loss of receptor blockade by dihydroazapetine is 0.096 min$^{-1}$ and serves as a minimum estimate of the rate constant for dissociation of the antagonist from the receptor. In figure 20 it may be seen that there exists one phase where the efflux of $^{3}H$-dihydroazapetine from the denervated tissue has a rate constant of 0.075 min$^{-1}$, a value not significantly different ($p > 0.05$) from the rate constant for the loss of receptor blockade. Whether this phase of efflux, either whole or in part, represents dissociation of the antagonist-receptor complex, or a phase in equilibrium with the drug-receptor complex, is not known at this time.

In summary, dihydroazapetine is a reasonably potent and selective antagonist of the alpha-adrenoreceptor. The characteristics of the receptor with respect to the kinetics of onset and offset of blockade by dihydroazapetine have been investigated in isolated and denervated aortic strips. Evidence was obtained that suggests that
the receptor is either buried within, or located behind, what Furchgott (1955) refers to as the "biophase." The logical outcome of these experiments is to carry the analysis one step further and investigate the binding characteristics of $^3$H-dihydroazapetine in hopes of identifying the alpha-adrenoreceptor. It was reasoned that the best chances of locating the alpha-adrenoreceptor would be to study $^3$H-dihydroazapetine binding in the rat vas deferens which has a dense sympathetic innervation and is believed to contain a higher receptor population.

D.3. Characteristics of $^3$H-dihydroazapetine binding to a membrane fraction from the rat vas deferens.

$^3$H-Dihydroazapetine binds to protein in membrane fragments from the rat vas deferens and at least one-third of this binding may be blocked by simultaneous exposure to the alpha-adrenoreceptor antagonist, phentolamine. In many respects, the binding has characteristics expected for the alpha-adrenoreceptor based on pharmacological data. $^3$H-Dihydroazapetine binds rapidly and reversibly to membrane fragments and is saturable. The labeled antagonist appears to bind to both high and low affinity sites, the latter of which has a dissociation constant of $3.7 \times 10^{-7}$M and is in good agreement with the value of $2.5 \times 10^{-7}$M (i.e., $pA_2 = 6.6$) determined pharmacologically on the rabbit aorta (see section R.2.1). The significance
of the higher affinity site is not known at the present time. $^3$H-Dihydroazapetine binding is inhibited by low concentrations of all alpha-adrenoreceptor antagonists tested, and those agonists of the imidazoline class, but is not affected by pharmacologically high doses of antagonists of the muscarinic, histaminic and beta-adrenergic receptors, or muscarinic, nicotinic and beta-adreno-receptor agonists. There is good correlation between agonist and antagonist affinity for the receptor in intact tissue preparations and ability to inhibit binding of $^3$H-dihydroazapetine. This correlation extends over a 10,000-fold range in affinities for the receptor.

Since alpha-adrenoreceptors in various tissues exhibit stereoselective pharmacological effects for agonists or antagonists [reviewed, Patil et al. (1974)] the binding of isomers to the subcellular fraction was explored. Due to the converging nature of the curves, differences between stereoisomers of antagonists at high concentrations were nonexistent, but did approach approximately 1 log unit at lower concentrations. The reason for convergence of the curves for the two pairs of stereoisomers is not known, but may possibly reflect more specific drug effects at lower concentrations and relatively less specific actions at the higher concentrations which may mask the selectivity.
The alpha-adrenoreceptor antagonists appear to produce parallel effects on $^3$H-dihydroazapetine binding. Likewise, analogous curves for agonists of the imidazoline class also appear to parallel one another. No significant differences in slopes were observed between this class of agonists and the antagonists, indicating that the mechanism for inhibition of binding was most likely the same, possibly competitive interaction with the same binding site.

Agonists of the phenylethylamine class did not produce a decrease in binding of $^3$H-dihydroazapetine. On the contrary, these compounds produced a stereoselective increase in specific binding at low concentrations with the (-)-isomer being more active than the (+)-form. The mechanism for this effect is not known, but it may represent an allosteric effect, after activation of a different binding site on the alpha-adrenoreceptor, by the structural analogs of norepinephrine. Whether this site is similar to the norepinephrine recognition site described by the Easson-Stedman hypothesis (Patil et al., 1974) remains to be seen.

Based on the data in figures 30 and 31, it was postulated above that alpha-adrenoreceptor antagonists, in addition to those agonists of the imidazoline class, possibly act on an identical binding site. In view of the structural dissimilarities between these agents and those agonists of the phenylethylamine class (Table 2,
Table 3 and figure 34), in addition to the apparent differences in effects on $^3$H-dihydroazapetine binding, the possibility of different binding sites, or different modes of binding with the receptor, seems likely.

Pharmacological studies by Swamy and Triggle (1972) and Janis and Triggle (1971) indicate that possibly two other binding sites exist on the alpha-adrenoreceptor in addition to the norepinephrine recognition site. The nature of these sites and possible interactions between them is not known with certainty, but one might hypothesize that they represent different sites of interaction for different classes of alpha-adrenergic drugs. The possibility exists that interaction at one site may have an allosteric effect on another site and thereby alter binding characteristics of the receptor. Fleisch and Titus (1973) reported evidence for possible allosteric effects of drugs on the alpha-adrenoreceptor. Consistent with the above hypothesis are the findings of Kalsner (1970, 1973) who demonstrated that blockade of the alpha-adrenoreceptor by the irreversible antagonist N-ethoxycarbonyl-2-ethoxy-1, 2-dihydroquinoline (EEDQ) was not decreased by prior exposure to phenylethylamines, but paradoxically increased. It was proposed that the amines acted, stereoselectively, at one binding site thereby inducing, via an allosteric mechanism, a conformational change at yet another site
allowing more EEDQ to bind and produce greater blockade. This effect was not seen with the imidazoline, tetrahydrozoline. Thus, there are striking similarities between the pharmacological observations by Kalsner (1970; 1973), and those reported here with respect to the interactions between $^3$H-dihydroazapetine and two classes of agonists, namely the imidazolines and the phenylethylamines.

Tuttle and Moran (1969) have investigated the ability of the alpha-agonist, norepinephrine, and the alpha-antagonist, phentolamine (an imidazoline), to protect the receptor against irreversible inactivation by phenoxybenzamine. It was observed that protection by the agonist required Ca$^{+2}$ while that by the antagonist was Ca$^{+2}$-independent. This led the investigators to propose that agonists require Ca$^{+2}$ to bind to the receptor whereas antagonists do not, supplying further evidence that the mode of binding of agonists and antagonists (or possibly phenethylamines and imidazolines) to the alpha-adreno-receptor need not be the same. Based on the present findings, it is probable that differences not only exist in the modes of binding between agonists and antagonists, as proposed by Ariëns and Simonis (1964), but also between agonists of different chemical classes.

Observations with group selective reagents indicate that an intact disulfide bridge, in addition to a free
sulfhydryl and carboxyl group, are present at or near the binding site and are required for maximal binding. Recently, Salman et al. (1976) have shown that N-ethylmaleimide markedly affects the \textit{alpha}-adrenergic response of the rabbit aorta which suggests the existence of a free sulfhydryl group. Similar conclusions have been presented by Lippert and Belleau (1973). Likewise, the presence of a critical disulfide bridge at or near the \textit{alpha}-adrenoreceptor has been proposed (Salman et al., 1976).

It is believed that the \textit{alpha}-adrenoreceptor possesses a negative charge which is available for interaction with the protonated nitrogen of phenylethylamine agonists (Swamy and Triggle, 1972). The source of the negative charge has not been established, but free phosphate and/or carboxyl groups have been postulated (Swamy and Triggle, 1972). The results of the present study indicate that a free carboxyl function is required for the binding of $^3$H-dihydroazapetine and is therefore possibly associated with the receptor.

The actions of the protein denaturants and trypsin indicate that the $^3$H-dihydroazapetine binding site(s) is, at least in part, protein in nature. Furthermore, the effects of phospholipase A and C on the binding suggest the involvement of phospholipids. This observation is
consistent with the model of the **alpha**-adrenoreceptor proposed by DeRobertis (1975) and with the findings of Dikstein and Sulman (1965).

It has recently been postulated, based on experiments in poikilotherms (Kunos and Nickerson, 1976), that there exists one interconvertable pool of adrenoreceptors. The assumption is that **alpha**- and **beta**-adrenoreceptors are one and the same macromolecule. The temperature and/or metabolic state of the tissue determine which form, **alpha**- or **beta**-, predominates. Generally, lower temperatures and metabolic states favor conversion to the **alpha**-form whereas higher temperatures and metabolic states shift the equilibrium toward the **beta**-configuration. These experiments have also been performed on mammalian homeotherms with similar results (Kunos *et al.*, 1973, 1974). It is tempting to postulate that the decrease in $^3\text{H}$-dihydroazapetine binding with increasing temperature is related to a shift in the equilibrium of the adrenoreceptor pool in favor of the **beta**-form which would not bind $^3\text{H}$-dihydroazapetine. However, further experiments must be performed, possibly demonstrating a simultaneous increase in binding of a labeled **beta**-adrenoreceptor antagonist with increasing temperature, before this can be established.
D.4. Desensitization experiments on the rat vas deferens.

Specific desensitization is one technique used to classify receptors (Barsoum and Gaddum, 1935). If, for example, one administers a large dose or repeated doses of drug A so that the tissue no longer responds to this drug (desensitization), and then determine on the desensitized tissue that the response to drug B, but not drug C, is reduced or abolished, then it may be concluded that B, but not C, act at the same site as A (Waud, 1968). In this example, drug B shows "cross-desensitization" with drug A.

From the discussion on $^3$H-dihydroazapetine binding to proteins from the rat vas deferens, it was apparent that marked differences existed between agonists of the phenylethylamine and imidazoline classes (see section D.3). It was proposed that these differences were manifestations of differences in the site or mode of interaction of these agonists with the receptor. The imidazolines may interact at the same site as $^3$H-dihydroazapetine and therefore have direct effects on the ligand whereas phenylethylamines may be acting at a different site and as such affect binding of the labeled antagonist allosterically.

It was reasoned that specific desensitization might be a useful technique to prove the postulate that phenylethylamines and imidazolines interact differently with the receptor. Oxymetazoline produces rapid desensitization of the rat vas deferens (Mujic and VanRossum,
1965). This drug was administered repeatedly to produce complete desensitization. Once desensitized, the rat vas deferens remained unresponsive to other imidazoline agonists. It was observed, however, that all phenylethylamine agonists tested, with the exception of dopamine, were resistant to desensitization suggesting, therefore, that they did not occupy the same binding site on the receptor as the imidazolines. Dopamine, which behaved in binding experiments like the imidazolines (i.e., reduced $^3$H-dihydroazapetine binding) likewise resembled the imidazolines in the desensitization experiments in that it showed "cross-desensitization" with oxymetazoline. The significance of the "imidazoline-like" properties of dopamine in these experiments is not understood.

D.5. Possible model for the alpha-adrenoreceptor.

It appears that the alpha-adrenoreceptor is protein in nature, possibly closely associated with various phospholipids, and intimately related to the "biophase." Although the nature of the "biophase" is not known, Furchgott (1955) has proposed that it may represent the lipoidal component of the cell membrane. There probably exist multiple substrate-recognition sites on the receptor (Swamy and Triggle, 1972; Kalsner, 1970, 1973; and the present investigation) and at least one site possesses a free sulfhydryl and carboxyl group. No doubt, at least
one of the recognition sites is the Easson-Stedman site mediating the alpha-adrenergic effects of beta-hydroxylated phenylethylamines and to which most interest over the years has been directed.

Kalsner (1970) reports an additional site for amines, different from that described by Easson and Stedman (1933), which apparently does not interact with the imidazolines. Swamy and Triggle (1972) and Janis and Triggle (1971) have proposed, aside from the two sites discussed above, a third site which they call the "calcium-mobilization" site, believed to be allosterically regulated by the Easson-Stedman site. The calcium-mobilization site might be the locus of the "pore" through which the divalent ion traverses during a phenylethylamine-induced action potential. In addition, these investigators propose that the long-lasting blockade produced by the irreversible alpha-blockers, such as dibenamine and phenoxybenzamine, is the result of alkylation at the calcium-mobilization site. Swamy and Triggle (1972) also acknowledge the fact that the irreversible antagonists react with the Easson-Stedman recognition site, but they believe that antagonism here is only short-lived.

It is quite obvious that $^3$H-dihydroazapetine does not interact directly with the Easson-Stedman recognition site since phenylethylamines do not inhibit binding.
However, the apparent allosteric effect of the phenylethylamines on \(^3\)H-dihydroazapetine binding (i.e., enhancement of ligand binding) might mean that the labeled antagonist is interacting at the calcium-mobilization site proposed by Swamy and Triggle (1972). In support of this hypothesis, consider the close structural similarity between dihydroazapetine and dibenamine (figure 43), the latter being believed to irreversibly alkylate the calcium-mobilization site. If the compound, N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), alkylates a carboxyl group at this site (which does not seem unlikely and is consistent with observations made in the present investigation), then even the enhancing effect of phenylethylamines on EEDQ blockade of the receptor, which has been proposed to be allosteric (Kalsner, 1970, 1973), also fits this receptor model.

Where, then, do the imidazoline agonists and the competitive antagonists fit into the model? Swamy and Triggle (1972) have proposed that a hydrophobic region exists on the receptor between the Easson-Stedman and calcium-mobilization sites. The existence of a hydrophobic region on the receptor may be inferred from observations in the present investigation. From figure 36 it may be seen that elevated pH, which favors the unionized and more lipophilic form of dihydroazapetine, enhances binding. Since the imidazolines and alpha-adrenoreceptor
FIGURE 43

Chemical structures of dihydroazapetine and dibenamine. The marked structural similarity between the two compounds is apparent. Dibenamine is believed to interact with the calcium-mobilization site of the alpha-adrenoreceptor.
antagonists are fairly liposoluble, it does not seem unreasonable to assume that these compounds interact with this hydrophobic region which perhaps possesses small regions where overlap with the Easson-Stedman and calcium-mobilization sites takes place.

Ariëns and Simonis (1964) have proposed that the alpha-adrenoreceptor blocking activity of chlorpromazine (and all alpha-adrenoreceptor antagonists for that matter) results not from interaction with the norepinephrine recognition site but from interaction at a poorly defined (hydrophobic?) site in close proximity to the Easson-Stedman site. When the highly liposoluble chlorpromazine molecule binds to this site, there exists overlap of the chlorpromazine side chain with a small area (anionic head) of the Easson-Stedman site believed to be the point of attachment of the protonated nitrogen of phenylethylamines. A slight overlap between agonist and antagonist would be sufficient to interfere with agonist interactions with the receptor and thereby produce the observed characteristics of competitive blockade (Furchgott, 1972).

Likewise, the structural dissimilarity between phenylethylamines and the imidazolines, as well as the results of the present investigation, suggest that these two groups of agonists interact at different points on the receptor. The hydrophobic nature of the imidazolines might permit them to bind in a similar manner and to a
similar site as the competitive antagonists, only in such a way, however, as to allow them to have agonistic activity. If this proposed hydrophobic region associated with the Easson-Stedman and calcium-mobilization sites (and partially overlapping these sites) is indeed the site of interaction of the imidazolines, as well as competitive antagonists, then the differential effects with respect to $^3$H-dihydroazapetine binding between the phenylethylamines and the imidazolines (and antagonists) is easily understood. Since dihydroazapetine should interact with the hydrophobic region as well as the calcium-mobilization site (see above discussion), the Scatchard plot indicating two sites of interaction for the ligand seems logical.

A possible model of the alpha-adrenoreceptor which would explain all the observations of the present investigation, in addition to those of Kalsner (1970, 1973) and Swamy and Triggle (1972), is presented in figure 44.

Recently, convincing evidence has been presented suggesting that one adrenoreceptor pool exists in which alpha- and beta-adrenoreceptors are only different configurations of the same macromolecule. The postulation is that low temperature or metabolic states favor conversion to the alpha-form whereas higher temperatures or metabolic states shift the equilibrium in favor of the beta-configuration (Kunos et al., 1973, 1974; Kunos and Nickerson, 1976). The observation that $^3$H-dihydroazapetine binding
FIGURE 44

A hypothetical model of the alpha-adrenoreceptor based on findings from the present study as well as those of other investigations. Experimental evidence is available to support (though not necessarily prove) the existence each of the binding sites presented.
decreases as temperature is increased (figure 37) is consistent with this hypothesis.

Beta-adrenoreceptor agonist activity of catecholamines may be increased by addition of bulky substituents to the side chain nitrogen. The implication is that the nature of the side chain, as opposed to the catechol ring, dictates which activity, alpha- or beta-, predominates. Since the catechol moiety is common to both alpha- and beta-adrenoreceptor agonists, it is possible that the region of the Easson-Stedman site that recognizes the catechol portion of catecholamines is also common to both forms of the receptor and that the orientation of the side chain determines which activity is observed. For example, a catecholamine may first bind to the catechol-recognition site of the adrenoreceptor complex. Binding of the side chain may depend upon its bulkiness and steric configuration. That is, bulky side chains may preferentially bind to a site that elicits beta-effects (i.e., a site coupled with adenylate cyclase) whereas, a relatively unhindered side chain, as in norepinephrine, may bind to a different site which elicits alpha-effects (i.e., a site possibly allosterically coupled with the calcium-mobilization site). Free rotation about the alpha-beta carbons of phenylethylamines could possibly direct the nitrogen function to different parts of the
receptor (i.e., adenylate cyclase vs. hydrophobic region) while the catechol portion and beta-hydroxyl group remain firmly anchored. This hypothesis has also been incorporated into the model presented in figure 44.

Beta-adrenoreceptor antagonists are structural analogs of norepinephrine and are therefore believed to interact at the Easson-Stedman recognition site of the beta-adrenoreceptor. If there is partial overlap between the two Easson-Stedman recognition sites for alpha- and beta-adrenoreceptors, then the well known effects of beta-adrenoreceptor agonists and antagonists (at higher concentrations) on the alpha-receptor, as well as the beta-adrenergic effects of alpha-agonists) may be explained. Similarly, the fact that all phenylethylamines possess some degree of activity on both alpha- and beta-receptors (although actions on one or the other receptor may predominate) is consistent with the model presented.

Imidazolines, which we propose to act at a site different from, but partially overlapping, the Easson-Stedman recognition site on the alpha-adrenoreceptor (but not overlapping the beta-recognition site of the receptor), only show alpha-effects and not beta. These observations are also consistent with the model of the adrenoreceptor presented in figure 44.

Although evidence exists for all facets of the model shown in the figure, it should be noted that the
model is still only speculative and requires further experimentation to establish its existence.
CHAPTER V

SUMMARY AND CONCLUSIONS

1. $^3$H-norepinephrine readily undergoes oxidation to form the ortho-quinone in the presence of tissue components. The ortho-quinone subsequently reacts with free nucleophilic sulfhydryl groups to form the sulfhydryl adducts. This reaction occurs to a far greater extent than interaction of the catecholamine with the alpha-adrenoreceptor, making identification of the latter impossible using $^3$H-norepinephrine as a ligand.

2. There is evidence to indicate that interaction of the ortho-quinone, resulting from oxidation of norepinephrine, with tissue sulfhydryl groups involves conformational changes in protein structure, which perhaps may be secondary to binding of the ligand.

3. The potent alpha-adrenoreceptor antagonist, azapetine, may be catalytically reduced with hydrogen or tritium gas to form dihydroazapetine or $^3$H-dihydroazapetine, respectively. The reduced compound retains alpha-adrenoreceptor antagonist activity and is approximately twenty times less potent than the parent compound.
4. $^3$H-Dihydroazapetine has been used in a kinetic investigation of the receptor in isolated rabbit aortic strips. The rate of onset of alpha-adrenoceptor blockade by the antagonist is equal to the rate of uptake of a rapidly filling compartment possibly representing the extracellular space and/or the "biophase." The implication is that diffusion of the drug into the extracellular space and/or "biophase" is the rate limiting step in the interaction of $^3$H-dihydroazapetine with the alpha-adrenoreceptor.

5. $^3$H-Dihydroazapetine binds to membrane fragments isolated from rat vas deferens. The binding of this ligand has many characteristics that one would expect if the alpha-adrenoreceptors were involved since many of the criteria in section I.2 have been satisfied. Binding of $^3$H-dihydroazapetine rapidly reaches equilibrium and is a reversible and saturable process. The dissociation constant for the ligand is nearly identical with that determined pharmacologically. All alpha-adrenoreceptor antagonists tested, and those agonists of the imidazoline class, inhibited the binding of $^3$H-dihydroazapetine and did so with affinities correlating well with known affinities for the receptor. Agonists and antagonists of other receptors were without effect.
6. **Alpha**-adrenoreceptor agonists structurally related to norepinephrine produced a stereoselective increase in binding at low concentrations. This effect has been attributed to an allosteric interaction between the Easson-Stedman recognition site for phenylethylamines and the $^3$H-dihydroazapetine binding site(s).

7. The results described above suggest that phenylethylamine agonists interact at a different site on the receptor than do the imidazolines. The structural dissimilarities between these two classes of agonists support this contention. Additional evidence has been obtained from desensitization experiments in which phenylethylamines were shown not to be cross-desensitized by the imidazolines. It must therefore be concluded that these two classes of agonists interact differently at the receptor.

8. A model of the **alpha**-adrenoreceptor has been presented which is consistent with all of the observations reported in the present study and by other investigators. The validity of this model, however, must await further investigation.
I. A first-order process is one in which the rate is dependent upon the concentration of one species and is described by the following equation:

\[
\text{rate} = \frac{d[X]}{dt} = k[X] \quad (1)
\]

where \([X]\) is the concentration of transferable drug at time \(t\), and \(k\) is the first-order rate constant in units of \(\text{time}^{-1}\). Upon integration, this equation yields:

\[
\ln[X] = \ln[X_0] - kt \quad (2)
\]

where \([X_0]\) is the total concentration of transferable drug. In terms of absolute concentrations, the above equation may be written as:

\[
\ln([A_t] - [A_\infty]) = \ln([A_0] - [A_\infty]) - kt \quad \text{for efflux data} \quad (3)
\]

or

\[
\ln([A_\infty] - [A_t]) = \ln([A_\infty] - [A_0]) - kt \quad \text{for accumulation data} \quad (4)
\]
where \( [A_0] \) is the initial concentration of drug, \( [A_t] \) is the concentration at time \( t \) and \( [A_\infty] \) is the concentration at infinite time. If the terms \( ([A_\infty] - [A_t]) \) and \( ([A_\infty] - [A_0]) \), in equation 4 are divided by \( [A_\infty] \) before natural logarithms are taken, the data are expressed as percent of the total transferable drug, as was done in figure 18. The half-time of a first order process is obtained from:

\[
t_{1/2} = \frac{0.693}{k}
\]

When more than one first-order process is present, the plot of \( \ln([A_\infty] - [A_t]) \) or \( \ln([A_\infty] - [A_\infty]) \) vs. \( t \) is a curve which may be resolved into its linear components by standard feathering techniques (Notari, 1975). The intercepts on the ordinate of each of the resolved phases are equal to the "sizes" of the compartments in terms of the percent of total transferable drug accumulated by each compartment, and not in terms of volume. Thus, a compartment that accumulates a greater percent of a compound is not necessarily the larger structure.

A second-order process is one in which the rate is dependent upon the concentrations of two species and is described by the following equation:
where \([X]\) and \([Y]\) are transferable concentrations of two different chemicals and \(k'\) is the second-order rate constant in units of concentration\(^{-1}\) time\(^{-1}\).

II. The compartmental model used in this experiment is similar to that discussed by Kramer et al. (1974) and consists of a central compartment (1) and two peripheral compartments (2 and 3).

This model was chosen because drugs cannot enter a tissue without first entering the extracellular space and/or "biophase" which is analogous to a central compartment. Equations describing this model have been reported by others (Kramer et al., 1974; Benet, 1972). The \(k\) values in the model are first-order microconstants which were obtained from the influx rate constants or efflux rate constants presented in figures 18 and 20, respectively, according to the technique of Benet (1972). Theoretical curves...
describing the accumulation and efflux studies, based on the proposed model, were obtained by solving pertinent equations (Kramer et al., 1974; Benet et al., 1972) with the nonlinear regression program NONLIN (Metzler, 1969) after substitution of the appropriate microconstants.

If the central compartment refers to the extracellular space and/or "biophase," then the receptor pool might possibly consist of one of the peripheral compartments or be in equilibrium with a peripheral compartment. It should be emphasized, however, that there are dangers in assigning compartments to anatomical structures since pharmacokinetic models, in many cases, are little more than empirical data-fitting (Thron, 1974). One should not accept the data presented in this investigation as proof of the existence of the model presented but only as findings which are consistent with the model.

III. The interaction of a drug \( D \) with its receptor \( R \) may be described as:

\[
\begin{align*}
D + R & \xrightleftharpoons[k_2]{k_1} D - R \\
\end{align*}
\] (7)
The rate of association may be expressed as:

\[ \text{rate} = k_1' \, [D] \, [R] \]  

(8)

where \( k_1' \) is a second-order rate constant. The rate of dissociation of the D-R complex is as follows:

\[ \text{rate} = k_2 \, [D-R] \]  

(9)

where \( k_2 \) is a first-order rate constant. At equilibrium, the rate of association equals the rate of dissociation, thus,

\[ k_1' \, [D] \, [R] = k_2 \, [D-R] \]  

(10)

which may be rearranged to:

\[ \frac{[D] \, [R]}{[D-R]} = \frac{k_2}{k_1'} = K_B \]  

(11)

where \( K_B \) is the dissociation constant of the drug-receptor complex.

From figure 22 it was observed that the rate constant for offset of receptor blockade by dihydroazapetine was 0.096 \( \text{min}^{-1} \) which is the lowest possible value that the rate constant for dissociation of the antagonist from the receptor (i.e., \( k_2 \)) could be. Knowing that the dissociation constant (\( K_B \)) for this antagonist is \( 2.5 \times 10^{-7} \text{M} \), it is possible to calculate
the minimum value that the second-order rate constant for interaction of dihydroazapetine with the receptor \( k'_1 \) can have, by substitution into equation 11 and solving for \( k'_1 \). This operation results in a minimum value of \( 3.84 \times 10^5 \text{M}^{-1}\text{min}^{-1} \) for the second order rate constant for association.
BIBLIOGRAPHY


