

ABSTRACT

Characterisation of particulate and soluble mammalian α_2 -adrenoceptors

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Particulate α_2 -adrenoceptors of several mammalian tissues have been quantified and characterized using radioligand binding techniques and selective antagonist ligands, ^3H -yohimbine and ^3H -rauwolscine. The results showed that both ligands specifically labelled an identical population of α_2 -receptors in all the tissues investigated. However, significant differences in drug affinities and relative drug potencies were observed between tissues within a given species or between species. The differences in pharmacological behaviour of the α_2 -receptors, particularly with respect to the relative potencies of the subtype selective antagonists, provided evidence for a possible receptor heterogeneity.

The effects of guanine nucleotides, cations and temperature on agonist/antagonist interactions with α_2 -receptors labelled by selective antagonist ligands have also been examined. The differential qualitative and quantitative influences of these modulators provided evidence for basic molecular differences in agonist and antagonist interactions with α_2 -receptors. The influence of endogenous or retained exogenous agonist on ^3H -yohimbine binding has further been evaluated in detail. The results revealed a 'pseudo non-competitive' agonist interference of ^3H -antagonist binding reversible under the influence of guanine nucleotides and Na^+ , and suggested the need for considering possible endogenous agonist influence when interpreting ^3H -antagonist binding data under certain assay conditions.

α_2 -receptors of the human platelet, rat and rabbit kidneys have been successfully solubilized using the glycoside detergent digitonin, and assayed with a polyethylene glycol precipitation-filtration method. The solubilized α_2 -receptors labelled by ^3H -rauwolscine generally exhibited reduced drug affinities but retained the pharmacological properties of the respective particulate receptors. The results discounted differences in membrane constraint/environment as a likely cause for pharmacological differences between α_2 -receptors and provided further evidence for the possible heterogeneity of α_2 -receptors.

**CHARACTERISATION OF PARTICULATE AND SOLUBLE
MAMMALIAN ALPHA₂-ADRENOCEPTORS**

**Thesis submitted to the University of Leicester, England,
for the degree of Doctor of Philosophy
in the Faculty of Medicine**

by

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- 2) Cheung, Y.D., Barnett, D.B. & Nahorski, S.R. (1982). 3H -Rauwolscine and 3H -Yohimbine binding to rat cerebral and human platelet membranes: possible heterogeneity of α_2 -adrenoceptors. Eur. J. Pharmacol. 84, 79 - 85.
- 3) Barnett, D.B., Cheung, Y.D. & Nahorski, S.R. (1983). Pseudo non-competitive interaction of endogenous and exogenous noradrenaline with cerebral α_2 -adrenoceptors. Br. J. Pharmacol. 80, 542 P.
- 4) Cheung. Y.D., Barnett, D.B. & Nahorski, S.R. (1984). Interaction of endogenous and exogenous norepinephrine with α_2 -adrenoceptor binding sites in rat cerebral cortex. Biochem. Pharmacol. 33, 1293 - 1298.
- 5) Cheung, Y.D., Nahorski, S.R., Rhodes, K.F. & Waterfall, J.F. (1984). Studies of the α_2 -adrenoceptor affinity and the α_2 - to α_1 -adrenoceptor selectivity of some substituted benzoquinolizines using receptor-binding techniques. Biochem. Pharmacol. 33, 1566 - 1568.

Chapter I
GENERAL INTRODUCTION

I.1. Receptors and drug actions

The term 'drug receptor' refers to the molecular component of a cell with which a drug interacts. Such an interaction usually leads to changes in the cellular functions. However, until more recently, 'receptor' appeared to be a rather abstract term, merely used for the convenience of describing drug action when the actual molecular site of action was uncertain or unknown.

The concept of 'receptor' was actually first introduced by Langley. In 1905, he postulated that effector cells have 'receptive substances' with which drug agents might react to produce responses. This view was extensively developed by Clark, and in 1933, he postulated that the actions of catecholamines and other drugs are mediated by interaction with specific cell surface structures called 'receptors' and that drug response is proportional to the number of receptors occupied by the drug. This 'occupation theory' still remains to be the keystone of most modern theories of drug action.

In brief, a drug receptor has at least two basic functions: the recognition of drug molecules, and the initiation of cellular changes in biochemical and physiological activities. The specific interaction of a drug with its receptor signifies the primary step of drug action. This interaction is generally thought to result in some conformational changes in the receptor molecule, initiating a 'signal' which serves to modulate the activity of an effector system. In many cases, a 'transducer' appears to be involved in conveying such a signal from the receptor unit to the effector unit. The final physiological response probably represents the resultant of a series of complex biochemical changes occurring within the biological system.

Drugs may be broadly classified into three categories, with respect to biological actions: agonists, antagonists, and partial agonists. An agonist is a drug which can bind to a specific receptor and also elicit a characteristic

response (ie having both affinity and intrinsic activity). In contrast, an antagonist can occupy a receptor but fails to facilitate its coupling with an effector system, and therefore produces no response (ie having affinity, but no intrinsic activity) (Ariens 1954). A partial agonist, as its name implies, is only partially 'active' and has an intrinsic activity between that of a full agonist and an antagonist (Stephenson 1956).

1.2. Catecholamines and adrenergic actions

Catecholamines are a class of amino compounds bearing a catechol moiety. The endogenous catecholamines: adrenaline, noradrenaline and dopamine, are all structurally similar and synthesized from the precursor amino-acid tyrosine by an enzymatic pathway. The steps involved in the biosynthesis of these catecholamines, originally proposed by Blaschko (1939), and subsequently confirmed, are shown in figure 1.1.

Endogenous noradrenaline is synthesized in the brain, adrenal medulla sympathetic ganglia, and particularly sympathetic nerve endings, where it is stored in granules or granulated vesicles, and released mainly as neurotransmitter (Wurtman, 1966; Euler, 1972). On the other hand, adrenaline is largely stored in the chromaffin cells of the adrenal medulla, where it is synthesized from noradrenaline, and represents a major source of circulating catecholamines (Wurtman, 1966; Cooper et al. 1978; Stjärne, 1972).

The catecholamines regulate a large variety of essential physiological activities. Their effects on the smooth muscles can be broadly classified into either excitatory or inhibitory. Amongst the catecholamines, noradrenaline is the most potent excitatory agent, but with poor inhibitory properties. Isoprenaline has just the opposite characteristics just the opposite to those of noradrenaline. Adrenaline is in the middle of the two extremes in that it possesses equally potent excitatory and inhibitory actions (Koelle, 1975). Catecholamines are in general stimulants of the cardiovascular system. Heart rate

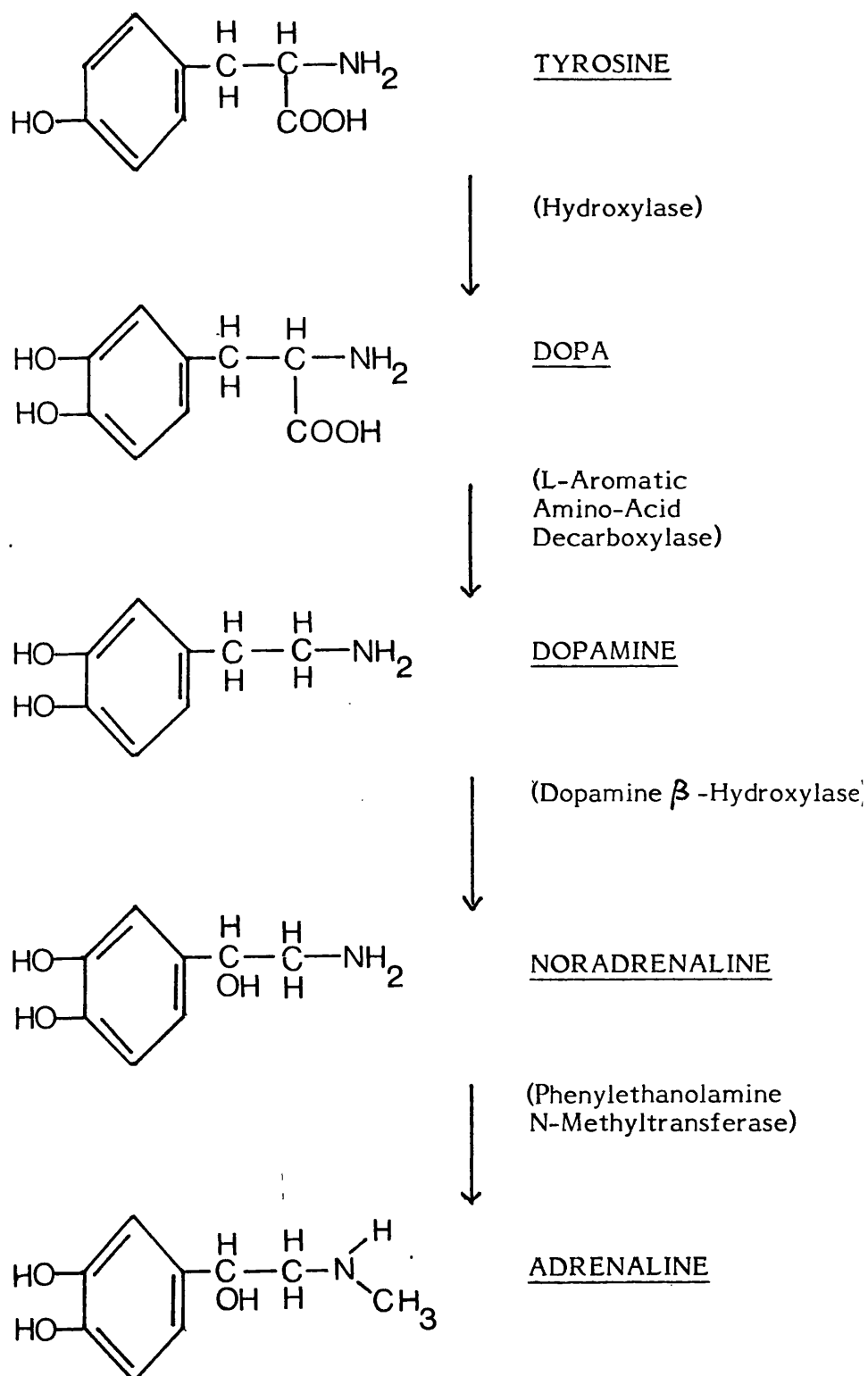


Fig. 1.1. Enzymatic Synthesis of Catecholamines

and contractility of the cardiac muscle are both enhanced (Innes & Nickerson, 1975). Depending on the relative excitatory and inhibitory actions of the particular catecholamine, vasoconstriction normally results in the skin, mucosa and kidney, whereas vasodilatation often predominates in the skeletal muscle (Innes & Nickerson, 1975). Adrenaline and isoprenaline are also potent relaxants of the bronchial muscles, and as such, have been particularly useful in the treatment of bronchial asthma (Innes & Nickerson, 1975; Aviado & Micozzi, 1981).

Besides their effects on the smooth muscles, the catecholamines exert significant influences on metabolic activities. They can stimulate glycogenolysis and gluconeogenesis in the liver and muscle (Innes & Nickerson, 1975). The release of insulin and glucagon from the pancreatic islets can also be stimulated or inhibited (Porte Jr. & Robertson, 1973; Porter, 1981), but their actions usually lead to hyperglycemia. Furthermore, lipid metabolism can be regulated under the influence of catecholamines (Burns et al. 1974; Innes & Nickerson, 1975) generally resulting in lipolysis and hyperlipidemia.

There are now many other physiological responses of different kinds known to be mediated by catecholamines. These include the regulation of renin release (Pettinger et al., 1976), inhibition of dispersion of melanocyte granules (Berthelson & Pettinger, 1977), inhibition of firing of the noradrenergic and serotonergic neurons in the brain (Svenssen et al., 1975), facilitation of noradrenaline release from sympathetic neurons (Céluch et al., 1978), and induction of platelet aggregation (Hsu et al., 1979; Barthel & Markwardt, 1974), to name but a few. Nevertheless, regardless of the exact mechanisms of action, all these responses appear to be mediated through the interactions of catecholamines with specific adrenergic receptors.

1.3. Classification of adrenoceptors

1.3.1. Divisions of adrenoceptors

The studies of adrenergic receptors began with attempts to explore the functions of the sympathetic nervous system. At the beginning, little was known about the nature of sympathetic neurotransmission. Nevertheless it was known quite early that the physiological effects elicited by sympathetic stimulation are in many respects similar to those produced by the extracts of adrenal medulla (adrenaline), though not qualitatively identical. For example, adrenaline exhibits equally potent excitatory and inhibitory responses, whereas sympathetic stimulation produces responses that are relatively excitatory in nature. In 1904, Elliot was the first to suggest that adrenaline might be the chemical stimulant released from the sympathetic nerve each time an impulse arrived at the periphery. This was the original concept of a chemical transmitter being involved in sympathetic neurotransmission. In 1905, Langley further introduced the idea that excitatory and inhibitory 'receptive substances' might be present on effector cells, which mediated the responses of adrenaline. In 1906, Dale discovered that ergot alkaloids could selectively block the motor responses elicited by adrenaline and sympathetic stimulation, but the inhibitor responses were relatively or absolutely unaffected. This was the earliest classical example of pharmacological antagonism of adrenergic actions, and strongly supported the view that different 'receptive substances' were involved in the adrenergic responses. Shortly afterwards, Barger and Dale (1910) further discovered that the actions of sympathetic stimulation more resembled those of the primary catecholamines such as noradrenaline than those of the secondary catecholamines such as adrenaline. However, it was not until the 1940s that Von Euler eventually identified noradrenaline as the sympathetic neurotransmitter.

The concept of cell surface receptors being the transducers of adrenergic actions, however, was not unanimously favoured at the early stage. This was not only because it was impossible to demonstrate the existence of the postulated post synaptic receptors, but it was not even clear about the identity of the neurotransmitter. Thus, some workers were still more enthusiastic about establishing a direct relationship between adrenergic actions and the chemical structures of the transmitter substances, rather than the end-organ receptors themselves. For example, Cannon and Rosenblueth (1937) introduced the term sympathin to refer to the sympathetic neurotransmitter. They postulated that upon being released from the nerve, sympathin was converted to sympathin I and sympathin E. These substances were in turn responsible for producing the inhibitory and excitatory responses respectively.

In 1948, Ahlquist examined the potencies of five structurally related catecholamines in eliciting physiological responses in various tissues. He found that the responses fell into two major categories on the basis of the order of potency of the agonists. One category of responses was mainly excitatory, and included such effects as vasoconstriction, stimulation of the nictitating membrane, dilator pupillae and uterus, but also inhibition of the gut. The order of agonist potency was L - adrenaline > L - noradrenaline > α -methylnoradrenaline > α -methyladrenaline > isoprenaline. The other group of responses was mainly inhibitory and included vasodilation, bronchodilation, relaxation of the uterus, but also stimulation of the heart. The order of agonist potency for these effects was isoprenaline > adrenaline > α -methyladrenaline > α -methylnoradrenaline > noradrenaline. Ahlquist thought that if the catecholamines structurally similar to adrenaline exhibited different potencies in producing the two types of responses, but maintaining the same rank orders, then the differences in activity must be attributed only to the differences in the chemical structure of the compounds. However, if the agonists differed not only in the relative potencies, but also in the rank orders between the responses or tissues, as was observed in this study,

then, such difference would strongly reflect difference in the receptors themselves. He therefore proposed the terms 'alpha' and 'beta' respectively to describe the 'excitatory' and 'inhibitory' adrenergic responses, and hence also the receptors mediating these responses. Although exceptions to this model of classification are obvious, namely the stimulation of the heart, and the inhibition of the gut, subsequent studies have confirmed that adrenergic responses generally fall into these two main categories. Ahlquist's proposal has also been substantiated particularly as the result of the development of new adrenergic antagonists which can exhibit selective blocking actions on the alpha or beta responses.

1.3.2. Sub-divisions of alpha and beta adrenoceptors

1.3.2.1. Beta-adrenoceptor sub-types

Following Ahlquist's classification of alpha and beta adrenergic responses, vigorous search for more selective adrenergic agonists and antagonists were underway. The consequence is not only that his dual adrenoceptor model has been strengthened, but that evidence has also emerged to indicate that this model needs to be expanded. For example, the beta-antagonist butoxamine has been developed and shown to produce selective blockade of the isoprenaline-induced vasodilation but not cardiac stimulation (Levy, 1966). Salbutamol, a beta-agonist, can selectively stimulate bronchial relaxation, but with little activity on the heart (Brittain, 1971). In contrast, the beta-antagonist practolol has been described to exhibit selective blockade on the cardiac actions of catecholamines without producing an equivalent degree of antagonism at the bronchioles (Dunlop & Shanks, 1968). Indeed, with the use of highly selective agonists and antagonists, beta-adrenoceptors in various tissues can be pharmacologically differentiated (Levy & Wilkenfeld, 1969) into the ' β_1 ' and ' β_2 ' subtypes. These terms were first introduced by Lands and colleagues in 1967. In their work, these workers studied

the relative potencies of a series of drugs in eliciting beta-adrenergic responses in a number of tissues and whole organs. The potencies were compared with those of isoprenaline. Correlations in relative potencies between pairs of tissue responses were assessed. The results clearly indicated that cardiac stimulation is quite different from the other beta adrenergic responses (Lands et. al., 1967 a,b.), thus giving rise to the idea of subdividing the beta-adrenoceptors into the 'beta₁' and 'beta₂' categories. Beta₁-receptors are located mainly on the myocardium and the adipose tissue. In contrast, beta₂-receptors are found mainly on the smooth muscles.

1.3.2.2. Alpha-adrenoceptor sub-types

Until recently, the alpha and beta adrenoceptors were thought to occur only on the postsynaptic effector cells, mediating adrenergic actions. Few people would expect that adrenergic receptors might also be present on the sympathetic nerve-endings, playing an 'auto-regulatory' role on its own release. The first evidence of the possible existence of such receptors was provided some years ago from the work of Gillespie and Brown (1957) when it was found that the alpha blocking agent phenoxybenzamine could enhance nerve-stimulated overflow of noradrenaline in the perfused cat spleen. Of course, besides being an alpha-adrenoceptor blocker, phenoxybenzamine is capable of blocking extraneuronal and neuronal uptake of noradrenaline (Iversen, 1965; Iversen, 1969; Eisenfeld et al., 1967). Therefore, it was once thought that the observed enhancement of noradrenaline overflow might be due to blockade of the uptake and metabolism of noradrenaline. However, drugs such as cocaine and the tricyclic anti-depressants such as desipramine, which block neuronal uptake of noradrenaline without causing alpha-adrenoceptor blockade, were found to produce little or no enhancement of the stimulation-evoked release of the transmitter, even at concentrations which produce maximal inhibition

of uptake (Boullin et al., 1967; Langer & Enero, 1974). Other alpha-adrenergic blockers such as phentolamine, were also shown to increase the release of noradrenaline upon nerve stimulation without blocking extraneuronal metabolism (Langer, 1970). Moreover, the phenoxybenzamine inhibition of metabolism of ^3H -noradrenaline released by nerve stimulation does not appear to sufficiently account for the large increase of noradrenaline output observed in the presence of this antagonist (Langer, 1970; Langer & Vogt, 1971). In fact, enhancement of stimulation evoked release of noradrenaline by the alpha-blockers can occur at concentrations which do not inhibit neuronal or extraneuronal uptake of the transmitter (Enero et al., 1972; Cebeddu et al., 1974). Finally, the alpha-blocker-induced increase in stimulated noradrenaline output does not appear to be functionally related to the blockade of postsynaptic alpha-adrenoceptors, since such effect could be observed in preparations in which the end-organ responses are mediated by the beta-adrenoceptors (Starke et al., 1971; McCulloch et al., 1972).

Therefore, these findings have led to the suggestion that an alpha-adrenoceptor may exist on the presynaptic membrane of the sympathetic nerve-terminals and regulate the release of noradrenaline by an 'auto-regulatory' feed-back mechanism (Langer, 1974; Langer, 1977; Starke, 1977). This hypothesis has been supported by the observations that while alpha-adrenergic antagonists increase the release of noradrenaline during nerve stimulation (Doxey et al., 1977; Weitzell et al., 1979), alpha-adrenergic agonists inhibit such release (Kirpekar et al., 1973; Starke et al., 1975). Results from studies of this kind also show that the alpha-adrenergic agonists and antagonists exhibit significant differences in potency and affinity at the pre-and post synaptic alpha-adrenoceptors (Dubocovich & Langer, 1974; Starke et al., 1975; Berthelson & Pettinger, 1977; Doxey et al., 1977; Weitzell et al., 1979), hence suggesting that the two alpha-adrenoceptors

are not structurally of the same type.

For instance, Starke et al. (1975) studied the potencies of a number of alpha-adrenergic agents in inhibiting the stimulation-evoked release of noradrenaline and in stimulating muscle contraction in the rabbit pulmonary artery. By calculating the relative potencies of the agonists in eliciting the pre-and postsynaptic responses, they found that these drugs could be classified into three main categories. One group had postsynaptic/presynaptic potency ratios of about 30 (ie they are more potent for the postsynaptic response). Methoxamine and phenylephrine fell into this category. In a second group, the potency ratios were close to 1.0 (ie the agonists are equipotent for the pre-and post-synaptic responses). This group comprised adrenaline, noradrenaline and naphazoline. The third group had post synaptic/pre synaptic ratios of less than 0.2 (ie the agonists are more potent for the pre-synaptic response). This group comprised oxymetazoline, α -methylnoradrenaline, tramazoline, as well as clonidine.

On the other hand, Doxey et al. (1977) and Weitzell et al. (1979) examined the relative potencies of the alpha-adrenergic antagonists in inhibiting the pre- and post synaptic adrenoceptor-mediated responses. The results demonstrated that yohimbine and its diastereoisomer rauwolscine were more selective for the presynaptic than the postsynaptic adrenoceptors. In contrast, corynanthine, phenoxybenzamine and prazosin were more selective postsynaptically.

In view of the anatomical and functional differences between the pre- and post synaptic alpha-adrenoceptors, it was suggested that they should be called ' α_2 ' and ' α_1 ' receptors respectively (Langer, 1974). However, it did not take long to realize that alpha-adrenoceptors with the α_2 -like pharmacological properties are also present in a variety of postsynaptic and non-neuronal locations.

For example, in addition to the postsynaptic α_1 -adrenoceptors, post synaptic α_2 -like adrenoceptors also appear to be involved in mediating

contraction of the vascular smooth muscle. Evidence for the presence of postsynaptic α_2 -adrenoceptors in the vascular smooth muscle was obtained in the cat, rat and dog (Drew & Whiting, 1979; Docherty & McGrath, 1980; Langer et al., 1980). The main findings from these studies were that, compared to the vasopressor effects elicited by the α_1 -agonists such as phenylephrine, the responses to the agonists with strong α_2 -activity (eg noradrenaline, clonidine, xylazine) were relatively resistant to prazosin, an antagonist with high α_1 -selectivity (Cambridge et al., 1977). However, the latter responses were more potently antagonized by the α_2 -antagonists such as yohimbine or rauwolscine.

Catecholamines are known to induce platelet aggregation or potentiate aggregation induced by other agents (Clayton & Gross, 1963; Mills & Roberts, 1967; Barthel & Markwardt, 1974; Grant & Scrutton, 1979; Lasch & Jakobs, 1979; Hsu et al., 1979). Such aggregatory action is also closely related to the inhibition of adenylate cyclase (Jakobs et al., 1976) and of the accumulation of cyclic-AMP (Marquis et al., 1970; Jakobs et al., 1976; Kafka et al., 1977). These effects appear to be mediated by α -adrenoceptors since only α but not β agonists and antagonists are effective in modifying the aggregatory and adenylate cyclase activities (Mills & Roberts, 1967; Marquis et al., 1970; Barthel & Markwardt, 1974; Kafka et al., 1977). Furthermore, the pharmacological specificity of the α -adrenoceptors suggests that they are of the α_2 -type. The ADP-induced platelet aggregation, for example, is much more potently enhanced by the α_2 -agonists, such as clonidine and α -methylnoradrenaline, than by the α_1 -agonists, such as phenylephrine and methoxamine (Hsu et al., 1979). In addition, yohimbine and phentolamine are much more potent than prazosin in antagonizing the adrenaline-induced aggregation (Hsu et al., 1979; Lasch & Jakobs, 1979).

Besides the vascular smooth muscle and the blood platelets, postsynaptic α_2 -adrenoceptors appear to occur in various other tissues, including, for example, the fat cells, frog skin melanocytes and the kidney, where they exert inhibitory effects on lipolysis, MSH-stimulated dispersion of melanocyte granules, and renin release respectively (Berthleson & Pettinger, 1977).

Clearly, alpha-adrenoceptors cannot be subclassified on the basis of physiological function or anatomical location. Therefore, it is now universally accepted that the terms ' α_1 ' and ' α_2 ' should still be used to differentiate the alpha-adrenoceptor sub-types, but that subclassification should be made with reference to pharmacological selectivity only (Starke & Langer, 1979). (Figure I.2. depicts the role and location of the alpha-adrenoceptors).

I.4. Approaches to characterising α_2 -adrenoceptors

The properties of a particular drug/hormone receptor can usually be described in two main aspects: the molecular interactions with drugs and the physiological responses elicited through the drug-receptor interactions. As with other receptor systems, both of these aspects have been the primary areas of study with the α_2 -adrenoceptors.

The characterisation of functional responses has, as described earlier, proved a major contribution to the original classification of adrenoceptors into the alpha and beta categories (Ahlquist, 1948), and also the subdivision of these receptors into the β_1 , β_2 , α_1 and α_2 sub-types (Lands et al., 1967 a,b; Starke et al., 1975; Wikberg, 1976; Doxey et al., 1977; Berthleson & Pettinger, 1977). The measurements of various pharmacological responses, such as changes in the extent of transmitter release (Starke et al., 1975), vasopressor responses (Drew & Whiting, 1979), sedative effect (Timmermans et al., 1981), vascular smooth muscle contraction (Docherty & Starke, 1981), induction of platelet aggregation (Hsu et al., 1979) etc. are still very useful for the characterisation of α_2 -adrenoceptors,

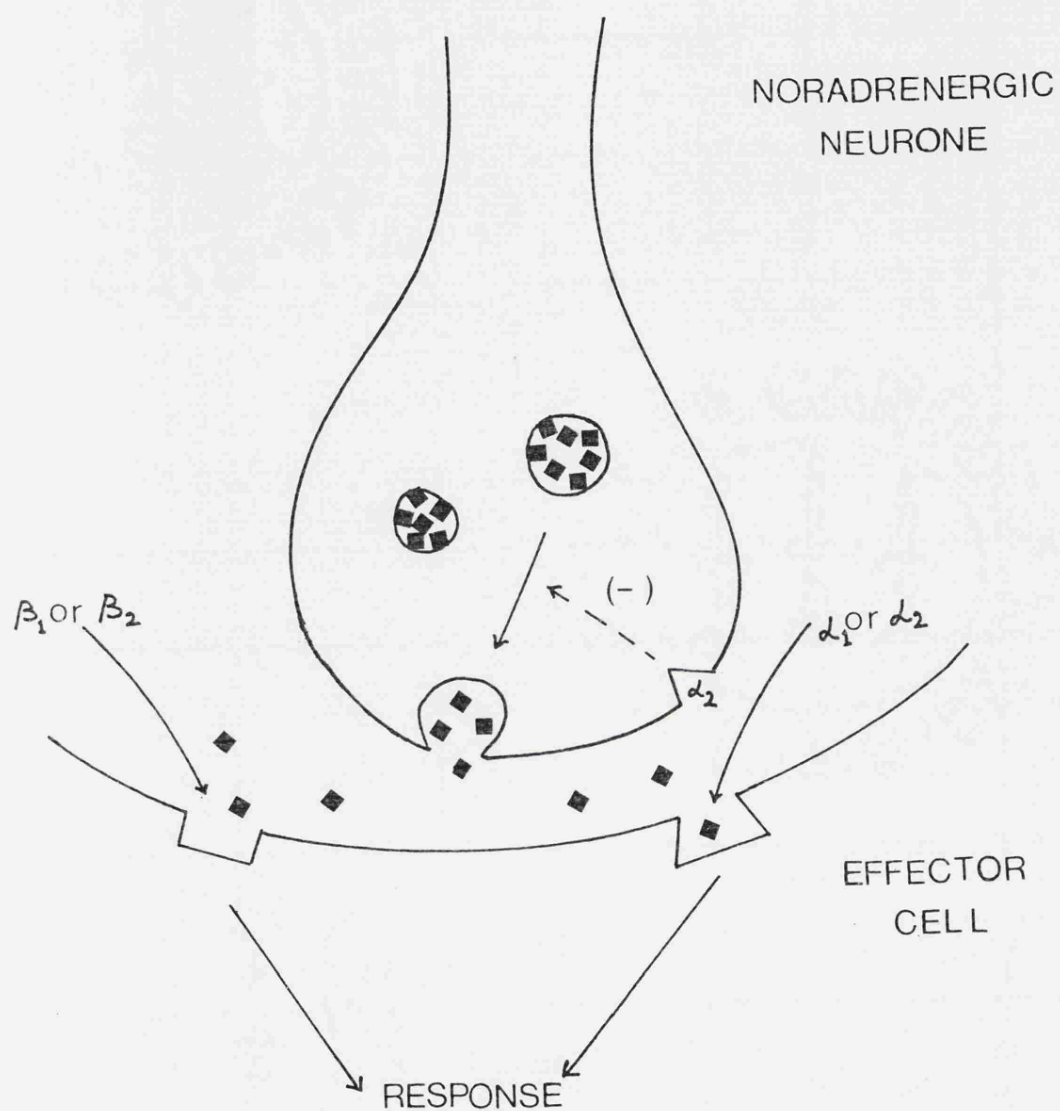


Fig 1.2. Location and role of adrenoceptors

although the use of radioligand binding assays has now become an additional approach.

Generally, the primary objective in the classification or characterisation of adrenoceptors is to determine their pharmacological specificities. In functional studies, this relies on two basic indices: relative agonist potencies, and relative antagonist affinities. To determine these indices it is necessary to develop appropriate experimental methods with which the affinities of drugs can be estimated. However, in usual practice, only the dissociation constants (which are proportional to the reciprocals of affinities) can be more directly determined. Determination of antagonist dissociation constants appears more straightforward, compared to the agonists. By definition, the dissociation constant of an antagonist is that concentration which causes a right-hand shift of the log dose-response curve of the agonist by two-fold. Usually, the antagonist dissociation constant is expressed as pA_2 value (negative logarithm of the dissociation constant). This can be conveniently determined from Schild plots (Schild, 1949) which relate the antagonist concentrations with the extent to which the agonist dose-response curve is displaced. However, in order to determine and compare antagonist affinities reliably, it is most important that experimental conditions are appropriately controlled and optimized (Furchgott, 1972). Another important consideration is that the experiment time should be long enough so that agonist-antagonist interactions at the receptor can reach an equilibrium state.

Compared to the antagonists, agonist affinities are technically even more difficult to determine directly. In many cases, only the EC_{50} values of the agonists are obtained, that is, concentrations which produce 50% of the maximal functional responses. Hence, agonist potencies, which are proportional to the reciprocals of the EC_{50} s, are determined. However, because different tissues may have different 'receptor reserve', and because

the response is not necessarily linearly proportional to the percentage of receptors occupied (see Starke, 1981; Ruffolo, 1982), EC₅₀ of an agonist can hardly ever be regarded as being equivalent to its dissociation constant. Relative potencies of agonists, therefore, are equivalent to their relative affinities only if the agonists are of equal efficacies (see Starke 1981; Morton & Halliday, 1981). Unfortunately, different agonists can have different intrinsic activities or efficacies within a tissue or between tissues (Stephenson 1956; Furchgott, 1966; Morton, & Halliday, 1981). Therefore when examining the rank order(s) or agonist potencies, the possible differences in efficacy between the drugs must be kept in mind.

There could be little doubt that the functional approach remains indispensable to studies of the adrenoceptors. However, the recent advances in radioligand binding assays have certainly provided an additional, simple approach to a better understanding of the molecular aspects of drug-receptor interactions, receptor regulation and receptor-effector coupling.

The main advantage of the radioligand binding approach, compared to the functional approach, is that, with a high-affinity and specific radioligand, the receptor under investigation can be identified and characterized even without the knowledge of its exact function and anatomical location. Direct equilibrium binding studies do not only provide information about the dissociation constant of the ligand, but also the density of the receptor. From the competition studies of radioligand binding to the receptor by various drug agents, the dissociation constants (K_i) of these drugs can be quite accurately estimated, assuming the drug-receptor interactions obey the law of mass action, and that the individual inhibitors are truly competitive (Williams & Lefkowitz, 1978; Cheng & Prusoff, 1973). The results of drug competition studies also generate a pharmacological profile which characterises the specificity of the receptor.

Until more recently, only radiolabelled agonist ligands, such as ³H-clonidine and ³H-adrenaline were available for direct labelling of the α_2 -adrenoceptors.

However, there are some problems regarding the use of agonist ligands. Compared to the antagonists, agonists interact with α_2 -adrenoceptors in heterogeneous affinity states, which can be modulated by various factors such as guanine nucleotides and mono- and divalent cations (Tsai & Lefkowitz, 1978; Glossmann & Presek, 1979; Tsai & Lefkowitz, 1979; also see Chapters II and VI). The ability of agonists to induce or stabilise high affinity states also implies that agonist ligands may only label a proportion of the guanine nucleotide-sensitive high affinity states of the receptor population (Hoffman et al., 1980b), thus providing quantitatively and qualitatively misleading information about the overall characteristics of the α -adrenoceptors. For these reasons, the use of antagonist radioligands appears to be more preferable and practical. Consequently, in the present studies, the characterisation of α_2 -adrenoceptors was carried out using the recently available α_2 -selective antagonist ligands, ^3H -yohimbine and ^3H -rauwolscine.

The development and progress of radioligand binding studies of the α -adrenoceptors, and in particular, the α_2 -adrenoceptors, will be discussed in further details in Chapter II.

Chapter II

MOLECULAR PHARMACOLOGY OF ALPHA₂-ADRENOCEPTOR

II.1. Radioligand binding studies of alpha-adrenoceptors: the general background

Until recently, classification of adrenoceptors relied exclusively on characterisation of physiological response to pharmacological agents. The success of this approach in turn depends on the development of appropriate experimental models which can identify and quantify specific receptor-mediated biological functions. Very often, it is also necessary to know, in advance, the anatomical locations or distributions of the receptors being investigated. Radioligand binding studies circumvent these problems, and provide a simple and direct method of characterizing and quantifying receptors.

Over the last decade or so, rapid progress has been made in the development of radioligand binding techniques for studying adrenoceptors. Naturally, the study of alpha-adrenoceptors has also received a great deal of impetus with the development and employment of this approach.

The alpha-adrenergic blocking properties of the ergot alkaloids were described by Dale as early as 1906. The incorporation of tritium into α -ergocryptine produces ^3H -dihydroergocryptine (^3H -DHE) which possesses high affinity and relatively higher specificity for alpha-adrenoceptors. In 1976, Lefkowitz and colleagues led the way to describe the characterization alpha-adrenoceptors in the uterus using ^3H -DHE. Since then, this ligand has been used quite extensively to study alpha-adrenoceptors in a variety of tissues, including the brain, liver, and platelets (see Table II). Being a non-selective alpha-antagonist, (Wood et.al., 1979; Lefkowitz & Hoffman, 1980_b) ^3H -DHE labels both the α_1 and α_2 subtypes of adrenoceptors. With the use of curve-fitting analyses on displacement of ^3H -DHE binding by selective agents such as prazosin (α_1) and yohimbine (α_2), proportions of alpha-receptor subtypes in a given tissue can also be estimated (Hoffman et. al., 1979). For instance, with this approach human platelets have been found to contain exclusively α_2 -adrenoceptors. In contrast, rat liver appears to contain alpha-receptors predominantly of the α_1 -subtype.

And, in the rabbit uterus, α_1 and α_2 -receptors appear to co-exist in the approximate ratio of two to three (Hoffman et. al., 1979). Before α_2 antagonist ligands were available, attempts were also made to investigate the functionally relevant modulation of agonist- α_2 -adrenoceptor interactions through examining the changes in characteristics of agonist displacement of ^3H -DHE binding (Tsai & Lefkowitz, 1978; Tsai & Lefkowitz, 1979; Hoffman et. al., 1980_a; Michel et. al., 1980; Giudicelli & Pecquery, 1981).

Despite its high affinity for the α -adrenoceptors, ^3H -DHE is in fact a rather difficult ligand to work with. It is poorly water-soluble, and has the tendency to adsorb onto various materials. Furthermore, it is a ligand not totally selective for the α -adrenoceptors. At higher concentrations, it can also label dopaminergic and serotonergic receptors (Caron et. al., 1978; Davis et. al., 1977). For these reasons, the applicability of ^3H -DHE for studying α -adrenoceptors, and particularly α_1 or α_2 -receptor subtype, is still rather limited.

While Lefkowitz and colleagues began using ^3H -DHE to study α -adrenoceptors in peripheral tissues, Snyder and co-workers also attempted to label α -adrenoceptors in the brain. Among the ligands used were the tritium-labelled partial agonist clonidine which has presynaptic α selectivity (Medgett et. al., 1978), and the antagonist WB4101, which appears to show in-vitro selectivity for the postsynaptic α receptors (Dubocovich, 1979; Langer et. al., 1980). However, in earlier studies, pre-treatment with 6-hydroxydopamine (which chemically destroys adrenergic neurons) did not produce any loss of the ^3H -clonidine and ^3H -WB4101 sites in the brain, suggesting that these sites might all be of a postsynaptic origin. Furthermore, agonists appeared to exhibit higher affinities for the ^3H -clonidine sites than the antagonists. Conversely, antagonists were more potent than the agonists at the ^3H -WB4101 sites. These results therefore led to the

hypothesis that the two ligands both labelled the same central postsynaptic alpha-adrenoceptor, existing in two distinct, non-interconvertible agonist and antagonist conformational states which show preference for agonist and antagonist agents respectively. However, in subsequent studies in which ^3H -clonidine and ^3H -WB4101 were used to label alpha-adrenoceptors in peripheral tissues, affinities of subtype selective agents at either receptor binding site were found to be in good correlation with those determined in the pre- or postsynaptic functional studies (U'Prichard & Snyder, 1979). Therefore, the indications are that ^3H -clonidine and ^3H -WB4101 might in fact label central α_2 and α_1 receptors respectively.

At the start, these two ligands looked promising as practical tools for selective labelling of the α_1 and α_2 -adrenoceptor subtypes. However, although WB4101 is relatively α_1 -selective in the brain, its selectivity is quite variable in the peripheral tissues. Indeed, the non-discriminatory use of ^3H -WB4101 for labelling α_1 -adrenoceptors has been cautioned by Hoffman & Lefkowitz (1980). Fortunately, prazosin, an antagonist with higher α_1 -selectivity, soon became available as a tritiated ligand (Greengrass & Bremner, 1979). Since then, ^3H -prazosin has proved its usefulness as the ligand of choice for studying α_1 -adrenoceptors in various tissues. In binding studies of α_2 -adrenoceptors, ^3H -clonidine has still remained to be one of the most widely used ligands. Other agonist ligands such as ^3H -adrenaline or ^3H -noradrenaline have also been used (see Table II). However, as mentioned before, there are disadvantages associated with the use of agonist ligands in general. Compared with the antagonist ligands, agonist binding to the α_2 -receptors can be significantly influenced by various factors, including the ions (Tsai & Lefkowitz, 1978; Glossmann & Presek, 1979; Rouot et. al., 1980; U'Prichard & Snyder, 1980), guanine nucleotides (Tsai & Lefkowitz, 1979; Rouot et. al., 1980; U'prichard & Snyder, 1980; Michel et. al., 1980), and even temperature (Barnett et. al., 1982). In addition, agonists, though not antagonists, can

interact with α_2 -receptors to form the guanine nucleotide-sensitive high-affinity states (Michel et. al., 1980; also see next Section and Chapter VI). Therefore, at lower concentrations, radiolabelled agonist ligands could preferentially label only the high-affinity sites of the α_2 -receptors (Hoffman et. al., 1980_b; Lefkowitz & Hoffman, 1980_b), thereby providing qualitatively and quantitatively misleading information which does not truly describe the overall characteristics of the α_2 -receptor population. This is why the antagonists appear to be more suitable ligands than the agonists as the α_2 -adrenoceptor probes.

Actually, besides ^3H -DHE, one other non-selective α antagonist, phentolamine, has also been tritium labelled, and used to study α_2 -adrenoceptors in tissues such as platelets and retina (Steer et. al., 1979; Bittiger et. al., 1980). However, despite of its relatively high affinity, this ligand has never been widely utilized, probably because subtype selective ligands, such as ^3H -clonidine and ^3H -prazosin have already been available.

The most suitable ligands for selective labelling of α_2 -adrenoceptors would undoubtedly be the α_2 -selective antagonists. The first ligand of this kind is ^3H -yohimbine which became available toward late 1970s. Since its first applications for studying α_2 -adrenoceptors on human platelets and rat brain (Motulsky et. al., 1980; Yamada et. al., 1980) this ligand has now become the ligand of choice for probing α_2 -adrenoceptors in the various tissues. Shortly after ^3H -yohimbine was introduced, its α_2 -selective diastereoisomer rauwolscine has also become available as a tritiated ligand. In the present studies, both ligands have been used to characterize α_2 -adrenoceptors. It will be demonstrated that both ligands label quantitatively and qualitatively identical sites of α_2 -receptor characteristics. However, ^3H -rauwolscine appears to be superior to ^3H -yohimbine, as it possesses a higher α_2 -selectivity, and also a consistently higher affinity for the α_2 -adrenoceptor sites in most tissues. As will be discussed

Table II Literature in earlier radioligand binding studies of alpha-adrenoceptors

Ligand	Tissue Source	Receptor type	References
<u>Agonists</u>			
³ H-Adrenaline	Calf brain membranes	alpha-noradrenergic 'agonist state'	U'Prichard & Snyder, 1977; U'Prichard & Snyder, 1978; Greenberg et. al., 1978
		alpha ₂	U'Prichard & Snyder, 1980
³ H-Noradrenaline	Rat liver membranes	alpha ₂	Hoffman et. al., 1980 _a
	Human platelet membranes	alpha ₂	Hoffman et. al., 1980 _a
	Calf brain membranes	alpha-noradrenergic 'agonist state'	U'Prichard & Snyder, 1977
	Rat liver membranes	alpha ₁ 'active' sites	Geynet et. al., (1980)
³ H-Clonidine	Calf brain membranes	alpha-noradrenergic 'agonist state'	Greenberg et. al., 1978;
		alpha ₂	U'Prichard & Snyder, 1979; U'Prichard & Snyder, 1980
	Rat brain membranes	alpha-noradrenergic 'agonist state'	Greenberg et. al., 1976; Greenberg & Snyder, 1978
		alpha ₂	Rouot et. al., 1980; Tanaka & Starke, 1980
		alpha ₂	Glossman et. al., 1980

CONTD.

Ligand	Tissue Source	Receptor Type	References
³ H-Clonidine contd	Rabbit duodenum, rat spleen, salivary gland membranes	alpha ₂	U'Prichard & Snyder, 1979
	Bovine retina membranes	alpha ₂	Bittiger et. al., 1980
	Guinea-Pig Ileum membranes	alpha ₂	Tanaka & Starke, 1979
	Guinea-Pig kidney membranes	alpha ₂	Summers, 1980
³ H-Para-Amino Clonidine	Rat brain membranes	alpha ₂	Rouot & Snyder, 1979
<u>Antagonists</u>			
³ H-DHE	Rabbit uterine membranes	alpha(1/2)	Williams & Lefkowitz, 1976, 77; Williams et. al., 1976; Hoffman et. al., 1979.
	Rat liver membranes	alpha (1/2)	Guellaen et. al., 1978; Clarke et. al., 1978; Hoffman et. al., 1979
	Human platelet membranes	alpha (1/2)	Kafka et. al., 1977; Alexander et. al., 1978; Newman et. al., 1978; Cooper et. al., 1978; Hoffman et. al., 1979; Tsai & Lefkowitz, 1979; Hoffman et. al., 1980 _a

CONTD

Ligand	Tissue Source	Receptor type	References
³ H-DHE contd	Rabbit platelet membranes	alpha (1/2)	Tsai & Lefkowitz, 1978
	Rat brain membranes	alpha-noradrenergic 'antagonist state'	Greenberg & Snyder, 1977; Greenberg & Snyder, 1978
		alpha (1/2)	Miach et. al., 1978
	Calf brain membranes	alpha-adrenergic	Titeler & Seeman, 1978
³ H-phentolamine	Human platelet membranes	alpha (1/2)	Steer et. al., 1979
	Bovine retina membranes	alpha (1/2)	Bittiger et. al., 1980
³ H-WB4101	Rat brain membranes	alpha-noradrenergic 'antagonist state'	Greenberg et. al., 1976; Greenberg & Snyder 1978
		alpha ₁	Tanaka & Starke, 1980; U'Prichard & Snyder 1979
	Calf brain membranes	alpha-noradrenergic 'antagonist state'	U'Prichard & Snyder, 1978
		alpha ₁	U'Prichard & Snyder 1979; Hoffman & Lefkowitz, 1980

CONTD.

CONTD.

Ligand	Tissue Source	Receptor type	References
³ H-WB4101 contd	Rabbit uterine membranes	alpha (1/2)	Hoffman & Lefkowitz, 1980
	Rat heart membranes	alpha ₁	Raisman et. al., 1979
	Rat heart, vas deferens, salivary gland & spleen membranes	alpha ₁	U'Prichard & Snyder, 1979
³ H-prazosin	Rat brain membranes	alpha ₁	Greengrass & Bremner, 1979; Miach et. al., 1980
³ H-yohimbine	Rat brain and kidney membrane	alpha ₂	Yamada et. al., 1980
	Human platelet whole cells and membranes	alpha ₂	Motulsky et. al., 1980

later, the relative differences in affinity between the two diastereoisomers between tissues may in part reflect structural differences among the α_2 -adrenoceptors themselves.

II.2. Coupling mechanisms of α_2 -adrenoceptors to effector systems

It is now well established that agonist activation of beta-adrenoceptors is coupled positively to adenylate cyclase, and that the increases in cyclic AMP level mediate the adrenergic effects at these receptors (Robison et. al., 1971; Lefkowitz et. al., 1976). In contrast, substantial evidence is currently available to indicate that the α_2 -adrenoceptors are negatively coupled to adenylate cyclase. α_2 -adrenergic inhibition of adenylate cyclase has been reported in a variety of tissues, including the platelets (Jakobs et. al., 1976; Steer & Wood, 1979; Tsai & Lefkowitz, 1979), adipocytes (Garcia-Sainz et. al., 1980; Aktories et. al., 1979), neuroblastoma x glioma cells (Sabol & Nirenberg, 1979 a, b), and the kidney (Woodcock et. al., 1980).

Over the recent years, considerably progress has been made toward gaining a better understanding of the mechanism by which agonist occupancy of adrenoceptors is coupled to adenylate cyclase. For both the α_2 and beta-adrenoceptors, agonist activated modulation of cyclase activity requires guanine nucleotides, such as GTP (Ross et. al., 1977; Jakobs et. al., 1978). The regulatory effect of GTP on the adenylate cyclase is mediated through a guanine nucleotide binding protein (also called G, N or G/F protein) which serves to transfer messages across the membrane from the receptor to the effector (Rodbell, 1980; Limbird, 1981; Spiegel & Downs, 1981). The formation of a ternary complex composed of the agonist, receptor site and the N-protein (H-R-N), appears to be pre-requisite to the modulation of adenylate cyclase (DeLean et. al., 1980). Evidence for the formation of such a ternary complex has come from the demonstrations that there is an increase in the molecular size of the solubilized beta or α_2 -adrenoceptor following agonist, but not antagonist occupancy

(Limbird et. al., 1980; Michel et. al., 1981). The binding of an agonist to the receptor also facilitates the exchange of GTP for GDP on the N-protein. GTP destabilizes the ternary complex, resulting in the dissociation of the N-protein from the receptor, which now has a low affinity for the agonist. The N-protein - GTP complex then acts on the catalytic unit of adenylate cyclase, and, in the case of the beta adrenoceptors, produces a stimulation of the enzyme activity. The stimulatory action on the cyclase is presumably turned off by means of a GTPase on the N-protein, with GTP being hydrolysed to form GDP. Thus, non-hydrolysable analogues of GTP, such as Gpp(NH)p, produce irreversible stimulation of adenylate cyclase (cuatrecasas et. al., 1975; Lefkowitz & Caron, 1975; Steer & Wood, 1979).

Whether a common N-protein, or separate N-proteins are involved in the adrenoceptor-mediated stimulation and inhibition of adenylate cyclase has always been an important question to be answered. From recent studies on the human platelets which contain different receptors coupled both positively and negatively to adenylate cyclase, there is now strong evidence that different N-proteins, N_s and N_i , are involved respectively in enzyme stimulation and inhibition. For instance, Steer and Wood (1979) noticed that while low concentrations of GTP enhanced prostaglandin E_1 , (PGE_1) stimulation of adenylate cyclase, much higher concentrations of GTP were needed to support α_2 -adrenoceptor-mediated enzyme inhibition activated by adrenaline. Hoffman et al. (1981_a) discovered that Mn^{++} ion had differential effects on adenylate cyclase stimulation and inhibition. Lower concentrations of Mn^{++} (0.4 - 1.6mM) preferentially 'uncoupled' the α_2 -receptor mediated inhibition of adenylate cyclase without affecting the characteristic agonist-receptor interaction as regulated by guanine nucleotides (see below), or altering the Gpp(NH)p and PGE_1 -stimulated cyclase activity. At much higher concentrations of Mn^{++} , stimulation of adenylate cyclase was also

uncoupled. Smith and Limbird (1982) have looked at the effects of cholera toxin on the stimulation of adenylate cyclase in platelet membranes, and on the agonist- α_2 receptor interactions and inhibition of cyclase. Cholera toxin was shown to stimulate the ADP-ribosylation of the stimulatory N-protein in platelets, which was paralleled by the increase in GTP-sensitive adenylate cyclase activity. However, cholera-toxin treatment neither modified the α_2 -receptor mediated inhibition of cyclase activity by adrenaline, nor the guanine nucleotide-sensitive agonist-receptor interaction. There was also no association of the ADP-ribosylated subunit of Ns with the agonist- α_2 receptor complex. More recently, Jakobs and colleagues (1983a) have further shown that preactivated Ns-protein produced stimulation of adenylate cyclase in platelet membranes and was associated with a marked increase in the enzymes affinity for Mg^{++} although there was no change in affinity for the substrate Mg^{++} -ATP. In contrast, Gpp (NH)p and adrenaline produced non-competitive inhibition of the Ns - stimulated adenylate cyclase activity, accompanied by a significant reduction of the enzyme's affinity for Mg^{++} . Therefore, such observations do not only suggest that stimulation and inhibition of adenylate cyclase are mediated through functionally distinct N-proteins, but that their differential effects on the enzymes affinity for Mg^{++} may represent the molecular mechanisms by which the activity of adenylate cyclase is regulated.

The modulatory effects of guanine nucleotides on adenylate cyclase are also reflected in the changes of the agonist-receptor interactions. Radio-labelled antagonist binding to beta or α_2 -adrenoceptors is displaced by agonists in a manner different from that by antagonists, as manifested by the difference in shape and extent of 'shift' of the displacement curves in the absence and presence of guanine nucleotides. In the absence of guanine nucleotides, agonist displacement curves are shallow, compared to the antagonist. Statistical analysis of such curves would indicate that agonists, but not antagonists interact with beta or α_2 -receptors in distinct high and low-affinity states (Kent et. al., 1980; Michel et. al.,

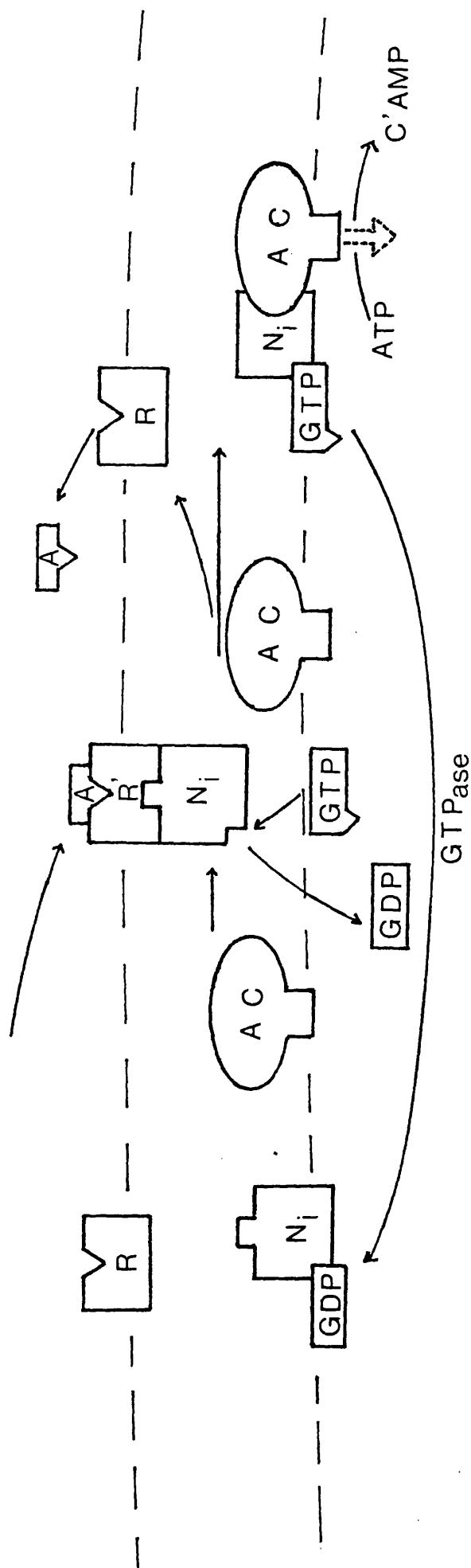


Fig. II. Postulated coupling mechanism of α_2 -receptor activation to adenylyl cyclase inhibition in membranes.

The binding of an agonist to α_2 -receptor (R) causes an initial conformational change in the receptor molecule (R'), allowing the binding of the agonist- R' complex ($A-R'$) to the inhibitory guanine nucleotide protein (N_i) to form a ternary complex ($A-R'-N_i$). The formation of this complex also simultaneously facilitates the exchange of GTP for GDP on N_i . This causes the dissociation of N_i from $A-R'$. The GTP- N_i complex then binds to and inhibits adenylyl cyclase (AC). The dissociation of N_i from $A-R'$ also converts R' back to R. The inhibition process is believed to be 'turned off' as a result of the hydrolysis of GTP to GDP by a GTPase.

1980). the high-affinity state corresponds to the ternary (H-R-N) complex described earlier. In the presence of high concentrations of guanine nucleotides, agonist displacement curves are shifted to the right, accompanied by steepening of the curves toward a slope of unity. Hence, agonists now interact with the receptor in a homogeneous low-affinity state, apparently as the result of the dissociation of the N-protein (Ns or Ni) from the ternary complex. The extents to which displacement curves of drugs can be shifted by guanine nucleotides appear to correlate with their intrinsic activities on the stimulation or inhibition of adenylate cyclase, and may reflect the differences in efficiency of receptor-effector coupling (Kent et. al., 1980; Tsai & Lefkowitz, 1979) (also see Chapter VI).

Besides guanine nucleotides, monovalent cations, such as Na^+ , also markedly reduce the affinities of agonists at α_2 -adrenoceptors (Tsai & Lefkowitz, 1978). The combined effect of the two modulators on agonist affinity is either additive or synergistic (Woodcock & Marley, 1981, Motulsky et. al., 1980; also see Chapters VI and VII). However, it has been further noticed that there seems to be difference between α_2 adrenoceptors (eg those in platelets vs. those in rat brain or kidney) with respect to the requirement for Na^+ in full expression of the regulatory effect of guanine nucleotides on agonist-receptor interaction. Whether or not such difference may imply difference in the molecular coupling mechanism will be discussed further Chapter VI.

Although inhibition of adenylate cyclase is believed to be the biochemical mechanism for many α_2 -receptor mediated effects, increase in cytosolic Ca^{++} appears to play a major mechanistic role in mediating some other α_2 -adrenergic actions, such as vascular smooth muscle contraction (Van Meel et. al., 1981; Godfraind et. al., 1982; Langer & Shepperson, 1982). In this respect α_2 -receptors resemble α_1 -receptors whose actions are generally associated with Ca^{++} influx and/or intracellular Ca^{++} mobilization (Exton, 1981; Morton & Halliday, 1981; Langer & Shepperson, 1982).

Since there is a good relationship between α_1 -receptor activation and the hydrolysis of membrane phosphatidylinositol (PI) and/or polyphosphoinositides (PPI), it has been suggested that the breakdown of PI and/or PPI may be directly involved in the Ca^{++} gating mechanism. (Michell, 1975; Michell, 1981; Fain & Garcia-Sainz, 1980). Recent evidence has further implicated that the metabolites of polyphosphoinositides - the soluble inositol phosphates (IP) may act as second messenger mediating intracellular Ca^{++} release (Burgess et. al., 1984). Whether or not PI and/or PPI breakdown represents the primary mechanism for increased cytosolic Ca^{++} upon α_1 and/or α_2 receptor activation remains to be clarified. Nevertheless, the similar functional significance of cytosolic Ca^{++} in mediating α_1 and α_2 adrenergic actions suggests that other than the inhibition of adenylate cyclase, facilitation of Ca^{++} fluxes may also be a mechanism coupled to some α_2 -receptor activation. After all, it is interesting to note that, although catecholamine-induced platelet aggregation is believed to be mediated through α_2 -receptors coupled to inhibition of adenylate cyclase, there is recent evidence suggesting that the catecholamine aggregatory action is also associated with an increase in Ca^{++} input which does not occur with other aggregatory agents, such as ADP (Owen et. al., 1980). Furthermore, the divalent cation ionophores, such as A-23187 was also shown to induce aggregatory response to catecholamines and imidazolines, an effect apparently mediated through the α_2 -adrenoceptors (Grant & Scrutton, 1980). Hence, in this system, α_2 -receptors may be coupled to both inhibition of adenylate cyclase and Ca^{++} influx. Full α_2 -receptor mediated aggregatory response may therefore involve the interaction between the two mechanisms. It is even tempting to believe that such possible interactive mechanisms may be involved in some other α_2 -adrenergic actions as well.

II.3. Study Objectives

This work will examine the molecular pharmacological properties of α_2 -adrenoceptors using radioligand binding techniques. There are four main objectives:

- 1 Initial studies will involve the characterisation of α_2 -adrenoceptors on human platelets using the highly selective antagonist ligands, ^3H -yohimbine and ^3H -rauwolscine. Platelets are a particularly useful system to study α_2 -receptors, as they can be conveniently obtained from human blood to yield relatively homogeneous preparations of intact cells and membranes.
- 2 The modulation of agonist and antagonist interactions with α_2 adrenoceptors by various factors (eg guanine nucleotides, temperature, cations) will be examined. The human platelet α_2 -adrenoceptor will serve as the basic study model.
- 3 Studies on the platelet α_2 -adrenoceptor will be extended to include α_2 -adrenoceptors in other tissues or species. The pharmacological properties of α_2 -adrenoceptors will be compared.
- 4 Attempts will also be made to solubilize α_2 -adrenoceptors from tissue membranes, and the possible differences in pharmacological properties of soluble and/or particulate α_2 -adrenoceptors will be evaluated.

Chapter III

METHODS

III.I. Tissue and membrane preparation

III.I.1 Preparation of platelet whole cells

Human whole blood was obtained from hospital source or from male volunteers. Blood was generally collected in Tri-Sodium Citrate 3-3.2% in a 9: 1 (v/v) ratio. Platelet-rich plasma (PRP) was separated from red and white cells by centrifugation at low speed ($270 \times g$) for 15 minutes at 10°C , and carefully harvested by transfer pipets. Subsequently, intact platelets were sedimented by centrifuging PRP at $27000 \times g$ for 10 minutes at 4°C . The supernatant, which is the platelet-poor plasma (PPP) was decanted, leaving behind the platelet pellet. In binding experiments with intact platelets, the platelet pellets were resuspended in isotonic buffer (50 mM Tris-HCl 20 mM EDTA, 150 mM NaCl pH 7.5) by gently smearing against the wall of the tubes with a glass-rod. This was followed by centrifugation at 4°C for 10 minutes. The platelet pellets were washed two more times by resuspension and centrifugation. At the end of the final wash, the platelets were resuspended in the same isotonic buffer at a suitable volume, and used for assays within the same day of preparation.

Provided the above procedures are carefully followed, and particularly if PRP is harvested with great care, high yields of sufficiently pure platelets can be obtained. Usual total platelet yields were >88-90% with negligible red and white cell contamination.

Storage of intact platelets at 4°C for 24 hours resulted in a loss of about 15% of binding sites, but 40-50% of sites were lost after a storage time of 72 hours.

III.I.2 Preparation of platelet membranes

- Intact platelets were isolated from PRP as described in section

III.I.1 The platelet pellet was resuspended in ice-cold hypotonic buffer (5 mM Tris- HCl 5 mM EDTA) and left for 1-2 minutes before being homogenized for 10 strokes with a motor-driven glass-teflon homogenizer. The suspension

was then centrifuged at 27000 x g for 10 minutes at 4°C. The pellet was washed once with the hypotonic buffer by gentle resuspension and then centrifugation at 27000 x g for 10 minutes at 4°C. A final washing was carried out by resuspension of the pellet in ice-cold assay buffer (50 mM Tris HCl, 0.5 mM EDTA, 0.1% ascorbate, pH 7.5), followed by centrifugation at 27000 x g for 10 minutes at 4°C. The final pellet was then resuspended in appropriate volume of assay buffer for binding assays. Membrane preparations could be stored frozen at -40 or -70°C for prolonged period of time (6 months or more) without significant loss of binding or change in overall pharmacological properties. However, after membranes had been frozen and thawed, characteristics of agonist- α_2 -receptor interactions appeared to be greatly altered (see Chapter VI).

III.1.3 Preparation of rat cerebral cortical membranes

Male Wistar/Sprague Dawley rats were killed by decapitation. Brains were removed. Cerebral cortex was dissected at 4°C and homogenized in 20 vol of ice-cold 5 mM Tris-HCl, 5 mM EDTA. Suspension was then centrifuged at 27000 x g for 10 minutes at 4°C. The resulting pellet was washed once more in the same buffer by homogenization followed by centrifugation at 27000 x g for 10 minutes at 4°C. One final washing was carried out by resuspension of the pellet in ice-cold 'assay buffer' (50 mM Tris-HCl, 0.5 mM EDTA, 0.1% ascorbate, pH 7.5) and then centrifuging at 27000 x g for 10 minutes at 4°C. The final pellet was resuspended in appropriate volume of assay buffer before being used in binding assays.

Extensive washing of membranes with the above hypotonic buffer containing EDTA was found to be essential in order to obtain more consistent results between preparations. There may be at least two good reasons for that. Firstly, the presence of endogenous divalent cations, which can be chelated by EDTA, could potentially reduce the affinity of ^3H -yohimbine or ^3H -rauwolscine as will be discussed in Chapter VI. Secondly, the use

of hypotonic buffer, rather than hypertonic sucrose buffer, could minimise the formation of synaptosomes when tissue is homogenized and washed. As will be shown in Chapter VII, cerebral membranes prepared in sucrose buffer contained a significantly high content of retained endogenous noradrenaline, the presence of which could produce pseudo non-competitive inhibition of ^3H -yohimbine (and probably also ^3H -rauwolscine) binding. Furthermore, the presence of residual divalent cations might also enhance the interaction of agonists with α_2 -adrenoceptors and potentiate the interference with ^3H -antagonist binding.

III.1.4 Preparation of rabbit cerebral cortical membranes

Male New Zealand white rabbits (2-3 kg) were killed by cervical dislocation. Cerebral cortex was dissected and chopped into smaller pieces before being homogenized in 20 vol. of ice-cold 5 mM Tris-HCl 5 mM EDTA buffer with an Ultra-Turrax homogenizer for 2 x 10 s. bursts. The suspension was then centrifuged at 27000 g for 10 minutes at 4°C. The pellet was washed and treated in the same manner as described in section III.1.3. The final pellet was resuspended in assay buffer.

III.1.5 Preparation of rat/rabbit kidney membranes

Rats and rabbits were killed as described in sections III.1.3. and III.1.4. Kidneys were removed bilaterally. Perirenal fat was removed and renal cortex was dissected. Tissue was cut into small pieces and homogenized in 20 vol. of ice-cold 5 mM Tris-HCl 5 mM EDTA buffer with an Ultra-Turrax homogenizer until a homogeneous suspension was obtained. The suspension was filtered through double-layer cheese cloth. The filtrate was centrifuged at 27000 x g for 10 minutes at 4°C. The resulting pellet was washed once more in the same buffer by homogenization, followed by centrifugation at 27000 x g at 4°C. One final washing was carried out in assay buffer (50 mM Tris-HCl, 0.5 mM EDTA, 0.1% ascorbate, pH 7.5). The resulting pellet was resuspended in appropriate volume of

assay buffer and used for assays.

III.1.6 Preparation of human cerebral cortical membranes

Human brain was removed and chilled as soon as possible post mortem. Cerebral cortex was dissected from the anterior portion of the temporal lobe within 24 hours and stored at -70°C prior to the membrane preparation procedures which took place within the same day of binding assays.

Cerebral cortical membranes were prepared in a way similar to that described for the rat and rabbit cerebral membranes.

III.2. Radioligand binding assays

III.2.1 General assay conditions and procedures

Radioligand binding assays were performed in a total assay volume of 250 microliters (μL) in 50 mM Tris-HCl, 0.5 mM EDTA, 0.1% ascorbate, pH 7.5, consisting of 50 μL radiolabel (^3H -yohimbine, ^3H -rauwolscine or ^3H -prazosin), 100 μL membranes, and either 100 μL buffer or 50 μL buffer plus 50 μL displacing drug.

Incubations were performed at room temperature ($22-25^{\circ}\text{C}$) or 37°C as appropriate. Upon reaching equilibrium (except for kinetics experiment) reaction was stopped by addition of 1.5 ml ice-cold assay buffer. Membranes were immediately collected on Whatman GF/B filters by filtration under vacuum and rapidly washed with 3 x 5 ml ice-cold buffer. The filters were then counted for radioactivity in FisoFluor 1 scintillation fluid (4-5 ml) using a LKB-Wallac Scintillation Counter at an efficiency of about 40%.

III.2.1.1 Special Considerations

A. Assay Buffer: There do not appear to be any strict criteria that must be met regarding the choice of assay buffers. The main aim is to adopt the best incubating conditions that optimise the specific radioligand

binding. Ideally, incubation should be carried out under more physiological conditions. However, the presence of metal ions was avoided in view of the potential interference of ^3H -yohimbine or ^3H -rauwolscine binding to α_2 -adrenoceptors by these ions (see chapter VI). Some workers suggested that Na-K phosphate buffer is more preferable to Tris-HCl buffer for the binding of ^3H -yohimbine or ^3H -rauwolscine to certain tissues (U'Prichard et al., 1983). However, in our hands, there appeared to be little difference between the two buffers with respect to the binding of these radioligands. For example, in parallel saturation experiments, ^3H -rauwolscine exhibited a K_d of 2.4 nM in Tris HCl buffer, compared to 2.7 nM in Na-K phosphate buffer; there was no difference in the B_{max} .

B. Assay pH: The assay pH was chosen to be as close to the physiological pH (7.4) as possible, although the binding of ^3H -yohimbine or ^3H -rauwolscine actually appeared to be higher around pH 7.0-7.2. However, there was not much difference in binding between pH 7.4-7.5.

C. Separation of free and bound radioligand: A number of methods are available for the separation procedure. These include equilibrium dialysis, centrifugation, and filtration. Separation by dialysis is often too time-consuming, whereas the centrifugation method may not sufficiently separate the bound ligand from the free, often resulting in high non-specific binding relative to specific binding. So far, millipore filtration has appeared to be the most efficient method. Rapid filtration under vacuum provides a rapid separation of bound ligand from the free, which is particularly essential when dealing with ligands with fast dissociation rate (e.g. binding of ^3H -yohimbine to rat kidney membranes). At suction pressure of 18-20 psi, separation was accomplished almost instantaneously.

D. Choice of membrane filters: The kind of filters chosen largely depends on the properties of the radioligand. Most commonly, Whatman GF/B or Whatman GF/C filters are used in assays with particulate receptors.

In dealing with ligands which tend to adsorb to various materials, such as ^3H -dihydroergocryptine, the use of thinner filters, like the Whatman GF/C, could reduce the high non-specific binding. In assays with ^3H -yohimbine or ^3H -rauwolscine, there appeared to be little difference between GF/B and GF/C filters with respect to the degree of non-specific binding. GF/B filters are less fragile and therefore practically easier to handle under strong suction pressure. Using double filters might theoretically enhance retention of membranes, but appeared to produce higher non-specific binding.

E. Washing of filters: Immediately after filtration, filters were quickly washed with cold buffer. This treatment could reduce non-specific ligand binding by removing free ligand trapped in the filter matrix. However, excessive washing of filters might wash off low-affinity specific binding (Boeynaems & Dumont, 1975). Moreover, non-specific binding might also be washed off with excessive washing, thus producing artefacts mistaken to be specific receptor binding. Generally, 2-3 times rapid washing with 5 ml buffer, which could be completed within 10 sec., was found to sufficiently minimize the non-specific binding without significant reduction of specific binding. Ice-cold buffer was also preferred for the washing, so that ligand dissociation could be minimized.

III.2.1.2 Determination of specific binding

Specific radioligand binding was defined as that binding displaceable by 5 μM Phentolamine. In other words, it was determined from the difference of total ligand binding in the absence and presence of 5 μM phentolamine. The choice of using this concentration of phentolamine to determine non-specific binding (NSB) was proved appropriate from the indication that maximal displacement of radioligand binding could be reached at micromolar concentrations of phentolamine, as should generally be expected of most selective antagonists acting at specific receptor sites (see Laduron, 1983; also see fig. III.1.).

Legends to figures III.1, 2 and 3

Figure III.1 Displacement of total ^3H -yohimbine binding by phentolamine and noradrenaline in human platelet membranes.

Figure III.2 Displacement of total ^3H -yohimbine and ^3H -rauwolscine binding to rat cerebral cortical membranes by phentolamine.

Figure III.3 Displacement of total ^3H -rauwolscine binding to rat renal cortical membranes by phentolamine.

The concentrations of phentolamine and noradrenaline required to define non-specific binding (NSB) for ^3H -rauwolscine or ^3H -yohimbine were evaluated by determining total equilibrium binding of radiolabel in the absence and presence of increasing concentrations of displacer. Binding decreased with increasing concentrations of displacer until all specific binding had been displaced. The residual binding represents NSB.

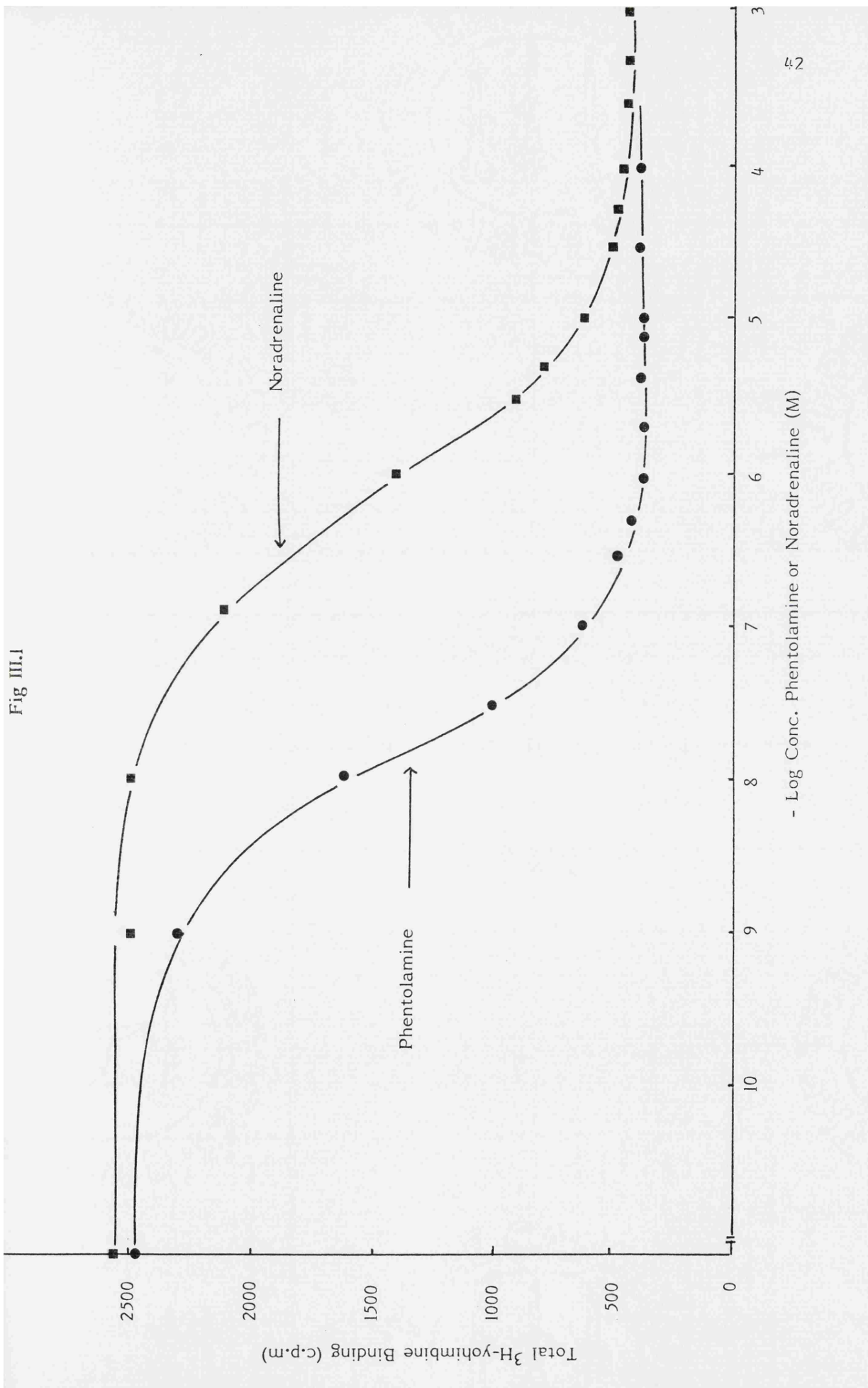


Fig III.2

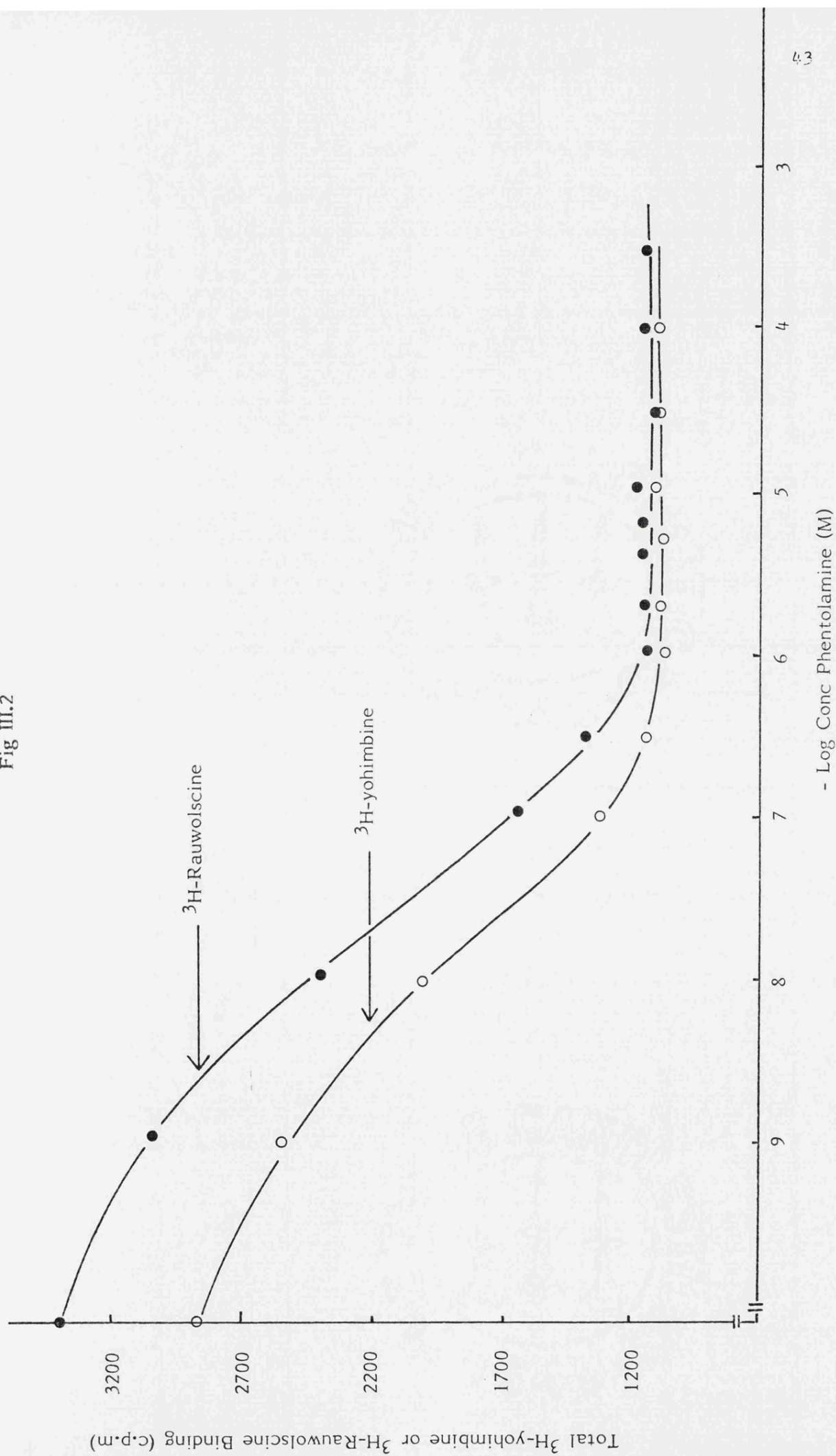
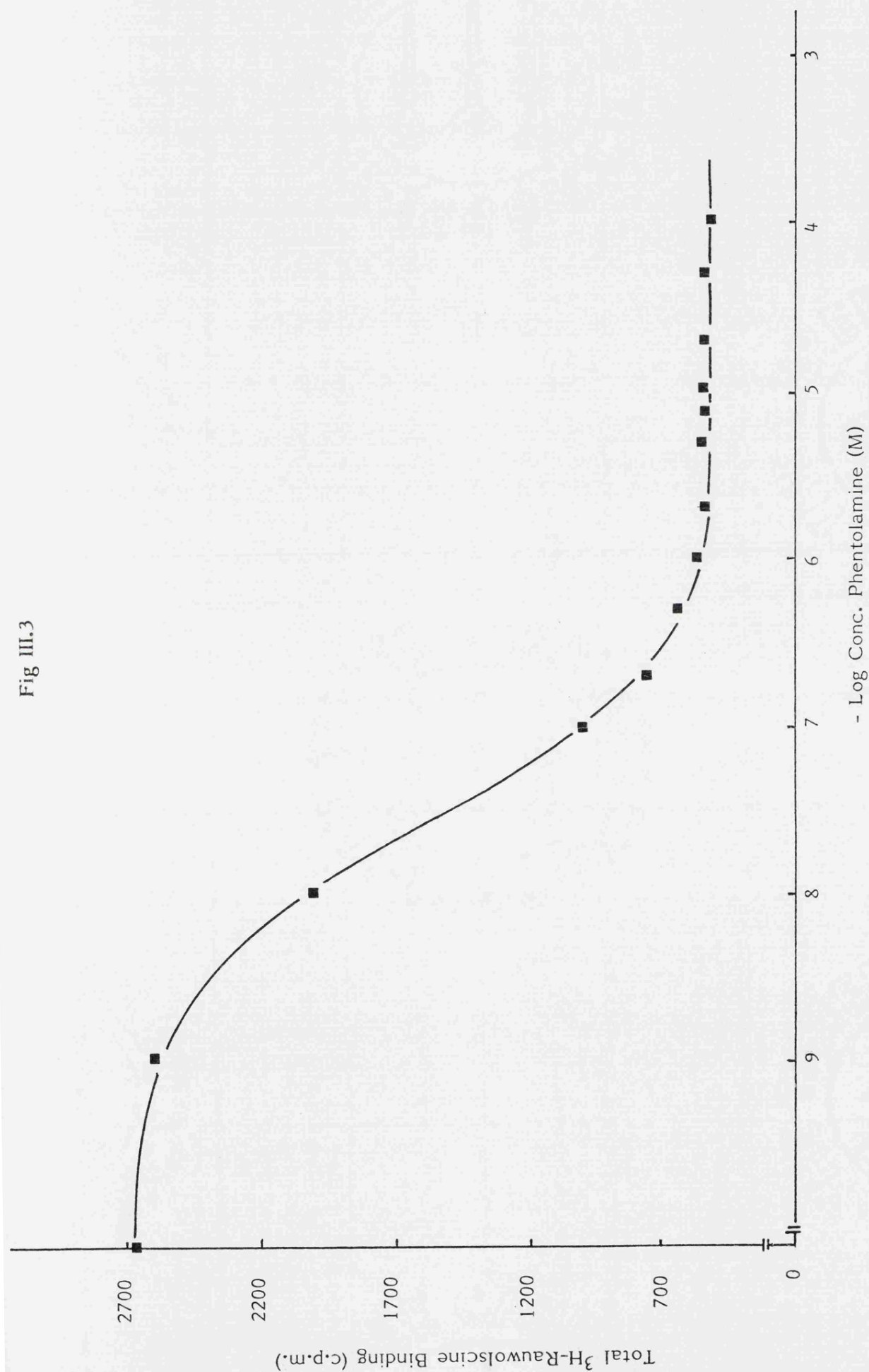


Fig III.3



III.2., and III.3.). Excessive displacer was not used, in order to avoid potential displacement of radioligand binding from non-specific membrane components (see Richardson & Nahorski, 1979; Richardson, 1979). In these studies, 1 mM noradrenaline appeared as effective as 5 μ M phentolamine to determine NSB. At 2-4 nM ligand concentration, specific binding ranged from about 45% (in rabbit kidney membranes) to about 90% (in human platelet membranes) of total binding. Specific binding activity for ^3H -yohimbine was significantly lower than ^3H -rauwolscine in tissues such as rat brain and kidney membranes, and rabbit kidney membranes.

III.2.2. Specific experimental procedures

III.2.2.1 Saturation experiments

These experiments were performed by incubating in groups of tubes an equal quantity of tissue membranes (under identical assay conditions) with increasing concentrations of radioligand. The incubations were carried out both in the absence and presence of NSB determinant at each radioligand concentration to determine specific binding. Maximal concentrations of ^3H -yohimbine or ^3H -rauwolscine were usually between 15-25 nM. Protein concentrations were in the range of 2-400 μg /tube for platelet membranes, and 4-700 μg /tube for other tissue membranes. Experiments were usually performed in duplicate or triplicate.

III.2.2.2 Competition experiments

These experiments were performed by first adding to a series of tubes (usually in duplicate) the same concentration of radiolabel and increasing concentrations of a displacing drug. Incubation then began upon the addition of an equal amount of membranes to all the tubes, and continued until equilibrium. In individual experiments, parallel incubations were also performed in the absence and presence of a NSB determinant to assess total and non specific radioligand binding.

III.2.2.3 Kinetics experiments

III.2.2.3.1 Association Kinetics experiments

In these experiments, groups of tubes in the absence and presence of NSB determinant were set up. An equal quantity of radioligand was added to all tubes. Membrane preparation was then added in equal quantity to the tubes group by group at various time intervals to initiate incubation. Incubations were allowed to proceed for increasing length of time (usually 30 seconds to one hour), and stopped by filtration. The timing of membrane addition and filtration was so designed such that identical length of incubation time was allowed for duplicate tubes. Hence, increasing amount of specific radioligand binding in relation to time was obtained.

III.2.2.3.2 Dissociation kinetics experiments

In these experiments, groups of tubes in the absence and presence of NSB determinant were set up. Radioligand, and then membrane preparation were added to all the tubes in equal quantities to initiate incubation. After incubation had proceeded well onto the equilibrium state (for 40-50 minutes in usual practice), an excess of phentolamine (final concentration = 5 μ M) was added to the tubes group by group at various time intervals to initiate the dissociation of radioligand binding. The dissociation was allowed to proceed for variable length of time (usually $t = 0$ to $t = 1$ hour) and stopped by filtration. The timing of phentolamine addition and membrane filtration was so designed such that identical length of dissociation time was allowed for duplicate tubes. Specific radioligand binding at zero dissociation time referred to the maximal specific binding at equilibrium, as determined from the difference between total and non-specific binding before the addition of excess of phentolamine.

III.3 Protein Determination

Protein contents were assayed according to the methods of Lowry et al (1951).

Bovine Serum Albumin was used as reference protein. Series of protein standards (12.5 - 300 $\mu\text{g/ml}$.) were prepared in distilled water. Sample preparations were also properly diluted in distilled water. Standards and samples were treated with reagents (listed in Appendix II), and after equilibration with Folin-Ciocalteu Reagent, absorbance was read at a wavelength of 750 nm with a pye-unicam Sp 6-500 spectrophotometer. Samples protein concentrations were determined from the regression lines from the standard (absorbance - concentration) curves.

III.4 Analysis of results

III.4.1 Saturation experiment data

Initial free radioligand concentrations were assessed by measuring radioactivity in extra 'Total' tubes containing membranes. This could provide better estimates of the amount of ligand truly 'free' for reaction. Equilibrium free ligand concentrations (F) were then calculated by subtracting total bound radioactivity from initial free ligand concentrations. Specific radioligand binding (B) was related to (F) by plotting B/F against F (Scatchard, 1949), or B against B/F . If B/F was plotted against B, the maximal binding capacity (B_{max}) and dissociation constant (K_d) were estimated from the X - intercept, and the reciprocal of the slope of the regression line respectively. If B was plotted against B/F , B_{max} and K_d were obtained respectively from the Y - intercept and the slope of the regression line.

Possible co-operativity or receptor heterogeneity was assessed from the slope (Hill coefficient) of the Hill plot ($\log B/[B_{\text{max}} - B]$ vs. $\log F$) (Hill, 1910)

Data were routinely analysed by computerized program based on the respective equations for the plots, and 'best-fitted' with regression.

Derivations of equations for Scatchard and Hill plots are presented in Appendix III.

III.4.2. Competition experiment data

Specific radioligand binding in the presence of inhibitor was compared with that in the absence of inhibitor, and % inhibition was calculated.

IC₅₀ values (concentrations of inhibitors displacing 50% specific ligand binding) were determined graphically from the displacement curves (% inhibition vs. log. conc. inhibitor) and from Hill plots ($\log \% I/[100 - \% I]$ vs. log. conc. inhibitor) from which the slope factors (nH) were also obtained from the slopes of the regression lines.

The computer interactive non-linear curve fitting program, LIGAND (Munson & Rodbard, 1980) was also used for more detailed analyses of some agonist displacement curves.

The inhibition constants (K_i) were calculated from IC₅₀ values using the relationship $K_i = IC_{50}/(1 + S/K_d)$ (Cheng & Prusoff, 1973), where S = concentration of the radiolabelled ligand used, and K_d = equilibrium dissociation constant of the ligand (see Appendix V).

III.4.3. Kinetics experiment data

The observed, or pseudo first-order rate constants (K_{obs}) for the association reactions were determined from the slopes of regression lines for the $\ln B_{eq}/(B_{eq} - B)$ vs. time plots, where B_{eq} = specific radioligand binding at equilibrium, and B = specific binding at time t.

First-order dissociation rate constants (K₂) were determined from the slopes of the regression lines for the $\ln (B/B_{eq})$ vs. time plots, where B = specific ligand binding after dissociation time t.

Second-order association rate constants (K₁) were calculated according to the relationship: $K_{obs} - K_2 = S (K_1)$, where S = concentration of the radioligand used.

Kinetically derived dissociation constants were calculated upon dividing K₂ by K₁.

Derivations of the kinetics equations are presented in Appendix IV.

III.5 Statistical treatment of results

Results were generally expressed as mean values \pm standard error of means, unless indicated otherwise. Comparison of results was tested for statistical significance using Student's 't' tests. Differences were considered significant when $p < 0.05$.

Chapter IV

CHARACTERISATION OF ALPHA₂-ADRENOCEPTORS ON INTACT HUMAN PLATELETS AND PLATELET MEMBRANES

IV.1. Introduction

Human blood platelets originate from the fragmentation of bone marrow megakaryocytes (Gordon, 1981; Weiss, 1982). These tiny circulating discs play an important physiological role in haemostasis, but are also an important factor for thromboembolic disorders (Zucker, 1981; Weiss, 1982). The stimulation of platelet activities is initiated through the interactions of platelet surface membranes with aggregating agents. Once stimulated, platelets undergo a sequence of morphological and functional changes (Varghaftig et. al., 1981). There is usually a rapid change in shape, associated with the formation of pseudopodes (Gordon, 1981; Varghaftig et. al., 1981). The membrane surfaces also become 'sticky', to which fibrinogen binds and facilitates the aggregation of platelets in the presence of calcium ions (Zucker, 1980; Shattil & Bennett, 1981). In addition, aggregating agents stimulate the intracellular mobilization of Ca^{++} , which facilitates the release of other chemical substances such as ADP, serotonin, etc from platelet granules, hence resulting in the recruitment and aggregation of additional platelets. Accompanying platelet aggregation is also the synthesis of chemical substances such as thrombin and thromboxane A_2 which further support the aggregatory and secretory activities (Shattil & Bennett, 1981; Varghaftig et. al., 1981; Zucker, 1980).

In-vitro platelet aggregation can be induced by a variety of soluble stimuli, each presumably interacting with a specific surface receptor. These include ADP (Gaarder et. al., 1961, Born, 1961; Born & Cross, 1963; Haslam, 1964; Mustard & Packham, 1970, Nachman et. al., 1974), serotonin (Mitchell & Sharp, 1964), thrombin (Grette, 1962; Clayton & Cross, 1963; Tollefsen et. al., 1974), and catecholamines (Mills & Roberts, 1967; Grant & Scrutton, 1979; Barthel & Markwardt, 1974; Scrutton & Wallis, 1981). Although catecholamines are only effective in stimulating full aggregation of human blood platelets, an effect which also only occurs at concentrations far beyond the physiological levels (Scrutton & Wallis, 1981), they do have

the ability to potentiate the aggregatory actions of other agonists, such as ADP (Drummond, 1976; Hsu et. al., 1979; Scrutton & Wallis, 1981).

This 'pro-aggregatory' effect of catecholamines therefore may be of greater physiological significance than the full aggregatory effect.

From functional studies, there is now substantial evidence to indicate that catecholamines induce platelet aggregation or pro-aggregation through interacting with an alpha-adrenoceptor (Clayton & Cross, 1963; Mills & Roberts, 1967). Moreover, such an alpha-adrenoceptor appears to be of the α_2 -subtype, as can be demonstrated by the selective effects of the α_2 -agents compared to the α_1 -agents (Grant & Scrutton, 1979; Grant & Scrutton, 1980; Hsu et. al., 1979). This has further been substantiated by radioligand binding studies in which the non-selective alpha-antagonist ligand, ^3H -dihydroergocryptine was used as the receptor probe (Alexander et. al., 1978; Newman et. al., 1978). With the use of computer-assisted curve-fitting analysis, Hoffman et al. have also reached the conclusion that human platelets contain alpha-adrenoceptors exclusively of the α_2 -subtype (Hoffman et. al., 1979).

The present study was undertaken with the primary aim of examining the pharmacological properties of receptor sites on human platelets labelled by the α_2 -selective antagonists ^3H -yohimbine and ^3H -rauwolscine. The results have demonstrated that both ligands label an identical population of homogeneous sites possessing pharmacological properties that are suggestive of the α_2 -adrenoceptors.

IV.2. Results

IV.2.1. Characterisation of α_2 -adrenoceptors in intact human platelets

IV.2.1.1. ^3H -yohimbine binding: Equilibrium and kinetics studies

^3H -yohimbine binding to intact human platelets was rapid and saturable, and of high affinity and high capacity. Equilibrium was reached in around 30 minutes at 22-25°C (Fig. IV.2). The maximal binding capacity

Legends to figures IV.1 and 2

Figure IV.1.

Specific binding of ^3H -yohimbine to intact human platelets. Platelets were incubated for 40 minutes at 22°C with ^3H -yohimbine (0 - 20 nM) in isotonic buffer (50mM Tris HCl, 0.15 M NaCl, 0.02 M EDTA, pH 7.5). Experiments were carried out similarly to that described in Methods (Chapter III). Left panel; saturation isotherm. Right panel: Scatchard analysis of binding data. Data illustrated is from one experiment representative of three performed in duplicate. $B_{\text{max}} = 178.3 \text{ f.mole/mg protein}$. $K_d = 2.07 \text{ nM}$.

Figure IV.2.

Kinetics of ^3H -yohimbine binding to intact human platelets. Kinetics experiments were carried out at 22°C , and results analysed as described in Methods (Chapter III). Left panel: association curve; the inset shows the pseudo first-order rate plot for the association reaction, the slope of which yields the observed rate constant, K_{obs} . Right panel: dissociation curve; the inset shows the first-order rate plot for the dissociation reaction, the slope of which yields the dissociation rate constant, K_2 ; the second-order association rate constant, K_1 was calculated from K_{obs} and K_2 as described in Chapter III.

Data presented is from one of two similar experiments performed in duplicate.

Fig. IV.1.

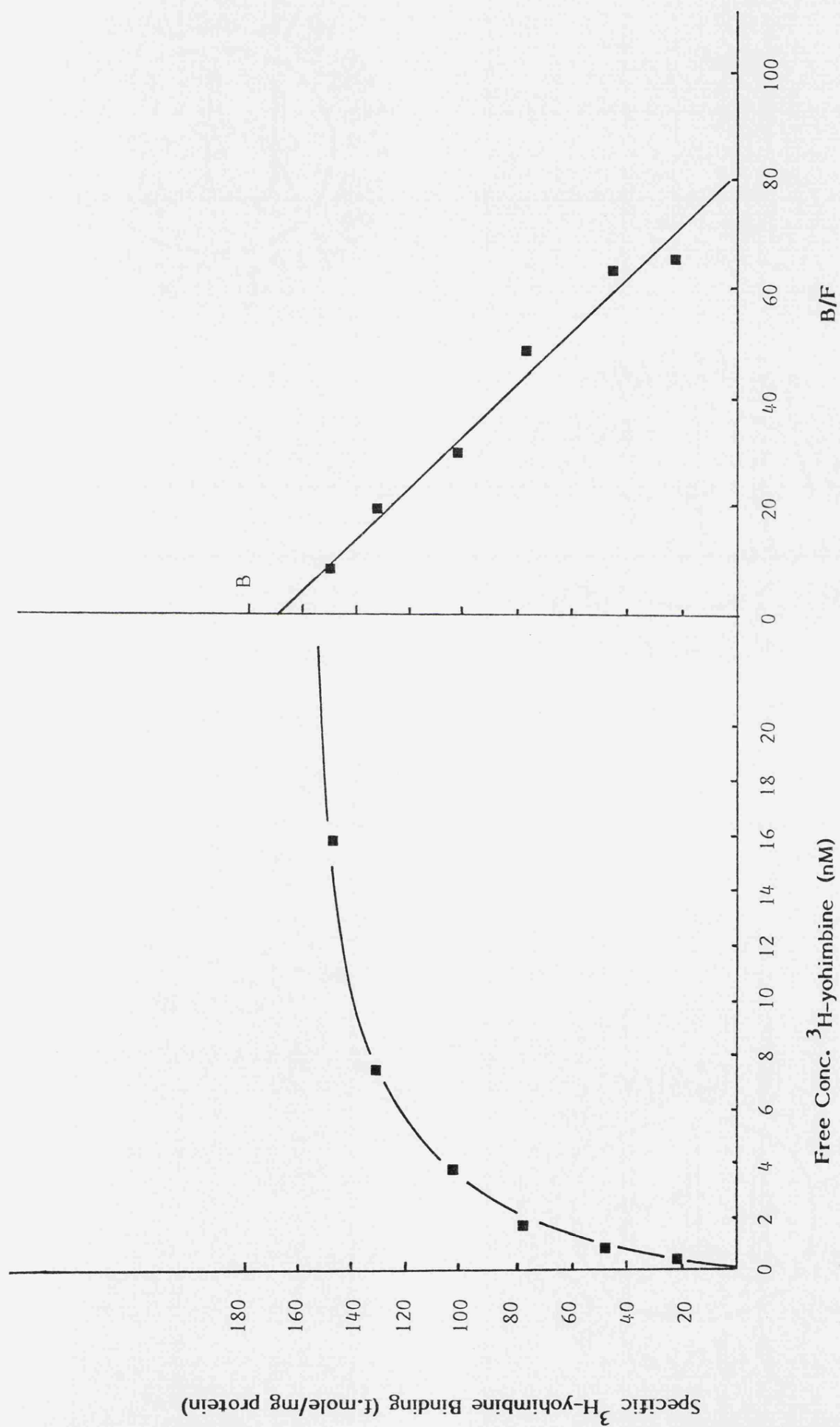


Fig. IV.2

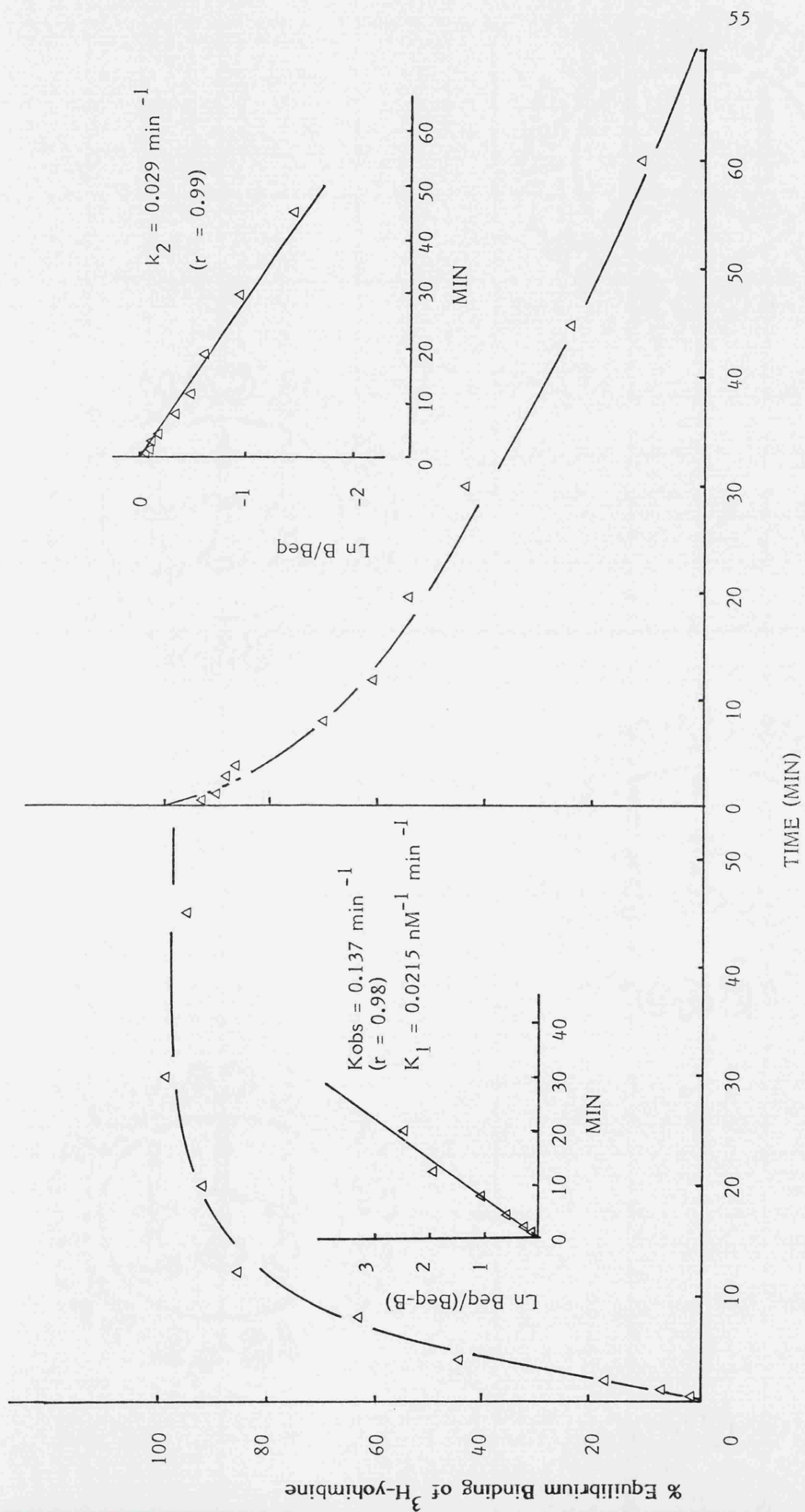


Table IV.1 Affinity of drugs at ³H-yohimbine binding sites on intact human platelets

Drug	Ki(nM)	nH
Rauwolscine	3.21 ± 0.17	1.07
Yohimbine	2.43 ± 0.26	1.06
Phentolamine	37.4 ± 2.8	0.98
Tolazoline	188 ± 22	0.94
Corynanthine	705 ± 117	0.93
Prazosin	1270 ± 210	0.96
Clonidine	67.1 ± 3.1	0.92
(-)-Adrenaline	2060 ± 160	0.82
(-)-Noradrenaline	6990 ± 350	0.79
(-)-Isoprenaline	53000 ± 5300	1.03
Dopamine	>100,000	
Serotonin	>100,000	

Experiments were performed at approximately 2nM ³H-yohimbine. Values shown are means ± S.E.M. of 3-6 separate experiments performed in duplicate. Ki values were calculated from IC50 values as described in Methods (Chapter III). nH values are mean slope factors of displacement curves.

(Bmax) was 187 ± 30 f.mole/mg protein or equivalent to 193 ± 23 sites/platelet ($n = 4$) (see figure IV.1). The equilibrium dissociation constant (Kd) of ^3H -yohimbine was 2.10 ± 0.13 nM. The Hill coefficient was 1.01 ± 0.004 , indicating that binding occurred at a single class of receptor sites in the absence of co-operative interactions.

From kinetics experiments, ^3H -yohimbine was found to bind to intact human platelets with an association constant (K_1) of $0.0215 \text{ nM}^{-1} \text{ min}^{-1}$. Binding was very stable and could be maintained for at least $1 - 1\frac{1}{2}$ hours. Dissociation followed a monophasic first-order kinetics and occurred at a relatively slow rate, with a dissociation constant (K_2) of 0.03 min^{-1} (Fig. IV.2). The kinetically derived Kd (k_2/k_1) was calculated to be 1.35 nM, which was in reasonably good agreement with those derived from equilibrium and competition studies (Table IV.1).

IV.1.2. Pharmacological Characteristics

^3H -yohimbine binding could be displaced by a variety of drugs. Listed in Table IV.1 are inhibition constants (K_i) of a number of drugs in competing against ^3H -yohimbine binding to intact human platelets. As also illustrated in figures IV.3.a.,b., the relative inhibitory potencies of adrenergic agents presented a pharmacological profile which is typical of one expected for the α_2 -adrenoceptors. Thus, the α_2 -selective antagonists yohimbine and rauwolscine appeared almost equipotent at these receptor sites, and both were considerably more potent than the non-selective antagonist phentolamine. The α_1 -selective antagonists corynanthine and prazosin were of even weaker affinities at the ^3H -yohimbine binding sites. With respect to the agonists, the α_2 -selective partial agonist clonidine was more potent than (-)-adrenaline and (-)-noradrenaline. The latter were at least ten-fold more potent than the (+)-isomers (data not shown). The beta agonist isoprenaline was very weak, so were dopamine and serotonin (data not shown). The Hill slope factors (nH) of the antagonist competition curves were all close to unity, which further indicated that

Legends to Figures IV.3.a. and b.

Displacement of Specific ^3H -yohimbine binding to intact human platelet by antagonists and agonists. Platelets were incubated in isotonic buffer room temperature ($22^{\circ} - 25^{\circ}\text{C}$) for 40 minutes with 2 - 3 nM ^3H -yohimbine in the presence of increasing concentrations of displacing drugs as described in Chapter III. Data points shown are means of 3 - 6 separate experiments performed in duplicate S.E.M. for each data point is generally less than $\pm 5\%$.

Fig. IV.3.a.

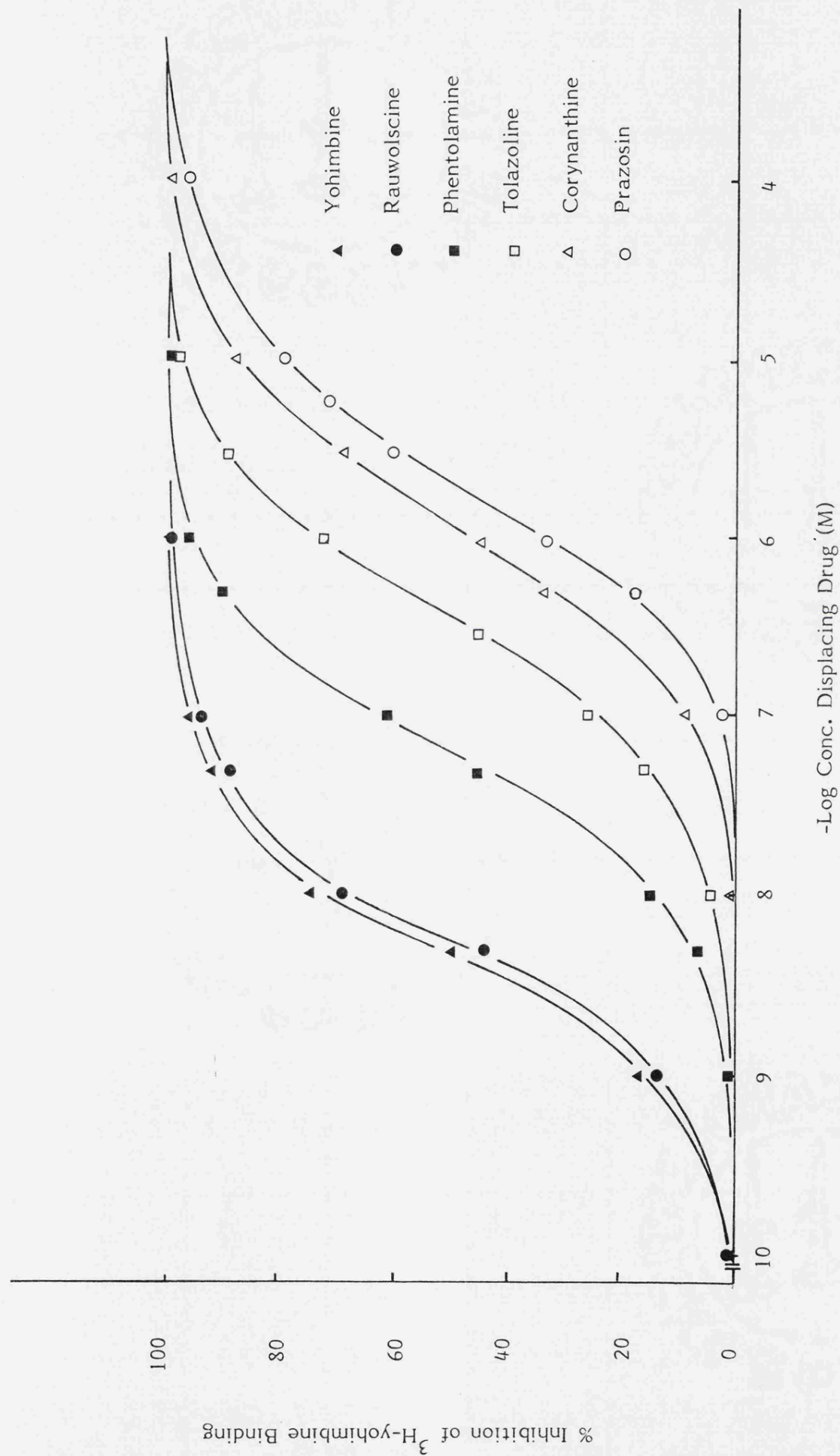
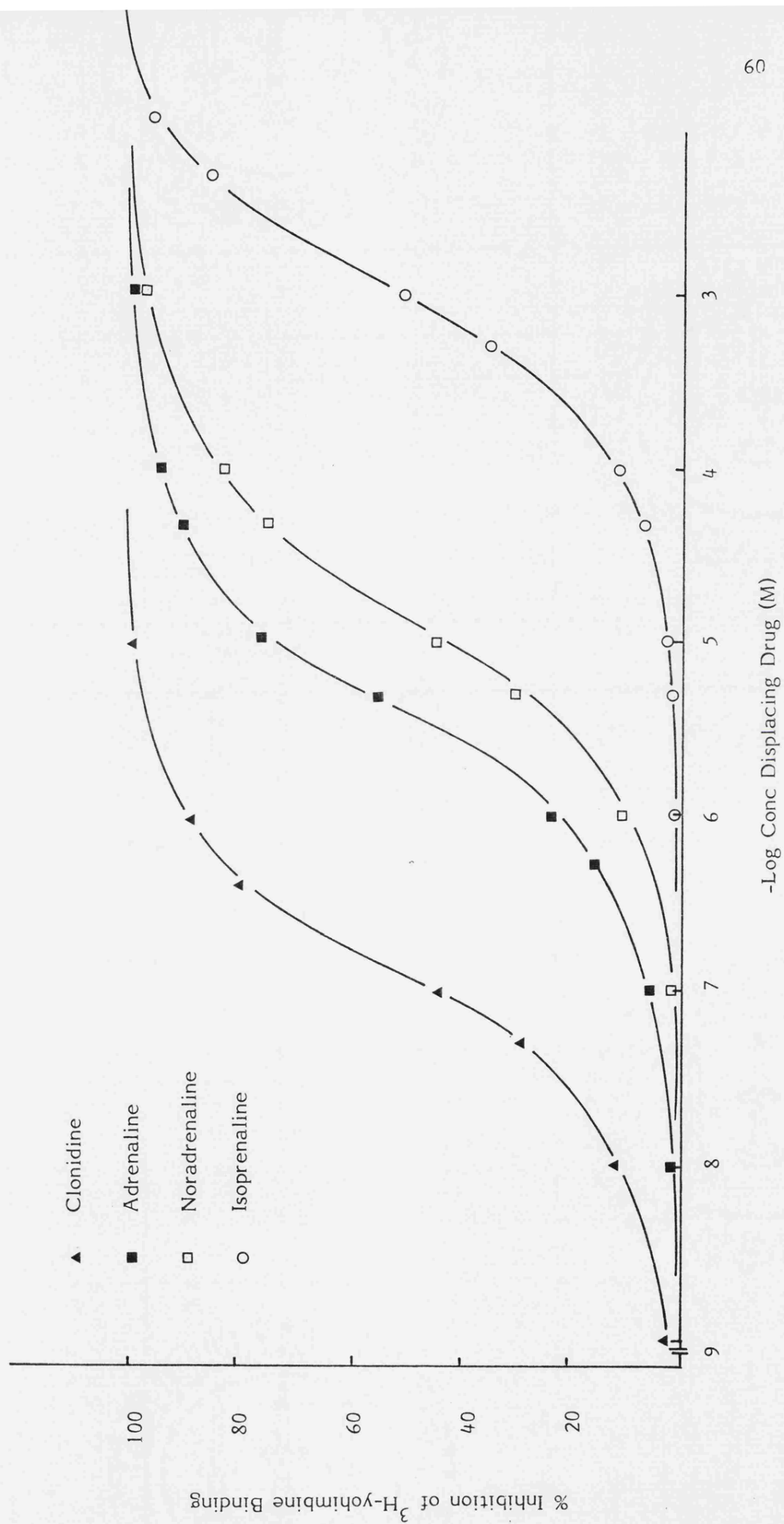


Fig. IV.3.b.



the interactions of drugs occurred at a single class of binding sites.

I V.2.2. Characterisation of α_2 -adrenoceptors on human platelet membranes

IV.2.2.1. ^3H -yohimbine and ^3H -rauwolscine binding: Equilibrium and Kinetics studies

Binding of ^3H -yohimbine and ^3H -rauwolscine to human platelet membranes was as rapid as to intact platelets, reaching equilibrium in 30-40 minutes at 22-25°C. In parallel studies, both ligands were shown to label an identical number of binding sites ($B_{\text{max}} = 148 \pm 9$ f.mole/mg. protein and 144 ± 5 f.mole/mg. protein ($n = 4$) for ^3H -yohimbine and ^3H -rauwolscine respectively). The equilibrium dissociation constants (K_d) were 0.59 ± 0.08 nM and 0.55 ± 0.06 nM respectively, which were also almost equal. The scatchard plots were linear, with Hill coefficients close to unity, suggesting that binding was to a homogeneous class of binding sites (Fig. IV. 4.)

^3H -yohimbine bound with a two-fold faster association rate to platelet membranes than to intact cells ($K_1 = 0.054 \text{ nM}^{-1} \text{ min}^{-1}$, compared to $0.0215 \text{ nM}^{-1} \text{ min}^{-1}$), and dissociated at a slightly faster rate ($K_2 = 0.04 \text{ min}^{-1}$ compared to 0.029 min^{-1}). Compared to ^3H -yohimbine, ^3H -rauwolscine binding to platelet membranes occurred at a slightly faster association rate and a slightly slower dissociation rate ($K_1 = 0.073 \text{ nM}^{-1} \text{ min}^{-1}$, and $K_2 = 0.03 \text{ min}^{-1}$). The kinetically derived dissociation constants for ^3H -yohimbine and ^3H -rauwolscine were 0.7 nM and 0.4 nM respectively, both being in good agreement with those derived from equilibrium studies (Fig. IV 5a and b).

IV.2.2.2. Pharmacological Characteristics

Table IV.2. shows the inhibition constants of various drugs in competing against ^3H -yohimbine and ^3H -rauwolscine binding to human platelet membranes in parallel studies. The data unequivocally confirmed that both ligands labelled pharmacologically identical sites on the human platelet membranes. The antagonist competition curves with either ligand

Legends to Figures IV.4, 5a and b

Figure IV.4

Specific binding of ^3H -yohimbine and ^3H -rauwolscine to human platelet membranes. Experiments were performed in parallel with both ligands (0 -20nM) in each membrane preparation as described in Chapter III. Data illustrated is from a single representative experiment performed in duplicate ($n = 4$). Left panel: Saturation isotherms. Right panel: Scatchard plots of binding data. B_{max} for ^3H -yohimbine was 160 f.mole/mg protein, with $K_d = 0.75$ nM. B_{max} for ^3H -rauwolscine was 154 f.mole/mg protein, with $K_d = 0.7$ nM.

Figures IV.5a and b

Kinetics of ^3H -yohimbine and ^3H -rauwolscine binding to human platelet membranes at R.T. FigIV.5a, ^3H -yohimbine binding. Fig.IV.5b, ^3H -rauwolscine binding. Experiment procedures and analysis of data were described in Methods (Chapter III). Left panels: association curves; the insets illustrate thepseudo first-order rate plots for the association. Right panels: dissociation curves; the insets illustrate the first-order dissociation rate plots. Data shown are from single experiments which were duplicated twice, with results differing by less than 5%.

Fig. IV.4.

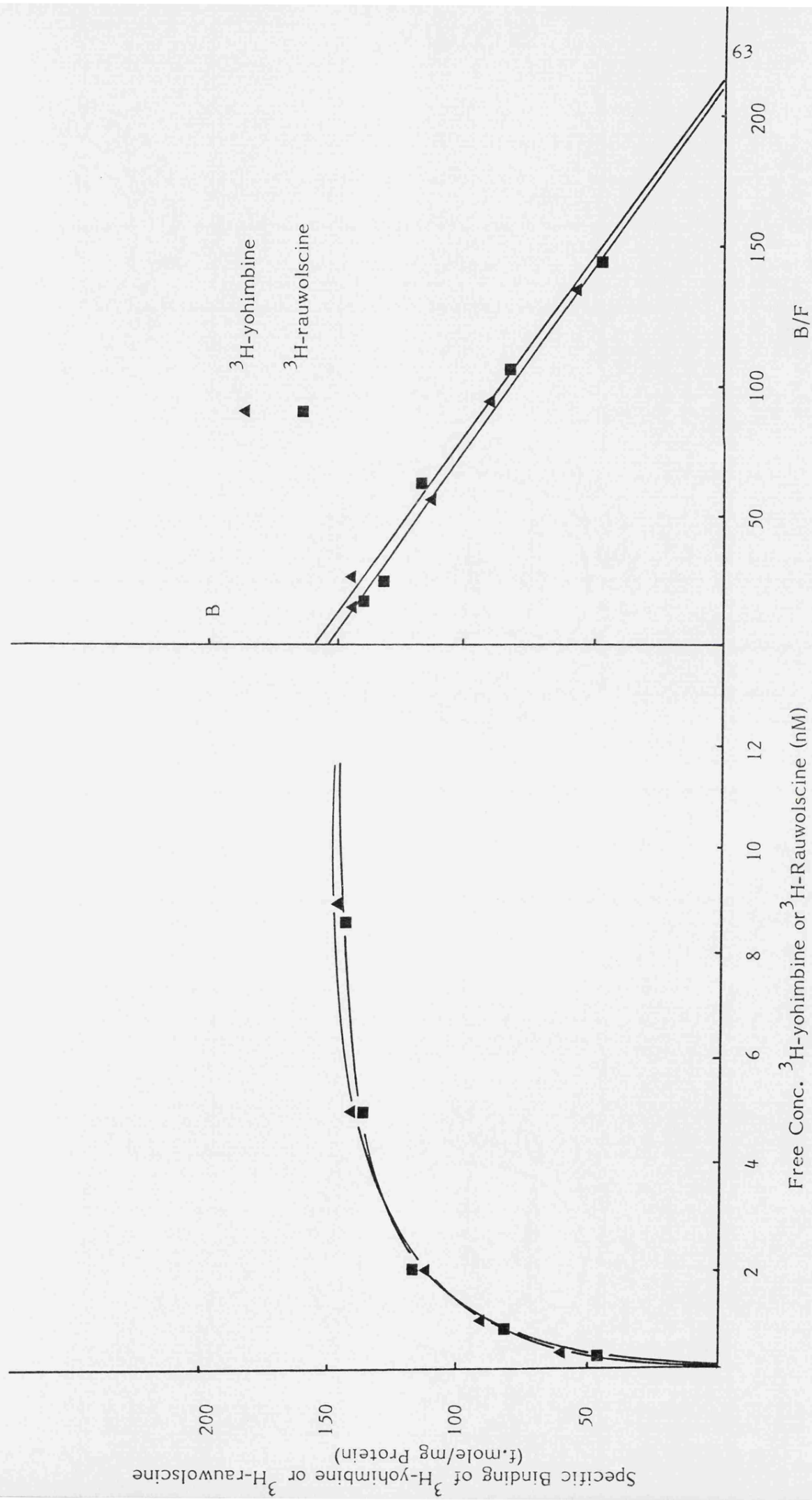


Fig. IV.5.a.

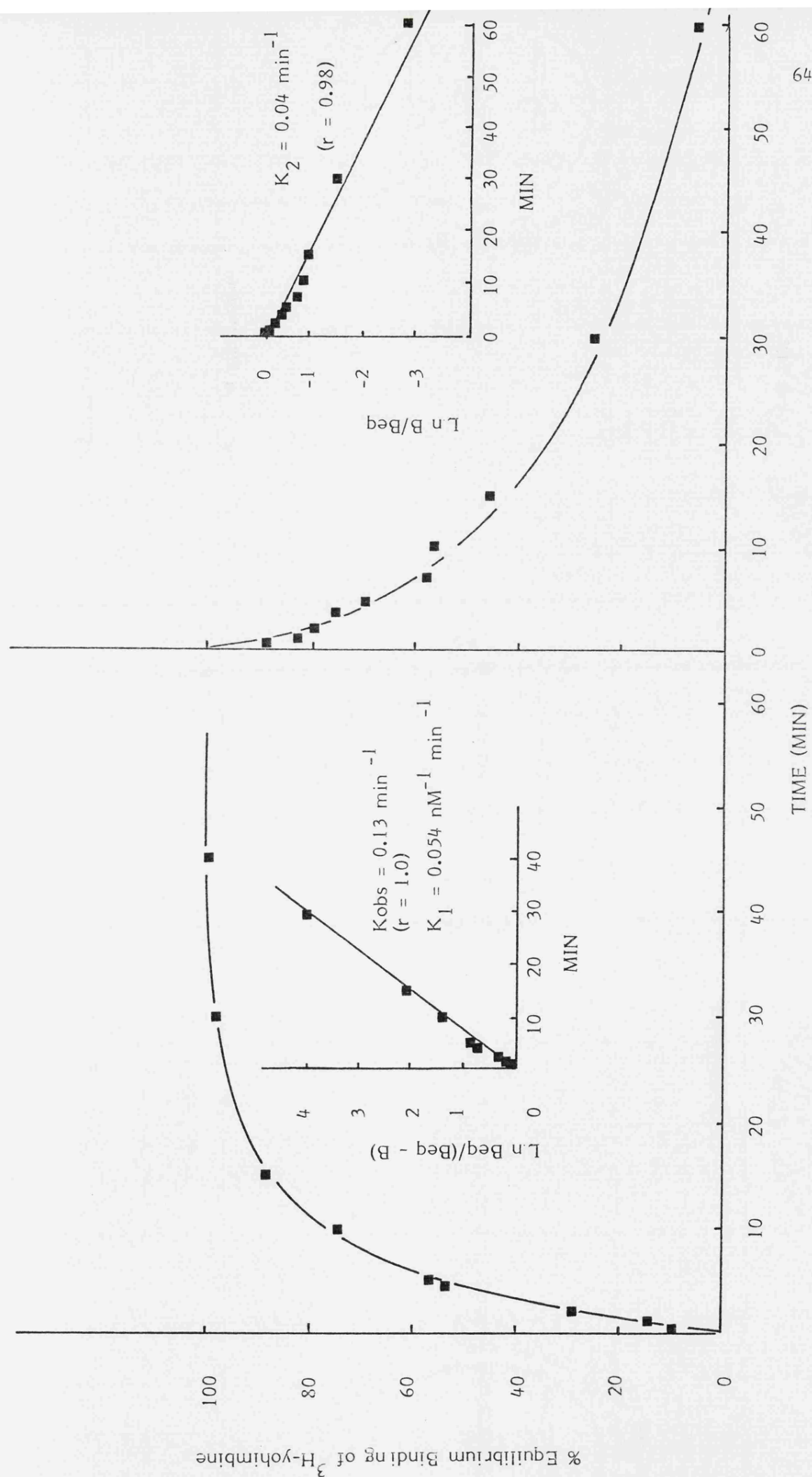


Fig. IV.5.b.

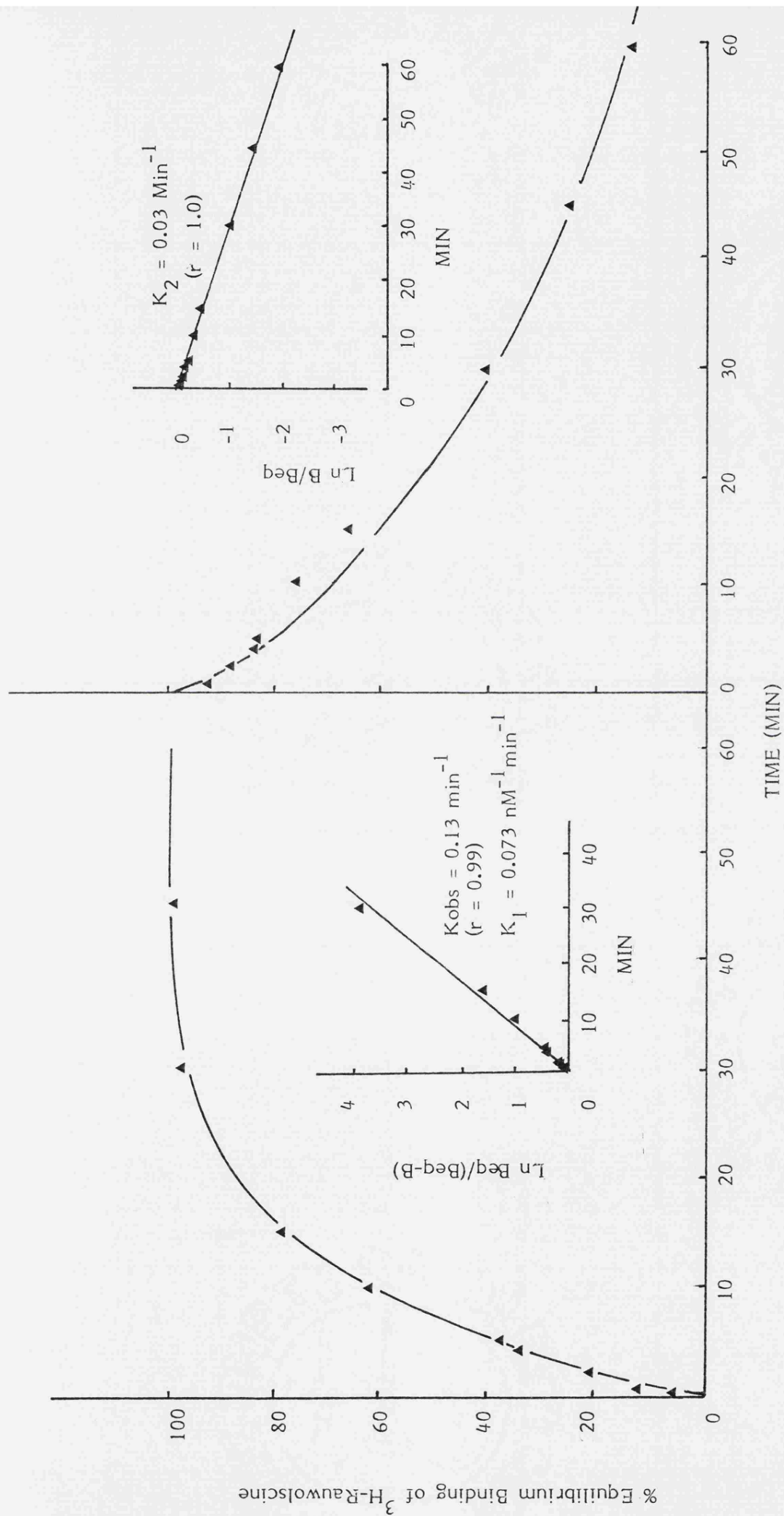


Table V.2. Affinity of various drugs at ^3H -Yohimbine and ^3H -Rauwolsine binding sites on human platelet membranes

Drugs	^3H -Yohimbine		^3H -rauwolsine	
	Ki (nM)	nH	Ki (nM)	nH
Rauwolsine	1.05 \pm 0.13	1.06	1.11 \pm 0.12	1.00
Yohimbine	0.99 \pm 0.07	1.11	0.85 \pm 0.09	0.97
Phentolamine	3.88 \pm 0.9	0.98	4.01 \pm 1.23	0.99
WB4101	3.97 \pm 0.5	1.05	3.52 \pm 0.2	0.96
Corynanthine	261 \pm 42	1.01	192 \pm 23	1.15
Prazosin	784 \pm 170	0.94	760 \pm 194	1.00
Clonidine	9.9 \pm 0.29	0.82	8.89 \pm 0.48	0.83
Adrenaline	29.9 \pm 7.7	0.79	28.9 \pm 4.9	0.79
Noradrenaline	89.7 \pm 4	0.82	88.7 \pm 13	0.91
Dopamine	1165 \pm 325	0.98	977 \pm 143	0.89
Isoprenaline	43400 \pm 4200	1.04	36850 \pm 10450	0.86

Experiments were performed at approximately 2nM ^3H -yohimbine and ^3H -rauwolsine. Specific binding was over 80 - 90% of total binding. Inhibition constants (Ki) were obtained and calculated as described in Methods (ChapterIII). Values shown are means \pm S.E.M. of at least 3 - 6 separate parallel experiments performed in duplicate. nH values are the mean slope factors of displacement curves.

were all steep, with slopes close to unity, indicating that interactions occurred at a single class of binding sites or states. The rank order of drug potencies at either ^3H -yohimbine or ^3H -rauwolscine binding sites was also one indicative of an α_2 -type of interactions. Hence, the rank order for the antagonists was yohimbine \gg rauwolscine $>$ phentolamine $>$ corynanthine $>$ prazosin. And, for the agonists, the order was clonidine $>$ adrenaline $>$ noradrenaline $>$ dopamine \gg isoprenaline (Fig. IV. 6a,b).

In general, the pharmacological behaviour of the ^3H -yohimbine or ^3H -rauwolscine binding sites on the platelet membranes appeared to be similar to that on intact platelets. The rank order of drug potencies was identical. However, the absolute inhibition constants of drugs appeared to be generally higher on intact platelets than on membranes, particularly for the agonists, such as adrenaline and noradrenaline, whose overall K_i values were at least one to two orders higher. This discrepancy might at least in part be attributed to the influence of sodium ion in the isotonic buffer (see discussion and Chapter VI).

Legend to figures IV.6.a. and b.

Displacement of specific ^3H -yohimbine binding to human platelet membranes by antagonists (IV.6.a.) and agonists (IV.6.b.). Membranes were incubated with 2nM ^3H -yohimbine in the presence of increasing concentrations of displacing drugs. Data points are means of 3 - 6 separate experiments performed in duplicate. S.E.M. of each point is generally less than $\pm 5\%$.

Fig. IV.6.a.

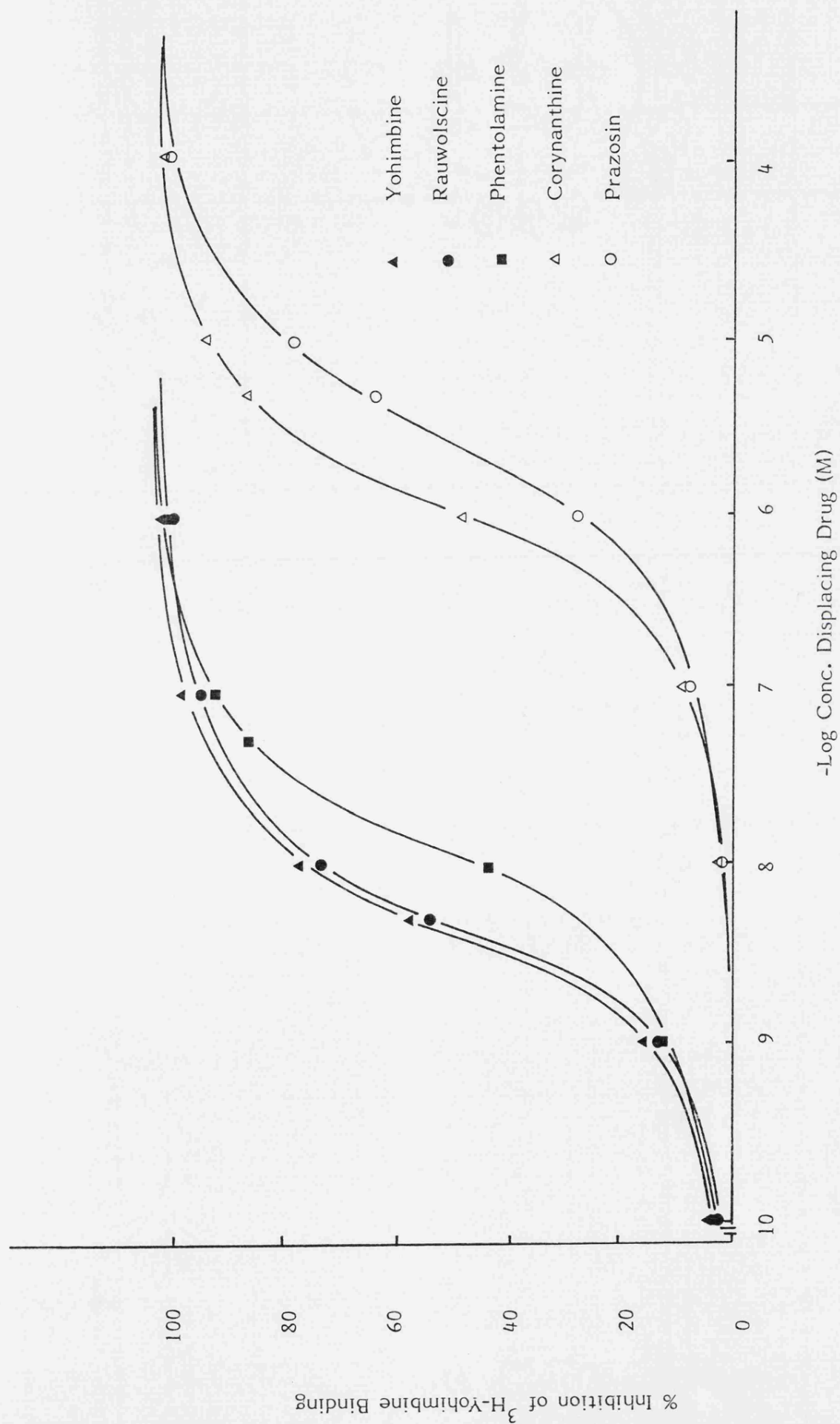
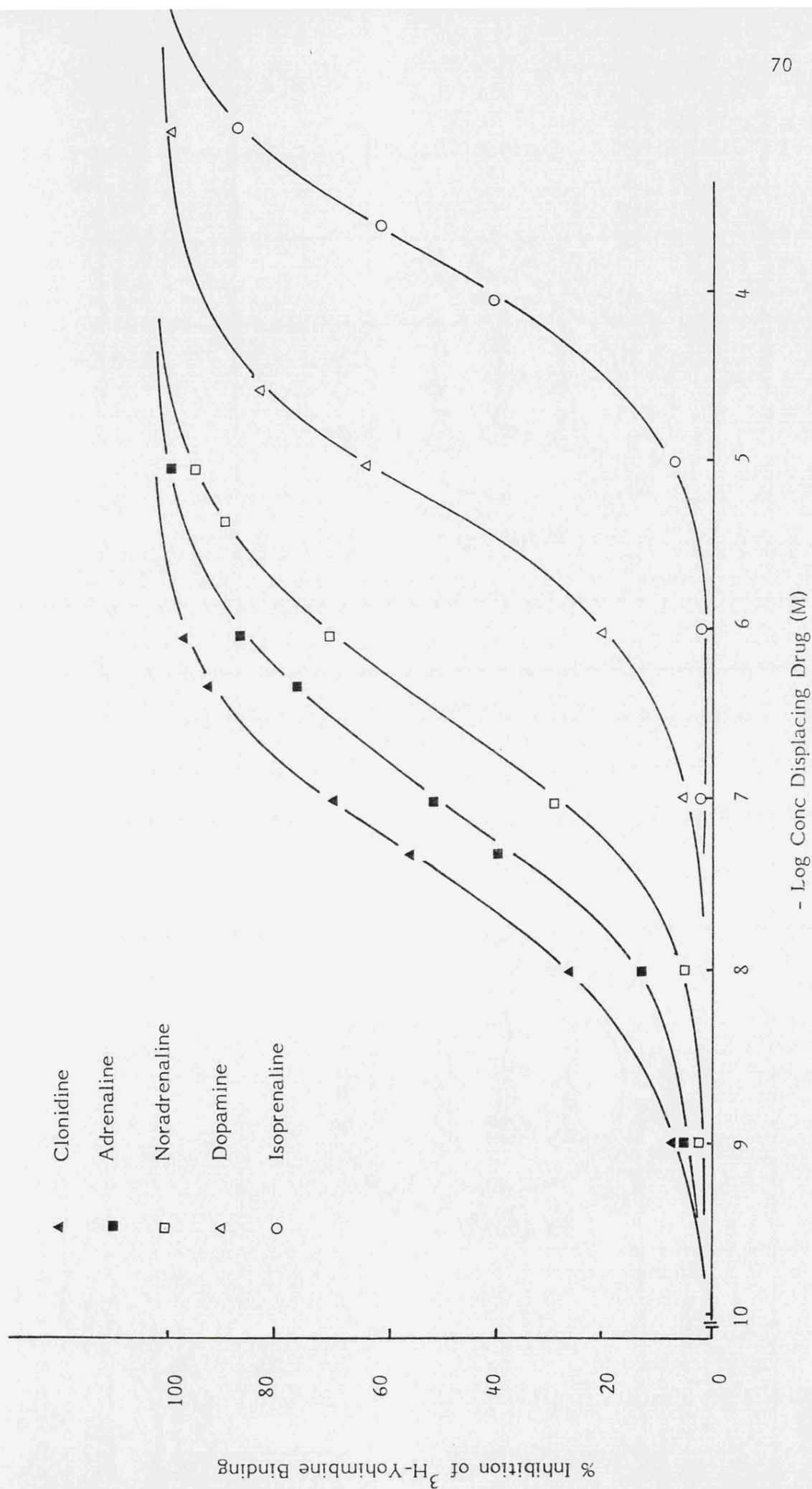


Fig. IV.6.b.



IV.3. Discussion

It is well established that catecholamines can induce in-vitro platelet aggregation, and also potentiate the actions of other aggregating agents. There is also sufficient evidence that such effect is mediated through α_2 -adrenoceptors. Inhibition of adenylate cyclase (and hence the reduction of cyclic AMP level), as well as calcium influx or mobilization have both been implicated as the biochemical mechanisms for the α_2 -adrenoceptor mediated platelet aggregation (Jakobs et. al., 1976; Owen et. al., 1980; Feinstein, et. al., 1981). Although the pathological significance of platelet α_2 -adrenoceptor in thromboembolic disorders has yet to be demonstrated, this receptor model does appear to be potentially useful in radioligand binding studies for examining the general molecular properties and mechanisms of α_2 -receptors. The easier accessibility of platelets as a tissue source is one major advantage; cell homogeneity is probably another benefit, compared to other tissues.

Earlier characterisation of platelet α_2 -adrenoceptor by radioligand binding studies was accomplished mainly with the use of non-selective antagonist ligands such as ^3H -DHE (Alexander et. al., 1978; Newman et. al., 1978; Hoffman et. al., 1980), and ^3H -phentolamine (Steer et. al., 1979) as receptor probes. Results from these studies are generally in good agreement which suggests that platelet α -adrenoceptors are exclusively of the α_2 -subtype (Elliot & Grahame-Smith, 1980; Hoffman et. al., 1979). However, studies of the molecular properties of α_2 -adrenoceptors would of course be more efficiently achieved by means of direct, selective labelling with an α_2 -selective ligand.

In this study, the newly available α_2 -selective antagonist ligands ^3H -yohimbine and ^3H -rauwolscine were used to characterize α_2 -adrenoceptors in intact human platelet and platelet membranes. While this study was in progress, a few reports were published which also examined the characterisation

of human platelet α_2 -adrenoceptors by ^3H -yohimbine (Motulsky et. al., 1980; Daiguji et. al., 1981). Results from this study confirm these earlier reports which indicated that ^3H -yohimbine selectively labels α_2 -adrenoceptor sites on human platelets. In addition, this work also shows that the diastereoisomer of yohimbine, rauwolscine, is an equally specific and effective ligand for labelling α_2 -adrenoceptors. Both ligands were shown to label an identical and homogeneous population of sites when examined in parallel studies. In comparison with ^3H -DHE, these two ligands appear to be much 'cleaner', and exhibit high affinity, lower non-specific binding and greater reproducibility and consistency (Motulsky & Insel, 1982). In fact, more recent assessments from this and other laboratories (Boon et. al., 1981; MacFarlane et. al., 1981; Motulsky et. al., 1982) have recognized that ^3H -DHE appears to label more binding sites on human platelets than ^3H -yohimbine or ^3H -rauwolscine. However, the exact nature of these extra ^3H -DHE binding sites remains unclear.

Since ^3H -DHE appears to bind to only a single class of sites, and non-adrenergic agents such as serotonin and dopamine compete weakly for the ^3H -DHE labelled sites (Motulsky & Insel, 1982), it seems unlikely that this ligand labels other types of receptors on platelets in addition to α -adrenoceptors. Nevertheless, ^3H -yohimbine and ^3H -rauwolscine have now proved to be more specific and preferable ligands for the studies of α_2 -adrenoceptors.

Although the overall pharmacological profiles of the α_2 -adrenoceptor sites on intact platelets and platelet membranes were identical, the affinities of drugs were obviously weaker on the intact platelets, particularly with respect to the agonists. Similar type of discrepancies in agonist affinity appears to occur also with the beta-receptors (Insel & Stoolman, 1978) and the α_1 -receptors (Sladeczek et. al., 1983). There was suggestion (Insel & Stoolman, 1978; Motulsky et. al., 1980) that in intact cells, intracellular guanine nucleotides such as GTP might be accountable for the overall low

affinities of the agonists for the beta and α_2 receptors, as most of the receptors might be expected to exist in a low-affinity state in the presence of sufficiently high concentrations of GTP (see Chapters II and VI). However, in membrane preparations, in which GTP concentrations are low, a significant proportion of the receptors is induced or stabilised in the high-affinity state, thus resulting in an overall higher affinity for the agonists. In the case of α_2 -adrenoceptors, the affinities of agonists, as well as some antagonists can also be significantly reduced by monovalent ions such as Na^+ (Tsai & Lefkowitz, 1978; Michel et. al., 1980, also see Chapter VI). Thus, it is indeed not unlikely that the weaker affinities of drugs on intact platelets might in part be due to the effect of sodium ion in the incubation buffer. As for the agonists, the combined influence of the intracellular GTP and extracellular Na^+ might at least partly account for the much lower affinities of agonists on intact cells, compared to membranes (Motulsky et. al., 1980). However, there appears to be some other possible explanations for the discrepancies in agonist affinity between intact cells and membranes, particularly when considering that such a phenomenon also occurs at α_1 -receptors at which no sufficient evidence is as yet available to indicate any significant effects of GTP or monovalent ions on agonist affinity. It has been found that, at least in the case of beta-receptors, discrepancies in agonist affinity between intact cells and membranes only occurred when lipophilic but not hydrophilic radioligands were used (Staehelin et. al., 1983). It was suggested that receptor internalization could occur in intact cells in the presence of agonists. Lipophilic ligands could 'penetrate' membranes and label these internalized receptor sites. Since agonists such as adrenaline or noradrenaline, are more hydrophilic, they would be expected to fail to compete with the lipophilic ligands for such sites, thus resulting in apparently weaker affinities in intact cells. There is no proof at the moment if this could provide a better explanation

for the discrepancies in agonist affinity at α_2 -receptors between intact platelets and platelet membranes. After all, in view of the close similarity of the agonist affinity in intact platelets and platelet membranes in the presence of Na^+ and GTP, it would seem likely that the weak affinity of agonists in intact platelets is more attributed to the presence of intracellular guanine nucleotides and extracellular Na^+ than to receptor internalization.

In summary, the α_2 -selective antagonists yohimbine and rauwolscine are both specific ligands for studying α_2 -adrenoceptors on human platelets. It appears that these two ligands will also be useful for studying α_2 -adrenoceptors in other tissues. In the next chapter, studies on α_2 -receptors in rat, rabbit and human cerebral cortical membranes, and rat and rabbit kidney membranes using these two radioligands will be described. Attention was focused on comparing the pharmacological properties of these receptors, as well as on comparing them with those of the platelet α_2 -receptors.

Chapter V

CHARACTERISATION OF ALPHA₂-ADRENOCEPTORS IN THE RAT, RABBIT AND HUMAN CEREBRAL CORTICAL MEMBRANES, AND THE RAT AND RABBIT RENAL CORTICAL MEMBRANES

V.1. Introduction

Alpha₂-adrenoceptors occur both in the peripheral and central noradrenergic neurons (Langer, 1977; Starke, 1977). The central alpha₂-autoreceptors may be present in the soma-dendritic regions and nerve terminals of noradrenergic neurons, and their activation can result in decreased impulse discharge and noradrenaline turnover (Cedarbaum & Aghajanian, 1977), as well as inhibition of transmitter release (Starke, 1981). Physiologically, central alpha₂-adrenoceptors have been implicated in a variety of central or peripheral effects. For examples, alpha₂-adrenoceptors have been suggested to mediate the central control of blood pressure (Berthelsen & Pettinger, 1977; McCall et al., 1983) and sedative effects (Drew et al, 1979; Timmermans et al., 1981). However, there is as yet no conclusive evidence that all the alpha₂-receptors involved in these effects are located on noradrenergic neurons (Starke, 1981). Indeed, although results from binding studies with ³H-clonidine confirmed the existence of central alpha₂- adrenoceptors, these results also suggested that most of the alpha₂- adrenoceptors in the brain are probably located post synaptically, since neurotoxic treatment with 6-hydroxydopamine resulted in no change in the ³H-clonidine binding capacity (U'Prichard & Snyder, 1979).

The kidney receives a rich supply of sympathetic innervation. Both subtypes of alpha-adrenoceptors are present in this tissue. Alpha₁-adrenoceptors appear to mediate such effects as renal arteriolar vasoconstriction (Schmitz et al., 1981), gluconeogenesis (McPherson & Summers, 1982a), as well as stimulation of sodium reabsorption (Di Bona, 1982). In contrast, the functional role of renal alpha₂-adrenoceptors is still not very clear, although there have been indications that they mediate an inhibitory effect on renin release (Pettinger et al., 1976). Auto radiographic and binding studies indicated that most of the alpha₂-adrenoceptors are located on the proximal tubules of the renal cortex (Young & Kuhar, 1980; McPherson & Summers, 1982b).

Cerebral and renal α_2 -adrenoceptors have been characterised by radioligand binding assays. Most of the earlier studies involved the use of ^3H -Clonidine (U'Prichard & Snyder, 1980; Glossmann et al., 1980; Summers, 1980). More recent studies have mainly employed the α_2 -selective antagonist ligand ^3H -yohimbine or ^3H -rauwolscine (Yamada et al., 1980; Perry & U'Prichard, 1981; Rouot et al., 1982; Cheung et al., 1982; Schmitz et al., 1981; Snavely & Insel, 1982).

This chapter describes studies on the characteristics of α_2 -adrenoceptors in a number of tissues, including the rat, rabbit and human cerebral membranes, and the rat and rabbit renal cortical membranes, using both ^3H -yohimbine and ^3H -rauwolscine as the receptor probes. Attention has been focused on the comparative pharmacological behaviour of the different α_2 -adrenoceptors. Since results from earlier work showed that both ^3H -yohimbine and ^3H -rauwolscine appear to label identical pharmacological sites on human platelet membranes, the pharmacological behaviour of α_2 -adrenoceptors in the other tissues has been studied primarily with the use of ^3H -rauwolscine. The results from these studies have revealed significant differences in pharmacological properties among α_2 -adrenoceptors. A possible heterogeneity of α_2 -adrenoceptors is discussed.

V.2 Results

V.2.1 Characterisation of α_2 -adrenoceptors in the rat cerebral and renal cortical membranes

V.2.1.1 Equilibrium binding studies with ^3H -yohimbine and ^3H -rauwolscine

^3H -yohimbine and ^3H -rauwolscine bound to rat cerebral and renal membranes in a saturable manner with high affinities and relatively low capacities. Scatchard analysis of specific binding of either ligand in both tissues suggest an interaction with a single population of non-cooperative binding sites (fig. V.1,2; table V.1), and in all cases, Hill coefficients were close to unity (data not shown). Results from parallel experiments also clearly indicated that both ligands label an identical number of sites in the rat brain or kidney, though there were differences in their affinities (table V.1). Thus, ^3H -rauwolscine appeared to possess a significantly higher affinity than ^3H -yohimbine in both tissues. Moreover, the affinities of both ligands were lower than those in the human platelet membranes where ^3H -yohimbine and ^3H -rauwolscine appeared to be of almost equal affinity (table V.1).

V.2.1.2 Kinetics studies

The specific binding of ^3H -yohimbine and ^3H -rauwolscine to either rat brain or rat kidney membranes at R.T. was rapid and reversible, reaching equilibrium in 5-10 minutes and 20-30 minutes respectively, and remained stable for more than 1 hour. In either tissue, significant differences in the apparent rate constants between the two ligands were observed. For example, in the rat cerebral membranes, although both ^3H -yohimbine and ^3H -rauwolscine appeared to exhibit similar association rate constants (K_1) at 22-25°C (0.089 $\text{nM}^{-1} \text{min}^{-1}$ and 0.076 $\text{nM}^{-1} \text{min}^{-1}$ respectively), the dissociation rate constant (K_2) for ^3H -yohimbine was significantly higher than that for ^3H -rauwolscine (0.42 min^{-1} and 0.19 min^{-1} respectively; fig. V.3,4). Thus, the kinetically derived dissociation constant ($\frac{K_2}{K_1}$) for ^3H -yohimbine

Ligands		Human Platelet	Human Cerebral Cortex	Rat Cerebral Cortex	Rat Renal Cortex	Rabbit Cerebral Cortex	Rabbit Renal Cortex
³ H-yohimbine	Bmax (fmol/mg.P.)	148 ± 9	-	125 ± 17	124.14 ± 19.31	134.6 ± 20.1	91.97 ± 5.27
	Kd (nM)	0.59 ± 0.08	-	4.7 ± 0.9	7.53 ± 0.09	5.97 ± 0.03	11.04 ± 0.83
³ H-rauwolscine	Bmax (fmol/mg.P.)	144 ± 5	135 ± 6	108 ± 7	120.38 ± 10.2	134.3 ± 8.2	76.73 ± 6.32
	Kd (nM)	0.55 ± 0.06	2.08 ± 0.3	1.8 ± 0.2	2.68 ± 0.29	6.31 ± 0.48	5.61 ± 0.55

Table V.I

The maximal binding capacities (Bmax, fmol/mg. Protein) and dissociation constants (Kd, nM) for specific ³H-yohimbine and ³H-rauwolscine binding to various tissue membranes. Data was analysed by Scatchard plots as described in Chapter III. Results presented are means ± S.E.M. of at least 3-4 separate experiments performed in duplicate or triplicate.

Legends to Figures V.1 and 2

Figure V.1.

Specific binding of ^3H -rauwolscine and ^3H -yohimbine binding to the rat cerebral cortical membranes. Saturation experiments for both ligands (0 - 20 nM) were carried out at R.T. as described in Methods (Chapter III). Left panel: Saturation isotherms. Right panel: Scatchard plots. Data from a single parallel experiment representative of three performed in duplicate. Bmax values for ^3H -rauwolscine and ^3H -yohimbine are 119 f.mole/mg protein and 127 f.mole/mg protein respectively. Kd values are 2.0 nM and 4.5 nM respectively.

Figure V.2.

Specific binding of ^3H -rauwolscine and ^3H -yohimbine binding to rat renal cortical membranes. Saturation experiments for both ligands (0 - 20 nM) were carried out at R.T. as described in Chapter III. Left panel: Saturation isotherms. Right panel: Scatchard plots of binding data. Data illustrated is from a single parallel experiment representative of four performed in duplicate. The Bmax values (f.mole/mg. protein) for ^3H -rauwolscine and ^3H -yohimbine are 132 and 136 respectively. The Kd values (nM) are 2.14 and 7.65 respectively.

Fig. V.1.

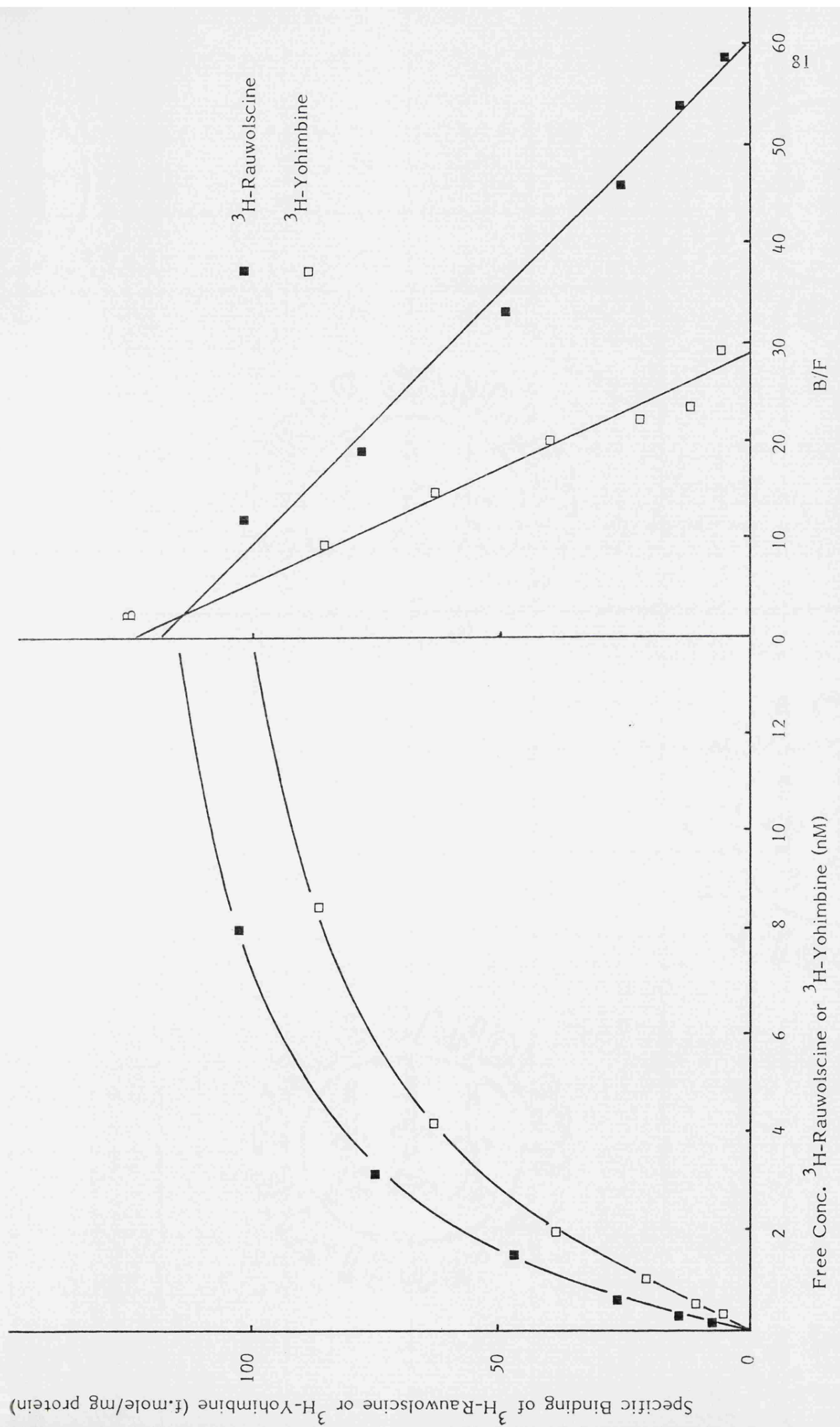
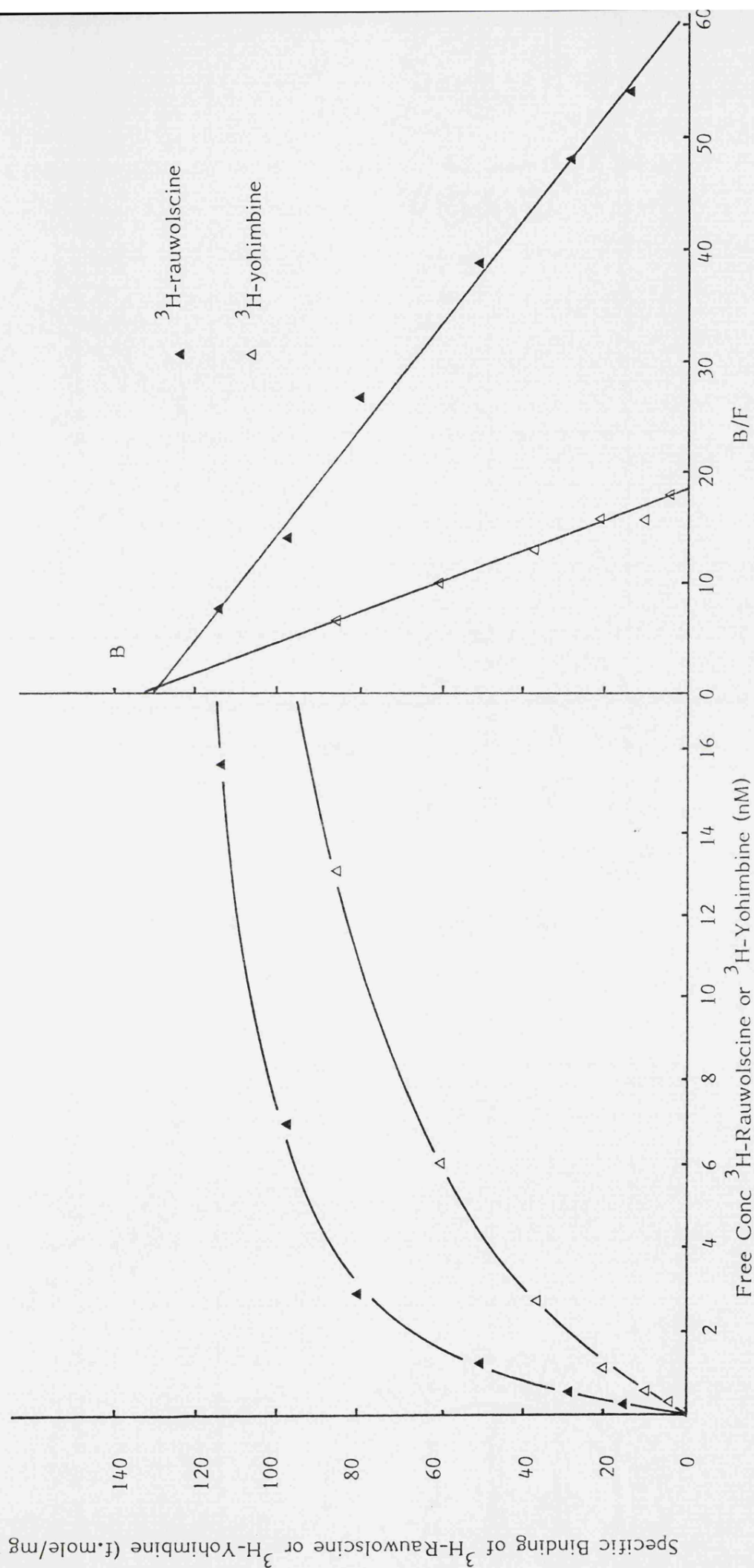


Fig. V.2.



Legends to figures V.3, 4, 5 and 6**Figures V.3 and 4**

Kinetics of ^3H -rauwolscine and ^3H -yohimbine binding to rat cerebral membranes at R.T. Fig. V.3, ^3H -rauwolscine binding; Fig. V.4., ^3H -yohimbine binding. Left panels: association curves; the insets show the pseudo first-order rate plots for the association reactions. Right panels: dissociation curves; the insets show the first-order rate plots for the dissociation.

Data are from single experiments, which were performed twice with duplicate determinations.

Figures V.5 and 6

Kinetics of ^3H -rauwolscine and ^3H -yohimbine binding to rat renal cortical membranes at R.T. Fig. V.5, ^3H -rauwolscine binding; Figure V.6., ^3H -yohimbine binding. Left panels: association curves, with the insets showing the pseudo first-order rate plots. Right panels: dissociation curves, with the insets showing the first-order dissociation rate plots. Data illustrated are from representative experiments, which were performed twice with duplicate determinations.

Fig. V.3.

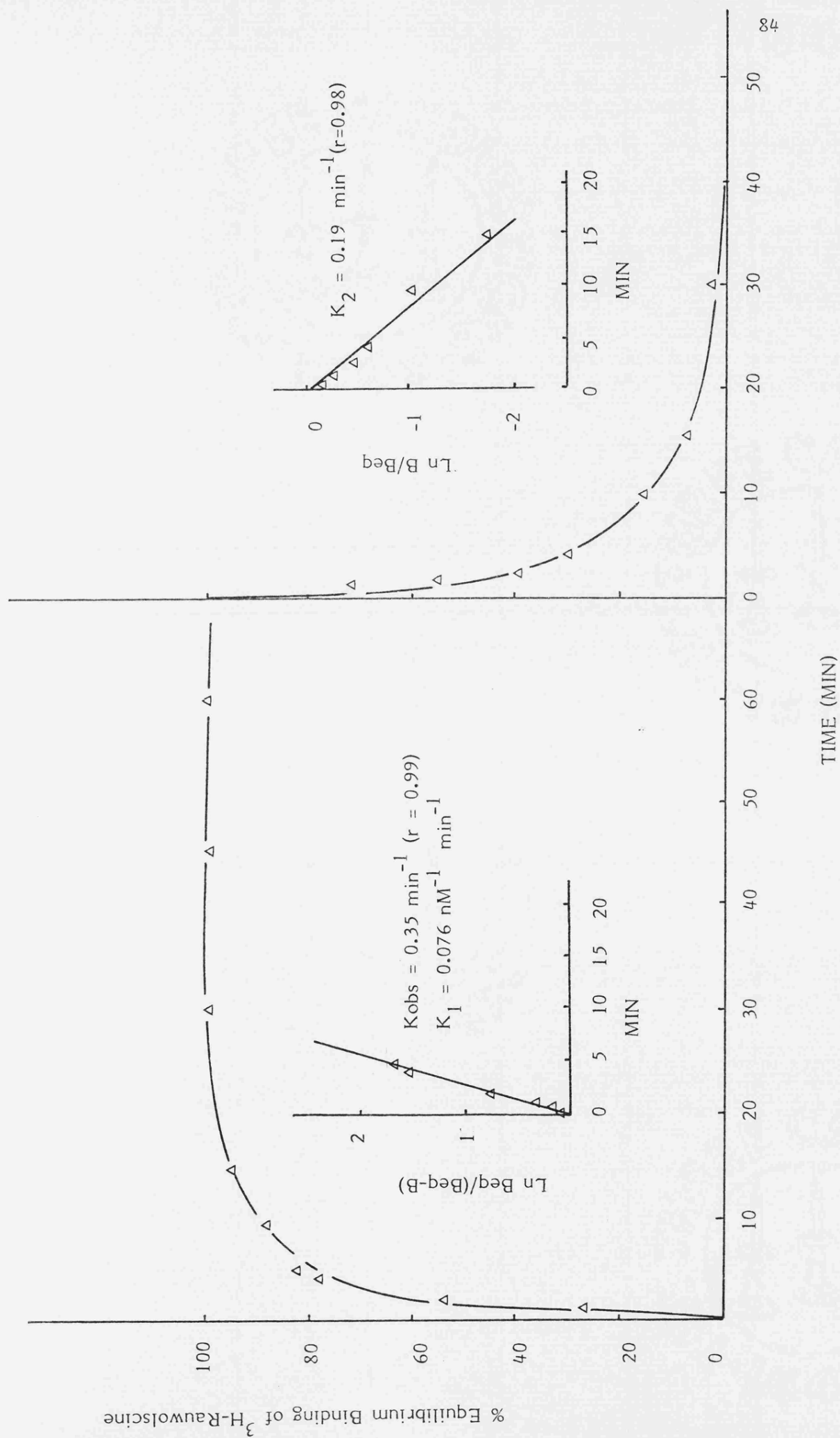


Fig. V.4.

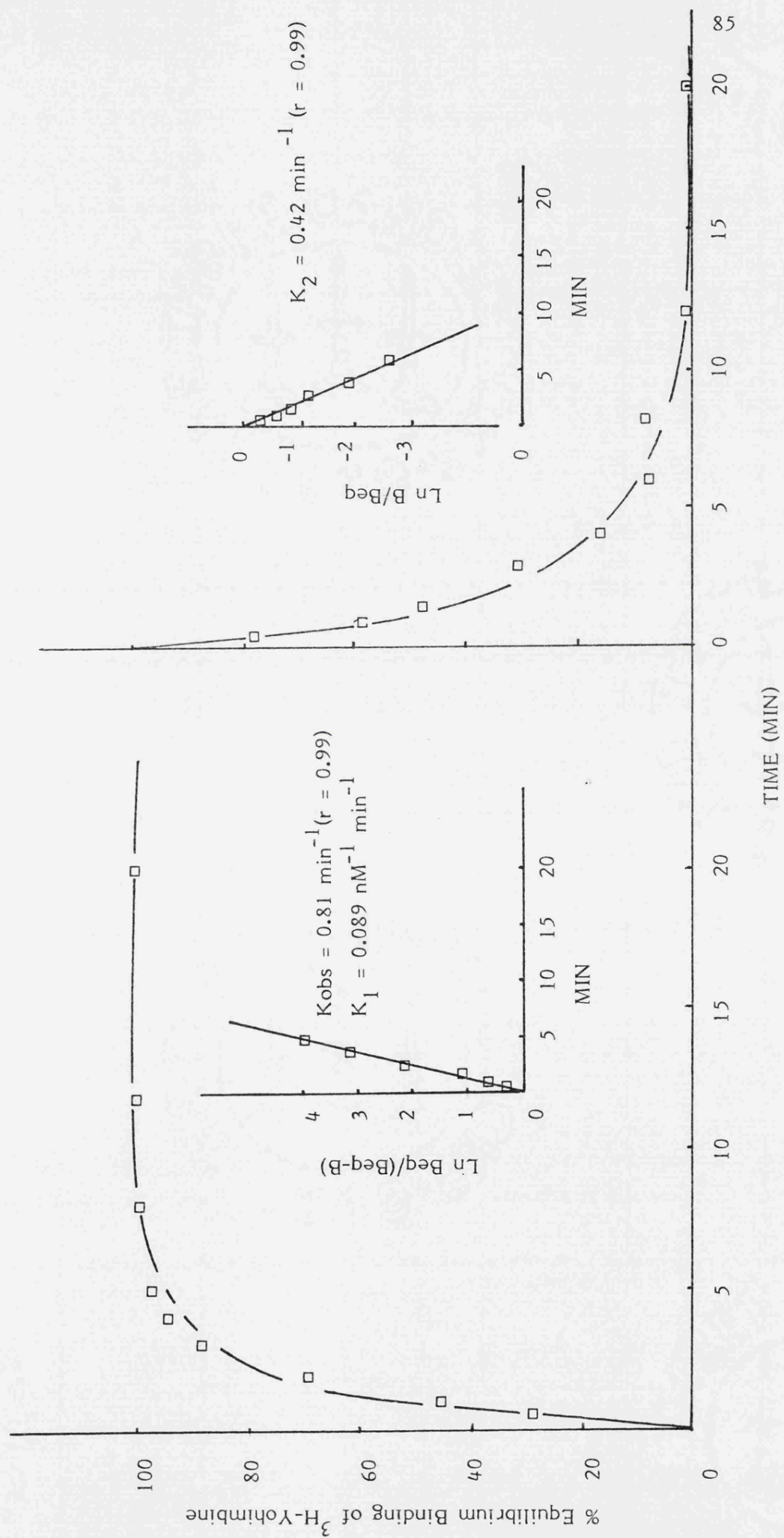


Fig. V.5.

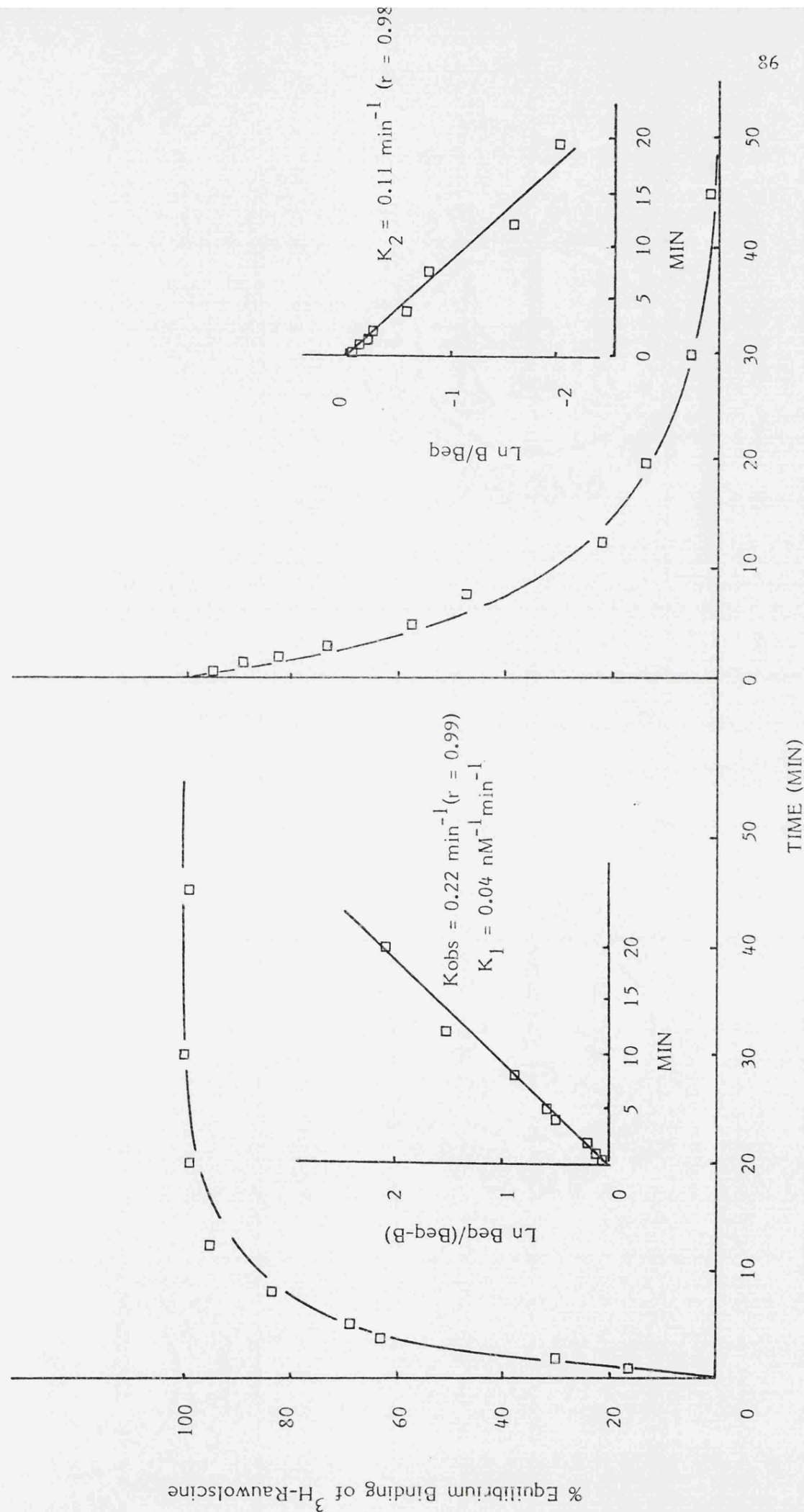
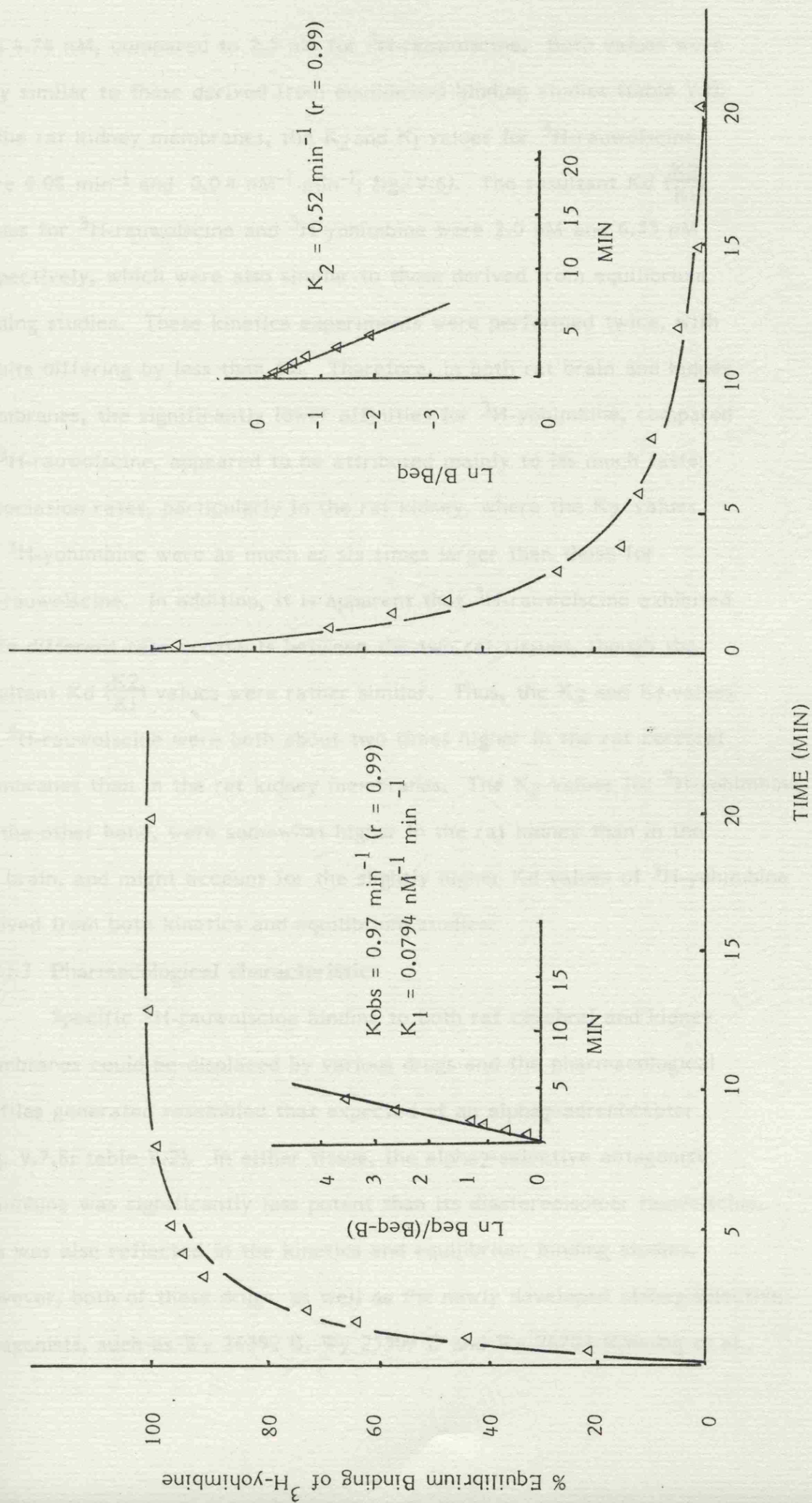


Fig. V.6.



was 4.74 nM, compared to 2.5 nM for ^3H -rauwolscine. Both values were very similar to those derived from equilibrium binding studies (table V.1). In the rat kidney membranes, the K_2 and K_1 values for ^3H -rauwolscine were 0.08 min^{-1} and $0.04 \text{ nM}^{-1} \text{ min}^{-1}$; fig. V.6). The resultant $K_d \left(\frac{K_2}{K_1} \right)$ values for ^3H -rauwolscine and ^3H -yohimbine were 2.0 nM and 6.55 nM respectively, which were also similar to those derived from equilibrium binding studies. These kinetics experiments were performed twice, with results differing by less than 5%. Therefore, in both rat brain and kidney membranes, the significantly lower affinities for ^3H -yohimbine, compared to ^3H -rauwolscine, appeared to be attributed mainly to its much faster dissociation rates, particularly in the rat kidney, where the K_2 values for ^3H -yohimbine were as much as six times larger than those for ^3H -rauwolscine. In addition, it is apparent that ^3H -rauwolscine exhibited quite different rate constants between the two rat tissues, though the resultant $K_d \left(\frac{K_2}{K_1} \right)$ values were rather similar. Thus, the K_2 and K_1 values for ^3H -rauwolscine were both about two times higher in the rat cerebral membranes than in the rat kidney membranes. The K_2 values for ^3H -yohimbine, on the other hand, were somewhat higher in the rat kidney than in the rat brain, and might account for the slightly higher K_d values of ^3H -yohimbine derived from both kinetics and equilibrium studies.

V.2.1.3 Pharmacological characteristics

Specific ^3H -rauwolscine binding to both rat cerebral and kidney membranes could be displaced by various drugs and the pharmacological profiles generated resembled that expected of an α_2 -adrenoceptor (fig. V.7,8; table V.2). In either tissue, the α_2 -selective antagonist yohimbine was significantly less potent than its diastereoisomer rauwolscine, this was also reflected in the kinetics and equilibrium binding studies. However, both of these drugs, as well as the newly developed α_2 -selective antagonists, such as Wy 26392 B, Wy 25309 C and Wy 26703 (Cheung et al.,

Drugs	Rat Cerebral Cortex		Rat Renal Cortex	
	³ H-rauwolscine	³ H-yohimbine	³ H-rauwolscine	³ H-yohimbine
Rauwolscine	6.28 ± 0.15 (0.94)	6.29 ± 0.49 (0.96)	3.64 ± 0.28 (1.06)	/
Yohimbine	9.52 ± 0.85 (0.91)	11.25 ± 0.85 (0.98)	7.98 ± 0.7 (0.92)	
Wy 26703	9.19 ± 2.98 (0.94)	-	12.43 ± 1.17 (1.03)	
Wy 26392B	5.47 ± 0.96 (1.02)	-	6.53 ± 0.03 (0.95)	
Wy 25309C	7.37 ± 1.62 (0.98)	-	11.31 ± 0.32 (0.95)	
Phentolamine	8.15 ± 1.16 (0.88)	7.94 ± 0.64 (0.88)	13 ± 0.2 (0.93)	
Prazosin	157 ± 17 (0.99)*	199 ± 36 (0.92)*	53 ± 6.14 (1.02)	
Corynanthine	5.64 ± 25 (0.97)	797 ± 182 (1.14)	336 ± 2.22 (0.93)	
Indoramin	973 ± 90 (0.99)	-	1023 ± 52 (0.97)	
Clonidine	24.6 ± 1.3 (0.78)	23.7 ± 5 (0.88)	38 ± 6.68 (0.93)	
Adrenaline	67.6 ± 5 (0.66)	64 ± 21 (0.59)	94 ± 5.42 (0.75)	
Noradrenaline	103.6 ± 10.4 (0.67)	91.5 ± 10.4 (0.57)	74 ± 4.73 (0.73)	
Dopamine	1796 ± 257 (0.88)	2236 ± 665 (0.82)	1702.6 ± 49.8 (0.80)	
Isoprenaline	16966 ± 5792 (0.93)	20233 ± 4441 (0.90)	16293 ± 1063 (0.68)	

Table V.2

Inhibition constants (K_i, nM) of drugs in competition with specific ³H-rauwolscine and ³H-yohimbine binding to rat cerebral cortical and renal cortical membranes. Experiments were performed at 2-3 nM ³H-rauwolscine or ³H-yohimbine. K_i values were determined as described in Chapter III. Results presented are means ± S.E.M. of 3-6 experiments performed in duplicate. Numbers in parentheses are mean slope factors for individual displacement curves. * indicates a statistical significance (p < 0.05) in comparison with value in the rat renal cortical membranes

Legends to figures V.7 and 8

Figure V.7 Displacement of specific ^3H -rauwolscine binding to rat cerebral membranes by antagonists.

Figure V.8 Displacement of specific ^3H -rauwolscine binding to rat renal cortical membranes by antagonists.

Experiments were performed as described in Chapter III at 2-3 nM of ^3H -rauwolscine. Each data point represents the mean of 3-6 separate experiments performed in duplicate. S.E.M. is generally less than $\pm 5\%$.

Fig. V.7.

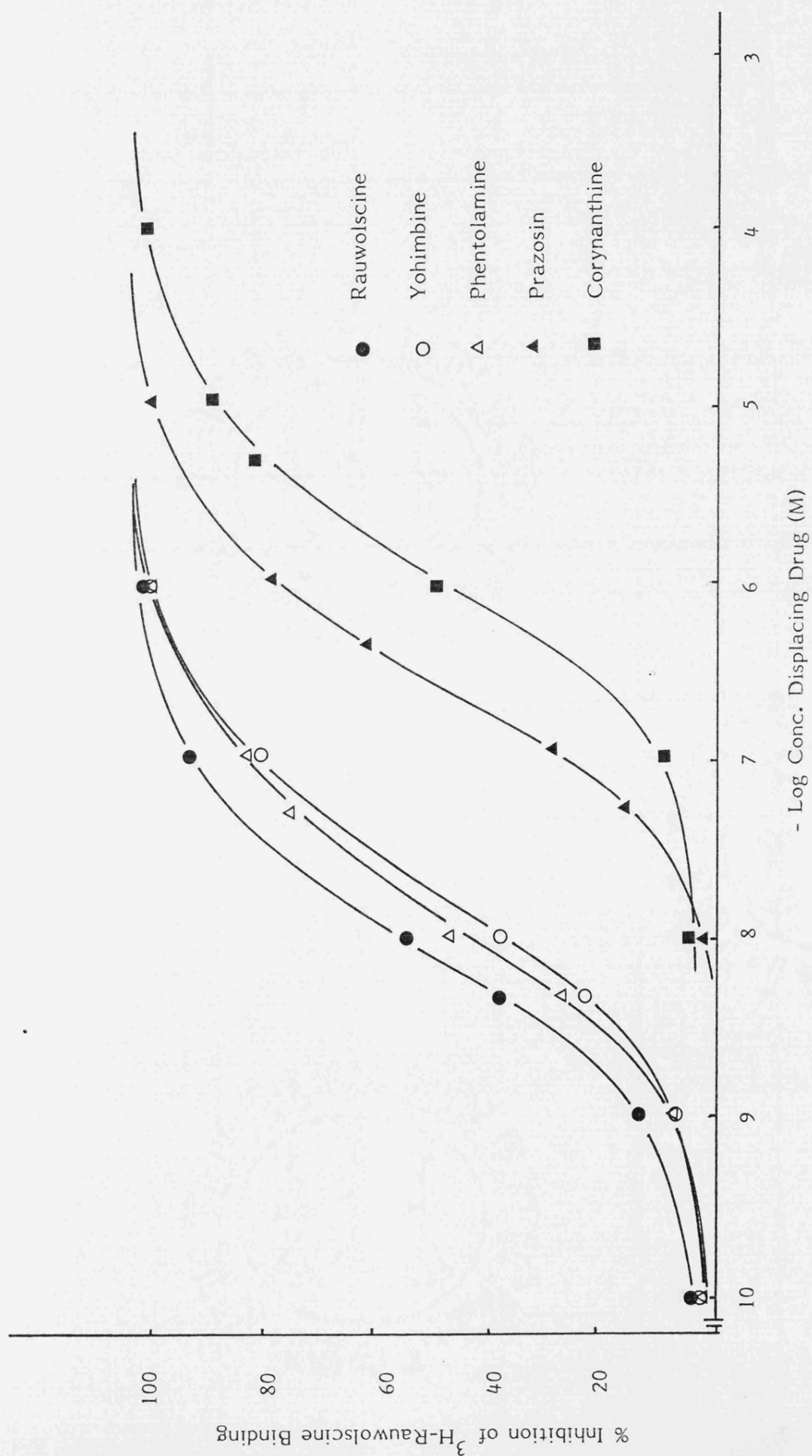
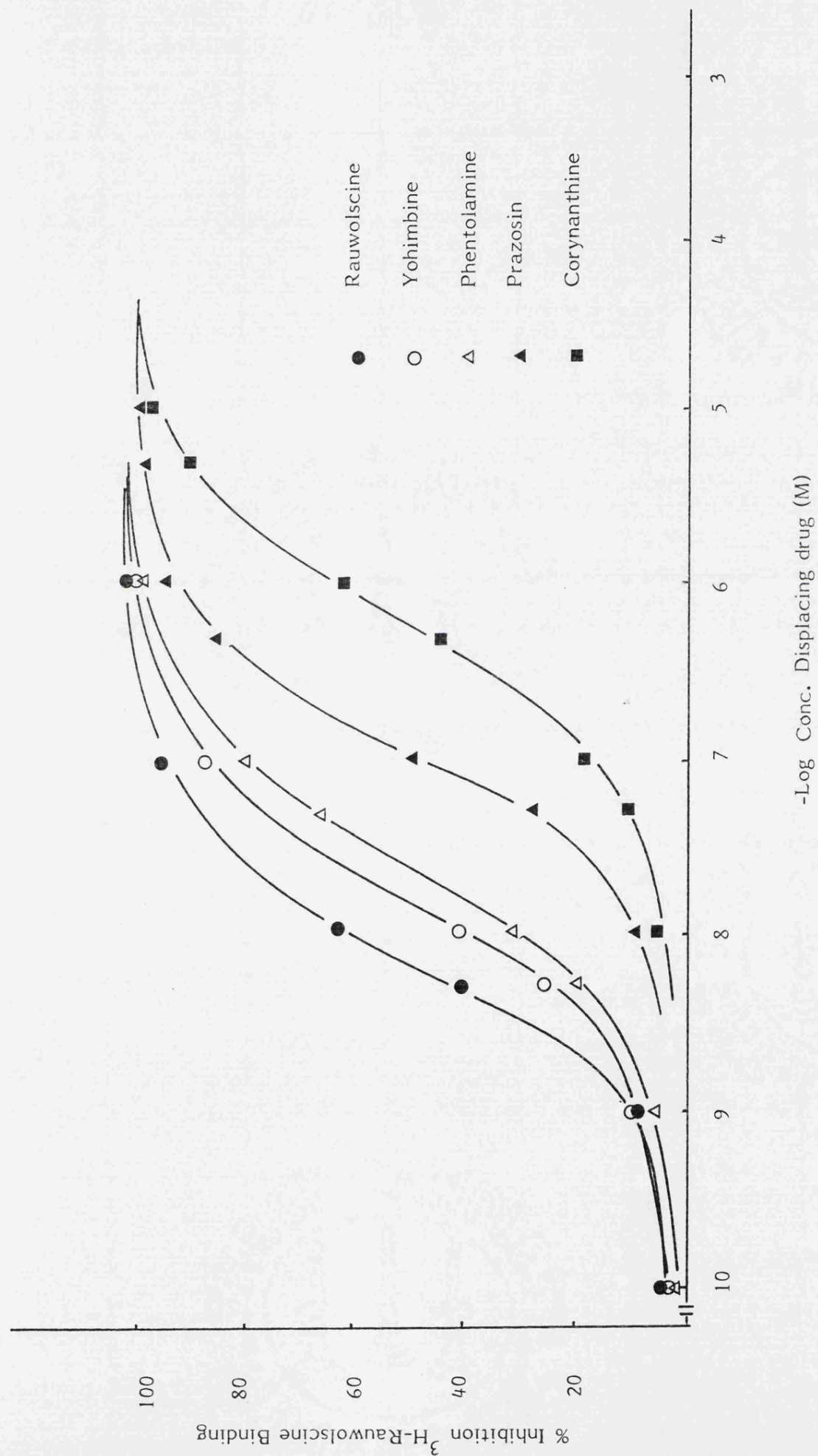


Fig. V.8.



1984) were all of relatively high affinities, compared to the α_1 -selective antagonists corynanthine (Weitzell et al., 1979), prazosin (Cambridge et al., 1977) and indoramin (Algate & Waterfall, 1978). Furthermore, the agonists adrenaline, noradrenaline and clonidine were much more potent than dopamine and the beta-adrenoceptor agonist isoprenaline. The close similarity of the K_i values of the drugs in competition with ^3H -rauwolscine and ^3H -yohimbine binding in the rat cerebral membranes further confirms that, as previously shown in the human platelet membranes, the sites labelled by both ligands are pharmacologically identical (table V.2). It is most noticeable that in the rat brain and rat kidney, prazosin displayed a high affinity relative to yohimbine or rauwolscine (fig. V.7,8; table V.2). This behaviour does not appear to be one normally expected of interaction with a 'typical' α_2 -adrenoceptor, since in the human platelet, prazosin possesses a much lower affinity (Chapter IV). It may also be worth noticing that, not only were the affinities of Wy 26703, Wy 26392B and Wy 25309C, in line with yohimbine and rauwolscine, significantly lower in the rat tissues than in the platelet, but the relative order of potency among these α_2 -antagonists looked quite different between the tissues (tables V.2,4). However, it must be emphasized that all the antagonists, including prazosin, generated curves with slopes close to unity, suggesting interaction with a homogeneous class of receptor sites. The agonist competition curves, in contrast, are of relatively shallow slopes. This probably reflects binding of the agonists to different affinity states of the α -adrenoceptor sites labelled by the antagonists with equal affinity. (See chapter VI)

V.2.2 Characterisation of α_2 -adrenoceptors in the rabbit cerebral and renal membranes

V.2.2.1 Equilibrium binding studies with ^3H -yohimbine and ^3H -rauwolscine

Specific binding of ^3H -rauwolscine and ^3H -yohimbine to rabbit cerebral and renal membranes was rapid, saturable, and reversible. Both

ligands bound with high and equal affinities to an identical number of sites in the rabbit brain membranes (fig.V.9; table V.I). In contrast, although ^3H -yohimbine and ^3H -rauwolscine also labelled an almost equal number of sites in the rabbit kidney membranes, the affinity of ^3H -yohimbine was significantly lower than that of ^3H -rauwolscine (fig. V.10; table V.I). Scatchard plots of both ligands to either tissue were linear, suggesting interaction with a single class of sites. It is also clear that the affinities of ^3H -yohimbine and ^3H -rauwolscine are, like those in the rat kidney and rat brain, significantly lower than those in the human platelets (table V.I).

V.2.2.2 Kinetics studies

^3H -yohimbine and ^3H -rauwolscine bound with a fast rate to both rabbit brain and kidney membranes at 22-25°C. The binding of ^3H -yohimbine to either tissue was faster, reaching equilibrium in 10-20 minutes, compared to 20-30 minutes for ^3H -rauwolscine (fig. V.11,12; fig. V.13,14). The association rate constants (K_1) for ^3H -yohimbine and ^3H -rauwolscine in the rabbit brain membranes were $0.134 \text{ nM}^{-1} \text{ min}^{-1}$ and $0.08 \text{ nM}^{-1} \text{ min}^{-1}$ respectively (fig. V.11,12), and the respective dissociation rate constants (K_2) were 0.36 min^{-1} and 0.27 min^{-1} . Therefore, the kinetically derived dissociation constants $\frac{K_2}{K_1}$ for ^3H -yohimbine and ^3H -rauwolscine, calculated to be 2.77 nM and 3.38 nM respectively, were not only similar to each other, but in reasonably good agreement with the K_d values derived from equilibrium binding studies (table V.I). In the rabbit kidney membranes, the K_2 values for ^3H -yohimbine were significantly higher than those for ^3H -rauwolscine, although the K_1 values for both ligands were very similar. For instance, the K_2 value for ^3H -yohimbine was 0.2 min^{-1} , compared to 0.06 min^{-1} for ^3H -rauwolscine (fig. V.13,14). The K_1 values for the two ligands were $0.016 \text{ nM}^{-1} \text{ min}^{-1}$ and $0.02 \text{ nM}^{-1} \text{ min}^{-1}$ respectively. Therefore, similar to that observed in the rat kidney membranes, the significantly higher K_d values of ^3H -yohimbine

Legends to figures V.9 and 10

Figure V.9 Specific binding of ^3H -rauwolscine and ^3H -yohimbine to rabbit cerebral cortical membranes. Experiments were performed in parallel with both ligands. Data illustrated is from one experiment representative of 3 performed in duplicate. Left panel: saturation isotherms. Right panel: Scatchard plots. Bmax values (Fmol./mg.protein) for ^3H -rauwolscine and ^3H -yohimbine are 118 and 110 respectively. Kd values (nM) are 5.7 and 5.98 respectively.

Figure V.10 Specific binding of ^3H -rauwolscine and ^3H -yohimbine to rabbit renal cortical membranes. Data illustrated is from one representative experiment performed in parallel (n = 3). Left panel: Saturation isotherms. Right panel: Scatchard plots. Bmax values (fmol./mg.protein) for ^3H -rauwolscine and ^3H -yohimbine are 68 and 81.5 respectively. Kd values (nM) are 4.78 and 10.88 respectively.

Fig. V.9

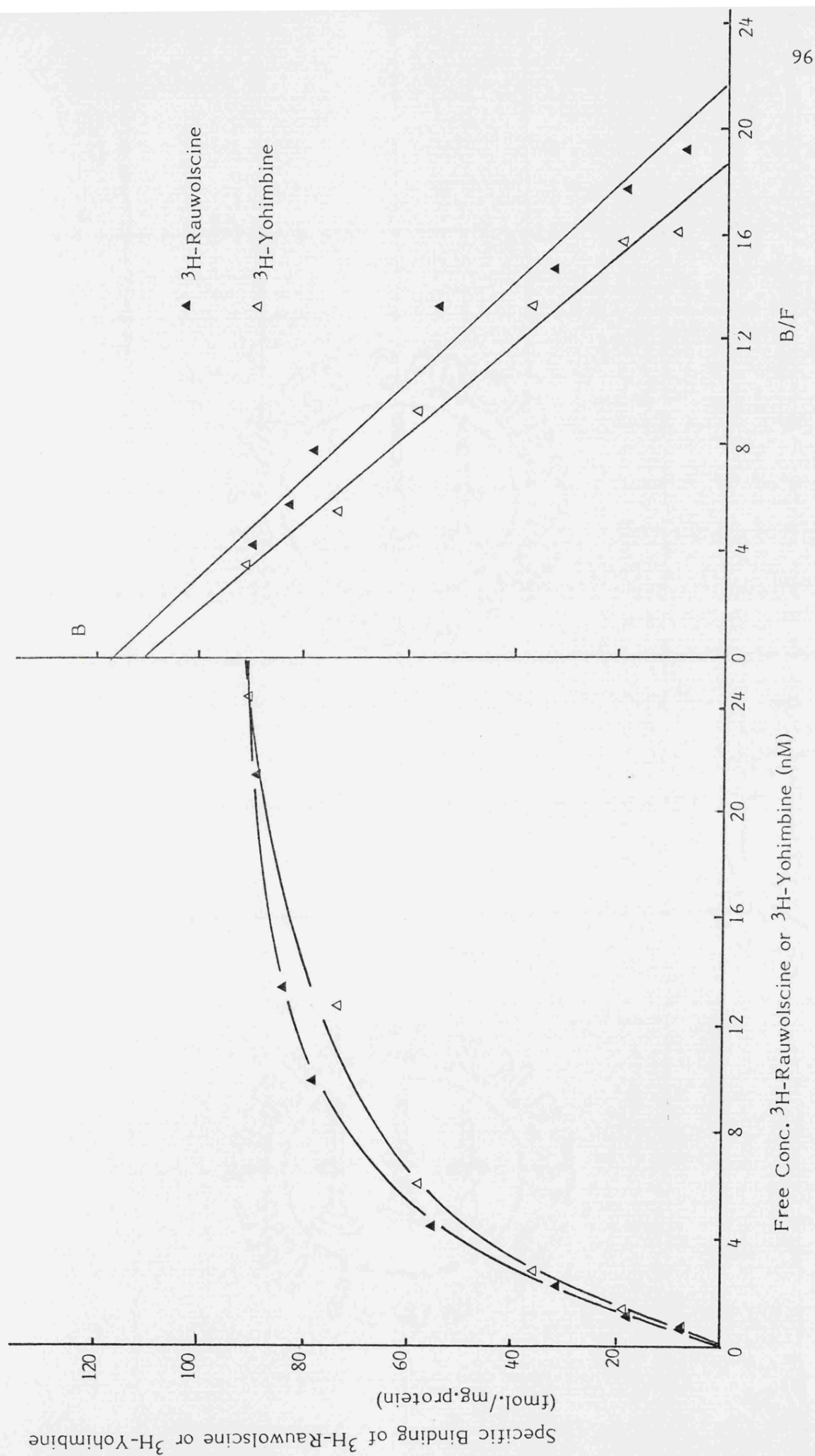
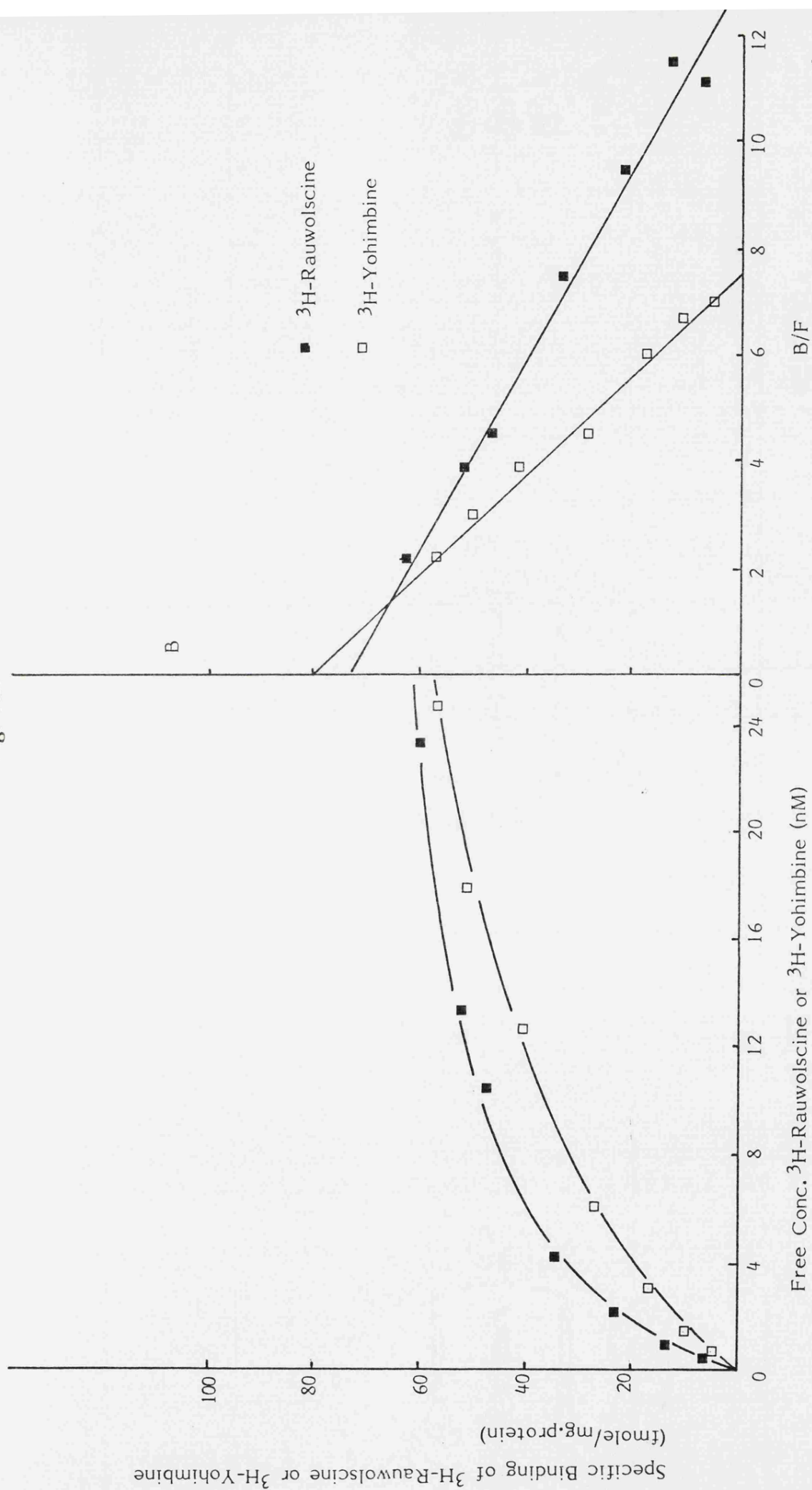


Fig. V.10



Legends to figures V.11, 12, 13 and 14

Figure V.11 Kinetics of ^3H -rauwolscine binding to rabbit cerebral membranes at R.T.

Figure V.12 Kinetics of ^3H -yohimbine binding to rabbit cerebral membranes at R.T.

Figure V.13 Kinetics of ^3H -rauwolscine binding to rabbit renal cortical membranes at R.T.

Figure V.14 Kinetics of ^3H -yohimbine binding to rabbit renal cortical membranes at R.T.

Experiments were performed as described in Chapter III.

Data presented are from one of two separate experiments performed in duplicate, with results differing by less than 5%. The left panels illustrate the association curves, with the pseudo first-order rate plots shown in the insets. The right panels illustrate the dissociation curves, with the first-order dissociation rate plots shown in the insets.

Fig. V.II

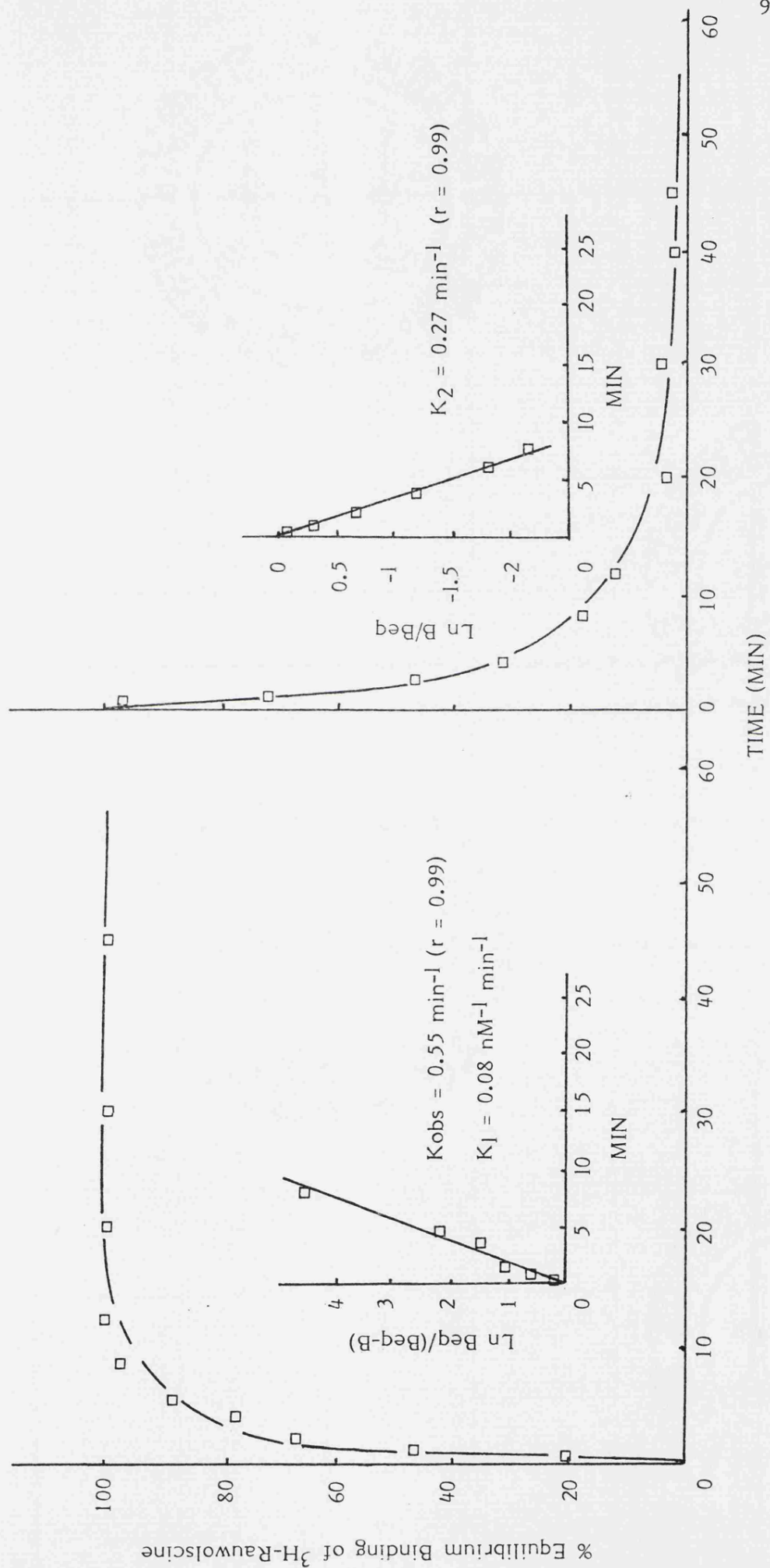


Fig. V.12

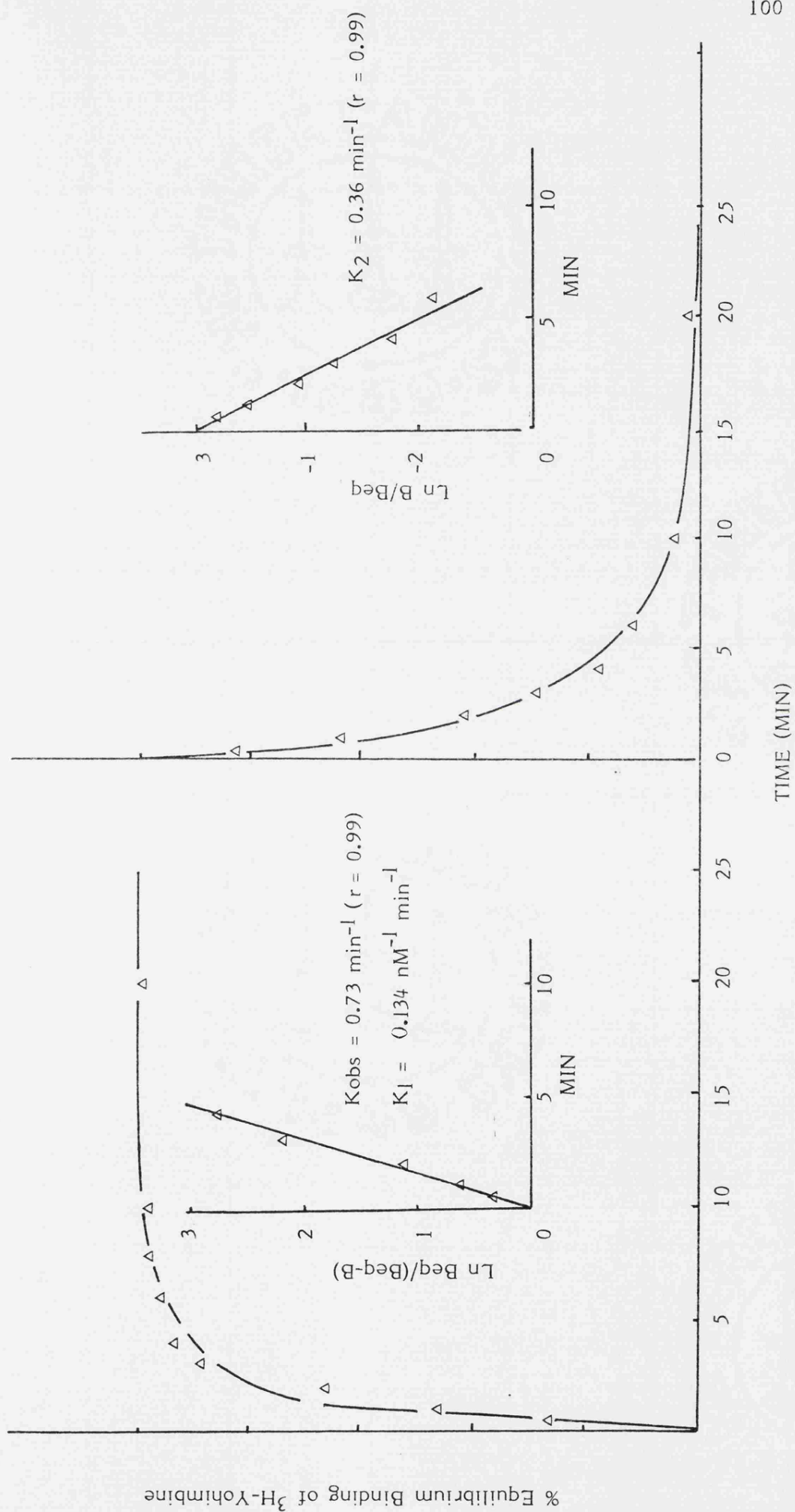


Fig. V.13

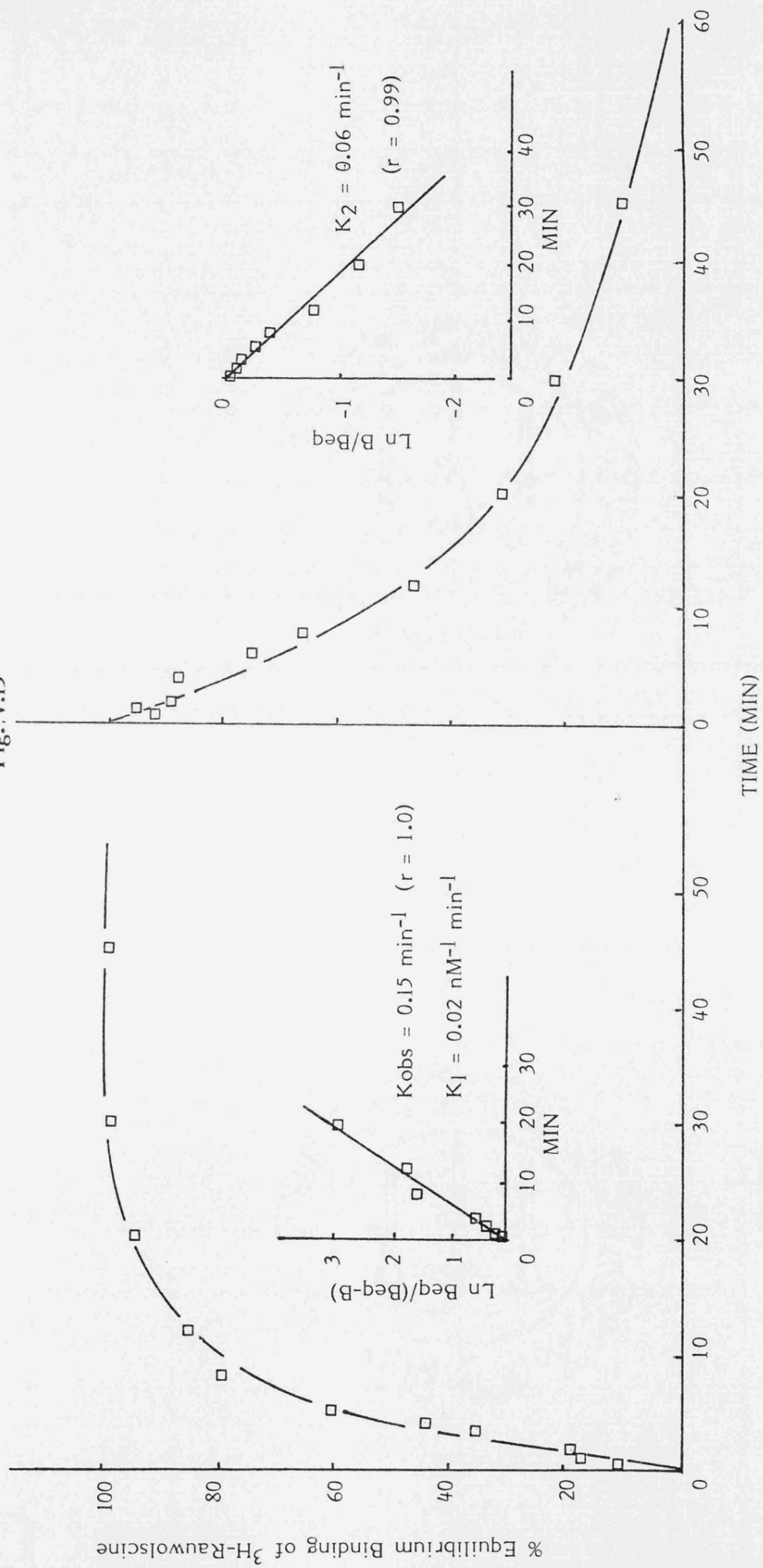
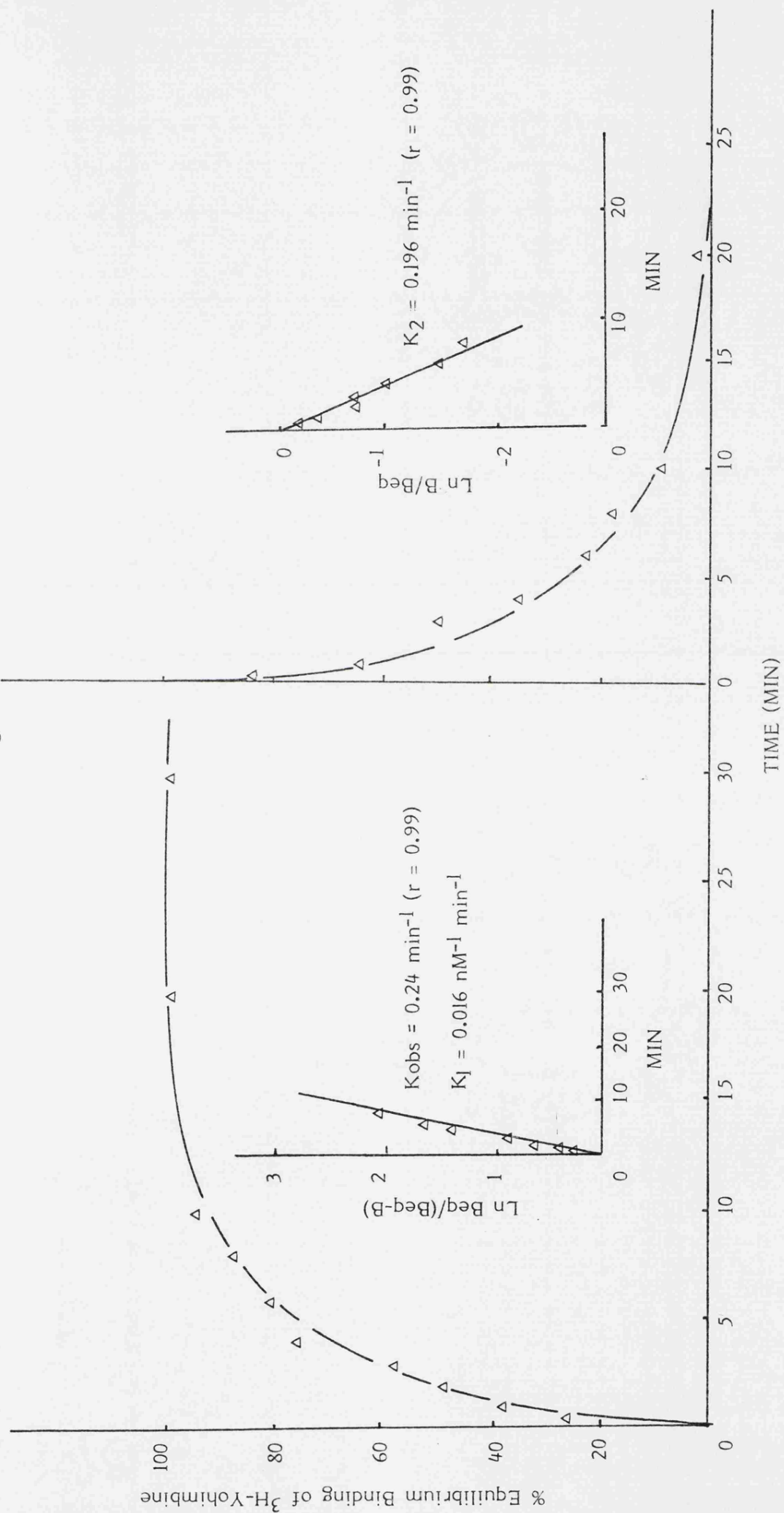


Fig. V.14



obtained from kinetics and equilibrium studies, compared to those of ^3H -rauwolscine, appeared to be mainly attributed to a faster dissociation of ^3H -yohimbine from the receptor sites. Where the affinities of the two ligands appeared similar, such as in the rabbit cerebral membranes, the respective association and dissociation rate constants were quite similar to each other. It is very apparent that ^3H -rauwolscine displayed very different binding kinetics between the rabbit brain and kidney membranes, with the K_2 and K_1 values about four times higher in the brain than in the kidney. The resultant K_d ($\frac{K_2}{K_1}$) values though, were very similar between the tissues. The rate constants for ^3H -yohimbine were also different between the two tissues. Although the dissociation rate constant (K_2) was somewhat higher in the brain than in the kidney (0.36 min^{-1} , compared to 0.2 min^{-1}), the significant difference in the resultant K_d ($\frac{K_2}{K_1}$) values between the tissues appeared to be mainly attributed to the more marked difference in the association rate constant (K_1) (e.g. $0.134 \text{ nM}^{-1} \text{ min}^{-1}$ in the rabbit brain, compared to only $0.016 \text{ nM}^{-1} \text{ min}^{-1}$ in the rabbit kidney).

V.2.2.3 Pharmacological Characteristics

The inhibition constants of various drugs in competition with ^3H -rauwolscine binding to rabbit cerebral and kidney membranes are listed in table V.3. It is evident that the pharmacological profiles generated for both tissues resembled that expected of an α_2 -adrenoceptor. Thus, in rabbit cerebral membranes, the α_2 -selective antagonists rauwolscine and yohimbine appeared equipotent (fig. V.15, table V.3), and both were significantly more potent than the α_1 -selective antagonists, corynanthine, indoramin and prazosin in that order. In the rabbit kidney membranes, rauwolscine was significantly more potent than yohimbine (fig. V.16, table V.3), an affinity difference which was also noted in the kinetics and equilibrium binding studies. The order of potency for other antagonists was phentolamine > prazosin > corynanthine > indoramin. The displacement curves for all the antagonists in either tissue had slopes close to unity,

	Rabbit Cerebral Cortex	Rabbit Renal Cortex
Rauwolscline	6.3 \pm 1.1 (1.0)	4.55 \pm 0.74 (1.1)
Yohimbine	6.9 \pm 0.98 (1.02)*	14.51 \pm 1.5 (1.03) ⁺
Phentolamine	11.3 \pm 0.5 (1.03)	13 \pm 0.64 (1.0)
Prazosin	4555 \pm 591 (1.03)*	303 \pm 11.3 (1.05)
Corynanthine	375 \pm 29 (1.0)	456 \pm 46 (0.97)
Indoramin	1662 \pm 45 (1.05)*	501 \pm 29.6 (1.04)
Clonidine	24 \pm 2.5 (0.68)*	70 \pm 7.3 (0.76)
Adrenaline	42.5 \pm 3.8 (0.67)*	254 \pm 16 (0.72)
Noradrenaline	59 \pm 5.3 (0.73)*	193 \pm 10.7 (0.68)
Dopamine	2633 \pm 275 (0.76)*	22833 \pm 1241 (0.64)
Isoprenaline	17717 \pm 1341 (0.65)*	61000 \pm 4501 (0.61)

Table V.3

Inhibition constants (K_i , nM) of drugs in competition with specific ^3H -rauwolscine binding to rabbit cerebral and renal cortical membranes. Experiments were performed at 2-4 nM ^3H -rauwolscine. Values presented are means \pm S.E.M. of 3-6 separate experiments performed in duplicate. Numbers in parentheses are mean slope factors of individual displacement curves. * Indicates a statistical significance ($p < 0.05$) in comparison with corresponding value determined in the rabbit renal cortical membranes. + indicates a statistical significant ($p < 0.05$) in comparison with K_i value for rauwolscine in the rabbit renal cortical membranes.

Legends to figures V.15 and 16

Figure V.15 Displacement of specific ^3H -rauwolscine binding to rabbit cerebral membranes by antagonists.

Figure V.16 Displacement of specific ^3H -rauwolscine binding to rabbit renal cortical membranes by antagonists.

Experiments were performed at 2-4 nM ^3H -rauwolscine.

Data points are means of at least 3-6 separate experiments performed in duplicate. S.E.M. for each point is generally less than $\pm 5\%$.

Fig. V.15

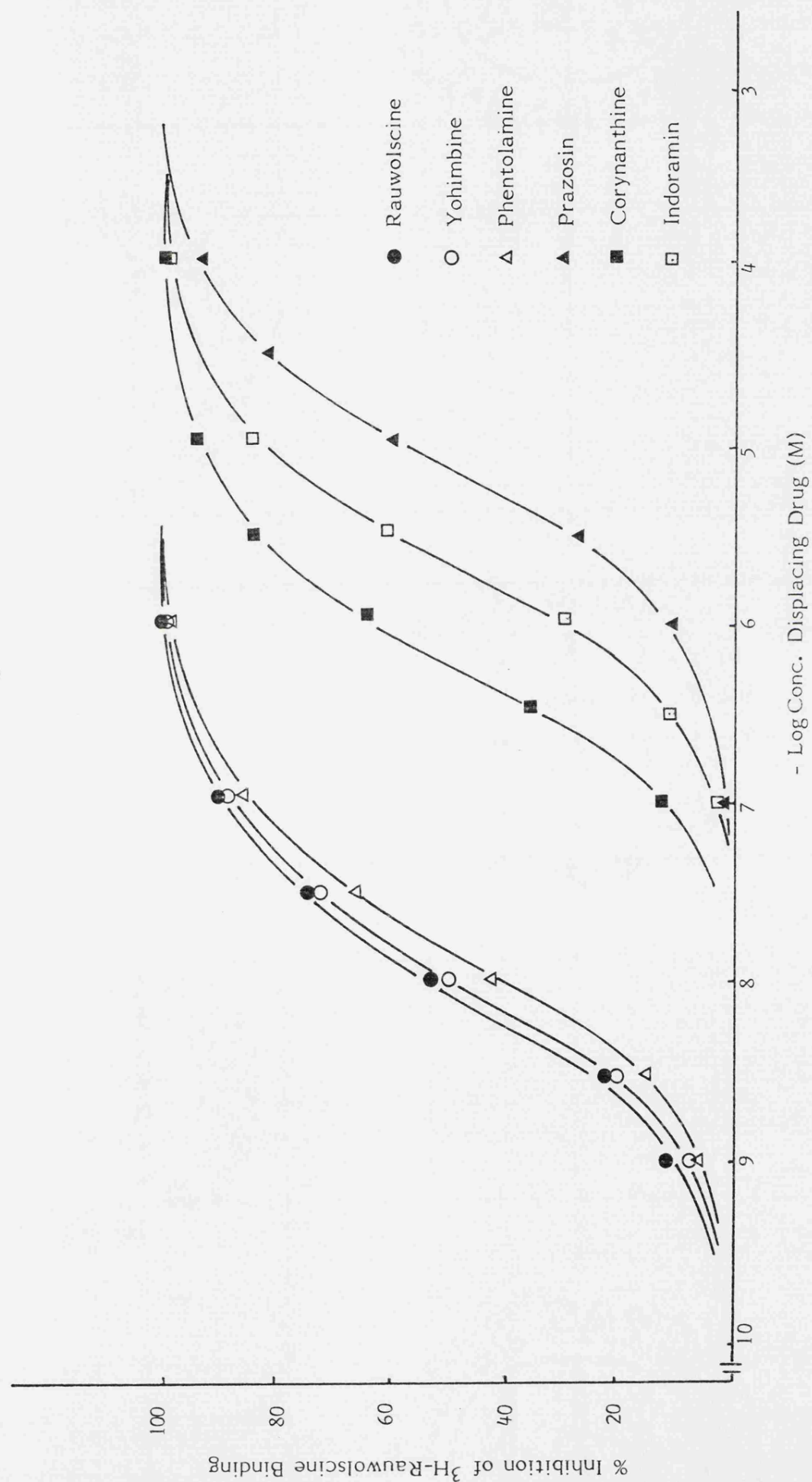
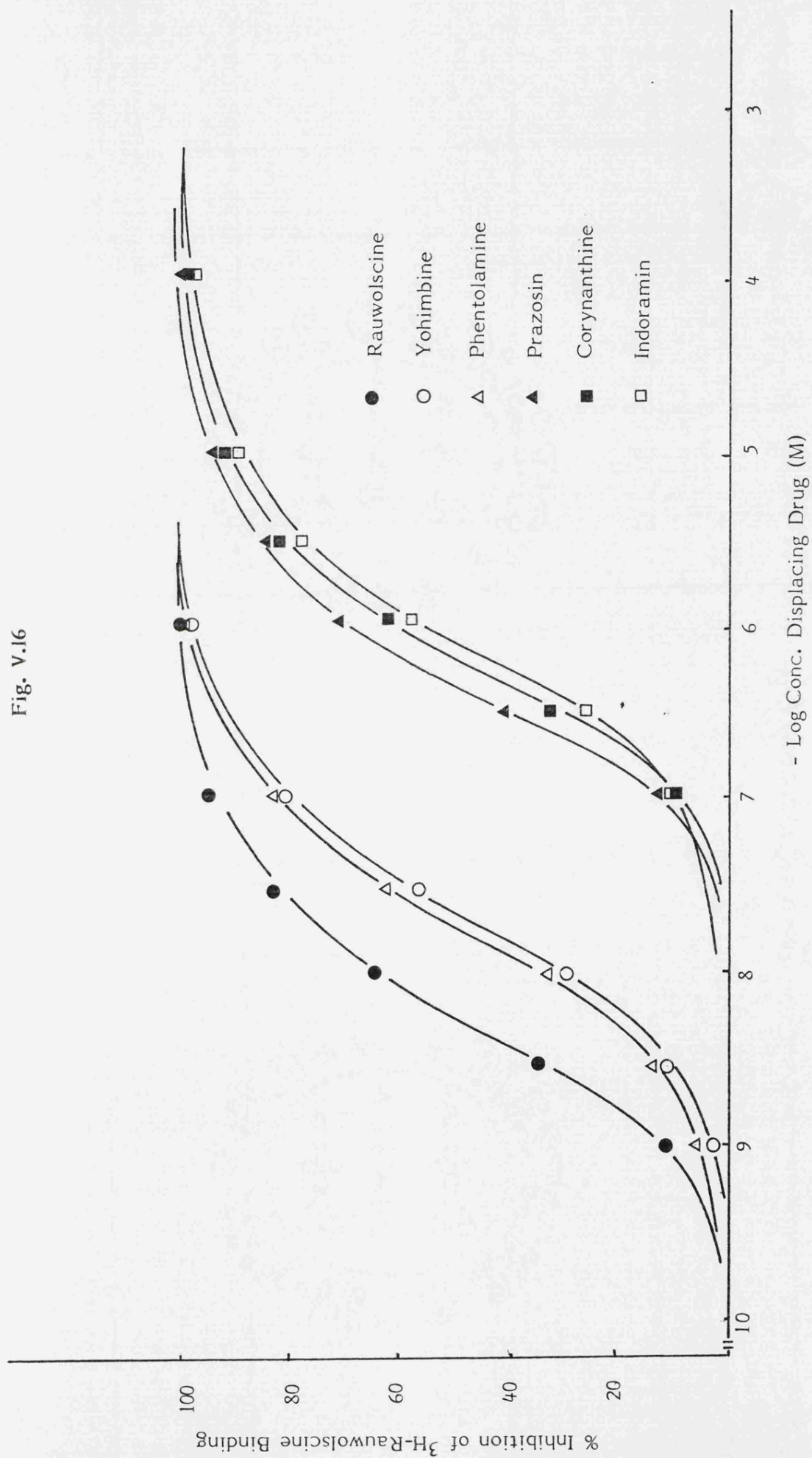


Fig. V.16



indicating interaction with a homogeneous class of sites or states (table V.3). However, it is very obvious that the affinity of the α_1 -antagonist prazosin was markedly higher in the rabbit kidney than in the rabbit brain (fig. V.15,16, table V.3). In addition, the less α_1 -selective antagonist, indoramin, also exhibited an affinity significantly higher in the rabbit kidney than in the rabbit brain.

In both of the rabbit tissues, the orders of agonist affinities were similar: clonidine > adrenaline > noradrenaline >> dopamine > isoprenaline (table V.3). The slopes of the competition curves of adrenaline, noradrenaline and clonidine were shallow, compared to those of the antagonists, suggesting agonist interaction with the receptor sites in heterogeneous affinity states (Hoffman et al., 1980_a).

V.2.3 Characterisation of α_2 -adrenoceptors in the human cerebral cortical membranes

V.2.3.1 Equilibrium and kinetics studies with ^3H -rauwolscine

^3H -rauwolscine exhibited high affinity binding to human cerebral cortical membranes in a saturable manner. The maximal binding capacity (B_{max}), determined from Scatchard analysis of equilibrium binding data, was 135 ± 6 f.mol./mg. protein ($n = 3$). The dissociation constant (K_d) of ^3H -rauwolscine was 2.08 ± 0.3 nM. The Hill Coefficient (n_H) was 1.06 ± 0.03 , indicating that binding was to a single population of non-interacting sites.

Kinetics studies showed that specific ^3H -rauwolscine and ^3H -yohimbine binding to human brain membranes was moderately fast at 22-25°C, reaching equilibrium in about 40-45 minutes. Compared to ^3H -rauwolscine, the association and dissociation rate constants for ^3H -yohimbine were both slightly higher. For example, the K_1 and K_2 values for ^3H -yohimbine were $0.028 \text{ nM}^{-1} \text{ min}^{-1}$ and 0.06 min^{-1} respectively, compared to $0.018 \text{ nM}^{-1} \text{ min}^{-1}$ and 0.04 min^{-1} for ^3H -rauwolscine. Similar experiments were

performed twice, each generating results that did not differ by more than 5%. The kinetically derived K_d values were 2.22 nM and 2.14 nM respectively for ^3H -rauwolscine and ^3H -yohimbine. These figures were in close agreement with the K_d or K_i values derived from saturation and competition experiments, which also indicated that the two ligands appeared to be of almost equal affinity in the human brain (table V.1,4). In comparison with the human platelet membranes, the K_2 values for both ligands were only slightly higher in the human brain membranes (e.g. 0.03 min^{-1} vs. 0.04 min^{-1} for ^3H -rauwolscine, and 0.04 vs. 0.06 min^{-1} for ^3H -yohimbine). The K_1 values though, were about 2-3 times lower for both ligands in the brain than in the platelets (e.g. $0.018 \text{ nM}^{-1} \text{ min}^{-1}$ vs. $0.073 \text{ nM}^{-1} \text{ min}^{-1}$ for ^3H -rauwolscine, and $0.028 \text{ nM}^{-1} \text{ min}^{-1}$ vs. $0.054 \text{ nM}^{-1} \text{ min}^{-1}$ for ^3H -yohimbine). Thus, the significantly lower K_d values for ^3H -rauwolscine and ^3H -yohimbine in the human platelet membranes, compared to human brain membranes, appeared to be mainly due to the higher association rate constants.

V.2.3.2 Pharmacological Characteristics

The pharmacological behaviour of the sites labelled by ^3H -rauwolscine in the human cerebral membranes was similar to that in the human platelet membranes. Table V.4 shows the K_i values of drugs in competition with specific ^3H -rauwolscine binding to the human cerebral membranes. The rank order of antagonist potency was clearly typical of one expected at an α_2 -adrenoceptor: rauwolscine \approx yohimbine > phentolamine > corynanthine > prazosin. The competition curves of the antagonists all generated slopes close to unity, suggesting interaction with a single class of binding sites. The agonists clonidine, adrenaline and noradrenaline, in that order of affinity, were all much more potent than dopamine or isoprenaline, but the slopes of their competition curves were quite shallow, possibly suggesting interaction with the labelled sites in heterogeneous affinity states.

The sites labelled by ^3H -rauwolscine in the human brain possessed

	Human Platelet			Human Cerebral Cortex		
Rauwolscine	1.11	± 0.12	(1.00)	2.96	± 0.02	(0.98)
Yohimbine	0.85	± 0.09	(0.98)	3.3	± 0.3	(0.97)
Wy 26703	2.16	± 0.31	(0.96)	-		
Wy 26309B	2.44	± 0.26	(0.97)	-		
Wy 25309C	3.38	± 0.45	(0.93)	-		
Phentolamine	4.01	± 1.23	(0.99)	2.25	± 3	(0.91)
Corynanthine	192	± 23	(1.15)	503	± 7.1	(1.03)
Prazosin	760	± 194	(1.00)	887	± 31	(0.93)
Indoramin	1062	± 28	(0.99)	2990	± 275	(1.02)
Clonidine	8.89	± 0.48	(0.83)	37.8	± 10.7	(0.68)
Adrenaline	28.9	± 4.9	(0.79)	69	± 10.7	(0.68)
Noradrenaline	88.7	± 13	(0.91)	245	± 45	(0.59)
Dopamine	977	± 143	(0.89)	3969	± 593	(0.81)
Isoprenaline	36850	± 10450	(0.86)	34353	± 13288	(0.72)

Table V.4

Inhibition constants (K_i , nM) of drugs in competition with specific ^3H -rauwolscine binding to human platelet and human cerebral cortical membranes. Experiments were performed at about 2nM ^3H -rauwolscine. Values presented are means \pm S.E.M. of 3-4 separate experiments performed in duplicate. Numbers in parentheses are mean slope factors of individual displacement curves.

a pharmacological behaviour very similar to that observed in the human platelet. In either tissue, yohimbine appeared equipotent with rauwolscine. Moreover, the relative order of potency between corynanthine and prazosin was similar in these two tissues, with corynanthine being more potent than prazosin. In contrast, prazosin was relatively more potent than corynanthine in the rat brain, rat kidney, as well as the rabbit kidney. Indeed, the significantly higher affinity of prazosin in these tissues, compared to the human tissues, seemed to be accompanied with a weaker potency of yohimbine relative to its diastereoisomer, rauwolscine. Finally, it is noteworthy that the absolute affinities displayed by most of the drugs examined were significantly lower in the human brain, compared to the human platelet, although the rank order of drug potency was the same in both tissues (table V.4)

V.3 Discussion

The results presented in these studies have provided further evidence that both ^3H -yohimbine and ^3H -rauwolscine can label an identical population of non-interacting sites when studied in parallel within a given tissue. Indeed, these two ligands have now been widely employed to study α_2 -adrenoceptors. In view of the reservations that are required concerning the use of ^3H -agonists (Hoffman et al., 1980_b), it would seem appropriate that both ^3H -yohimbine and ^3H -rauwolscine should be considered the ligands of choice at the moment for probing α_2 -adrenoceptors.

The recent suggestion by Perry and U'Prichard (1981) that ^3H -rauwolscine may be a more suitable ligand than ^3H -yohimbine for central α_2 -adrenoceptors receives no support in the present studies. It has been clearly demonstrated that, when these ligands were studied in parallel in either the human platelet or rat cerebral membranes, they labelled an equal quantity of receptor sites exhibiting identical pharmacological properties. Nevertheless, a significant affinity difference between the two ligands does exist in some, though not all the tissues. Thus, ^3H -rauwolscine and ^3H -yohimbine labelled sites with almost equal affinity in the human platelets, human brain, as well as the rabbit brain, but ^3H -rauwolscine exhibited an affinity 2-3 times higher than that of ^3H -yohimbine in the rat brain, rat kidney and rabbit kidney. Moreover, both ^3H -yohimbine and ^3H -rauwolscine displayed affinities significantly higher in the human platelet membranes than in the other membranes examined.

The affinity differences between these ligands within a given tissue or between different tissues are also reflected in the differences of their binding kinetics. As described earlier, in tissues such as the rat brain, rat kidney and rabbit kidney, where ^3H -yohimbine displayed a higher affinity than ^3H -rauwolscine, the dissociation rate constants for ^3H -yohimbine were significantly higher than those for ^3H -rauwolscine. Yet, differences

in the rate constants for either ligand also occurred between tissues within a given species, or across different species. For example, the K_2 values for both ^3H -yohimbine and ^3H -rauwolscine were significantly higher in the rat and rabbit tissues than in the human tissues, the differences being more pronounced with ^3H -yohimbine. Characteristically, the K_2 and K_1 values for ^3H -rauwolscine were also significantly higher in the rat and rabbit brain membranes, compared to the kidney membranes of respective species. In addition, the markedly lower association rate constant for ^3H -yohimbine in the rabbit kidney, compared to the rabbit brain, appeared accountable for its significantly higher K_d value. These inter- and intra-tissue differences in the binding kinetics, as well as the dissociation constants of ^3H -yohimbine and/or ^3H -rauwolscine have provided evidence for molecular differences among the α_2 -adrenoceptors.

Further support for the possible heterogeneity of α_2 -adrenoceptors comes from the results of the drug displacement studies. It appears certain that, in each of the tissues examined, the sites labelled by ^3H -yohimbine and ^3H -rauwolscine satisfy the criteria for classification as α_2 -adrenoceptors. However, the pharmacological profiles generated can be broadly, but distinctly, divided into two main categories. One category is chiefly characterised by the equal potency/affinity of the α_2 -diastereoisomers, yohimbine and rauwolscine, as well as the low overall affinities for the α_1 -antagonists, such as prazosin and indoramin. The α_2 -adrenoceptors in the human platelets and human brain can be described by this pharmacological behaviour. The other category is characterised by the differential affinities of the yohimbine and rauwolscine diastereoisomers, which apparently is also associated with higher affinity and/or relative potency for the α_1 -antagonists, of which prazosin is a distinctive example. The α_2 -adrenoceptors of the rat brain and rat kidney are best described by this pharmacological pattern. Apparently, although the overall pharmacological profiles for

the α_2 -receptors in the human platelet and human brain are similar, the absolute affinities of a number of drugs, including yohimbine and rauwolscine are significantly lower in the brain than in the platelet (table V.4). At least some differences in the binding kinetics of ^3H -yohimbine and ^3H -rauwolscine are observed between the two tissues. However, when the ratios of the K_i values of individual drugs relative to that of rauwolscine are compared between the two receptors, they exhibit a rather remarkable similarity. Hence, the apparent differences in the absolute affinities of the drugs between these tissues might partly be attributed to some differences in the membrane environment or constraint, though minor molecular differences in the receptor proteins cannot yet be ruled out. The α_2 -adrenoceptor in the rat brain resembles that in the rat kidney not only in the rank order of drug potency, but also in the absolute affinities of the drugs. A more apparent difference may perhaps be the relatively higher affinity of prazosin in the rat kidney than in the rat brain.

Species difference becomes obvious when the pharmacological behaviour of the α_2 -adrenoceptors is compared between the human and rat tissues (tables V.2,4). A more pronounced contrast may be observed between the human platelet and the rat kidney (fig. V.17). It is clear that the affinities of rauwolscine and yohimbine in the rat kidney are not only different, but they are both significantly lower than those in the human platelet. In addition, the affinities of phentolamine, clonidine and adrenaline are all significantly lower in the rat kidney than in the human platelet. The strongest contrast, however, is the more significantly reduced affinity of yohimbine, and the markedly higher potency of prazosin in the rat kidney, compared to the human platelet. Although the affinity of indoramin (also α_1 -selective) in the rat kidney is not apparently different from that in the human platelet membranes, it is quite significantly higher than that in the human brain membranes. Moreover, when the potency of indoramin relative to rauwolscine is compared between the platelet membranes and

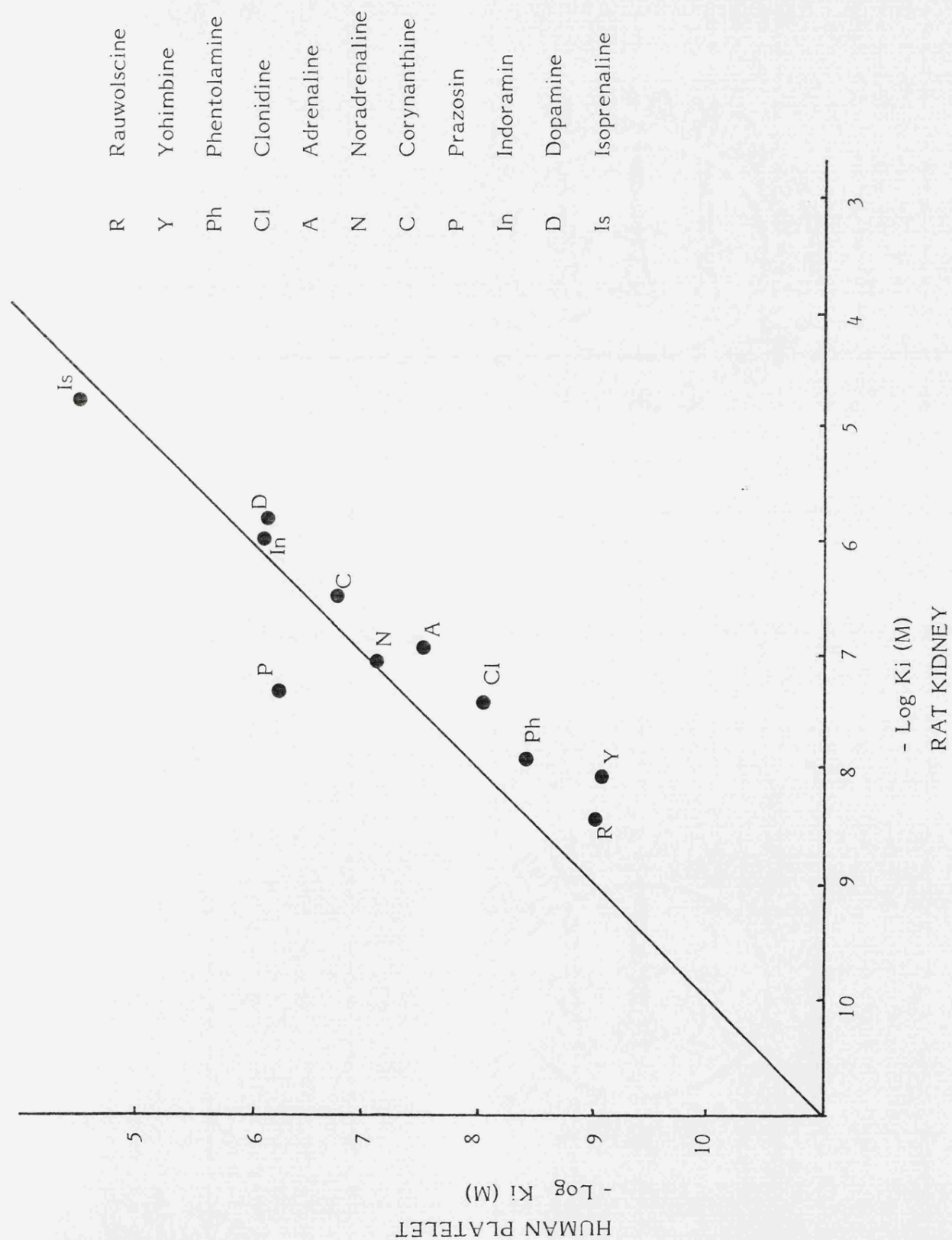
Legends to figures V.17 and 18

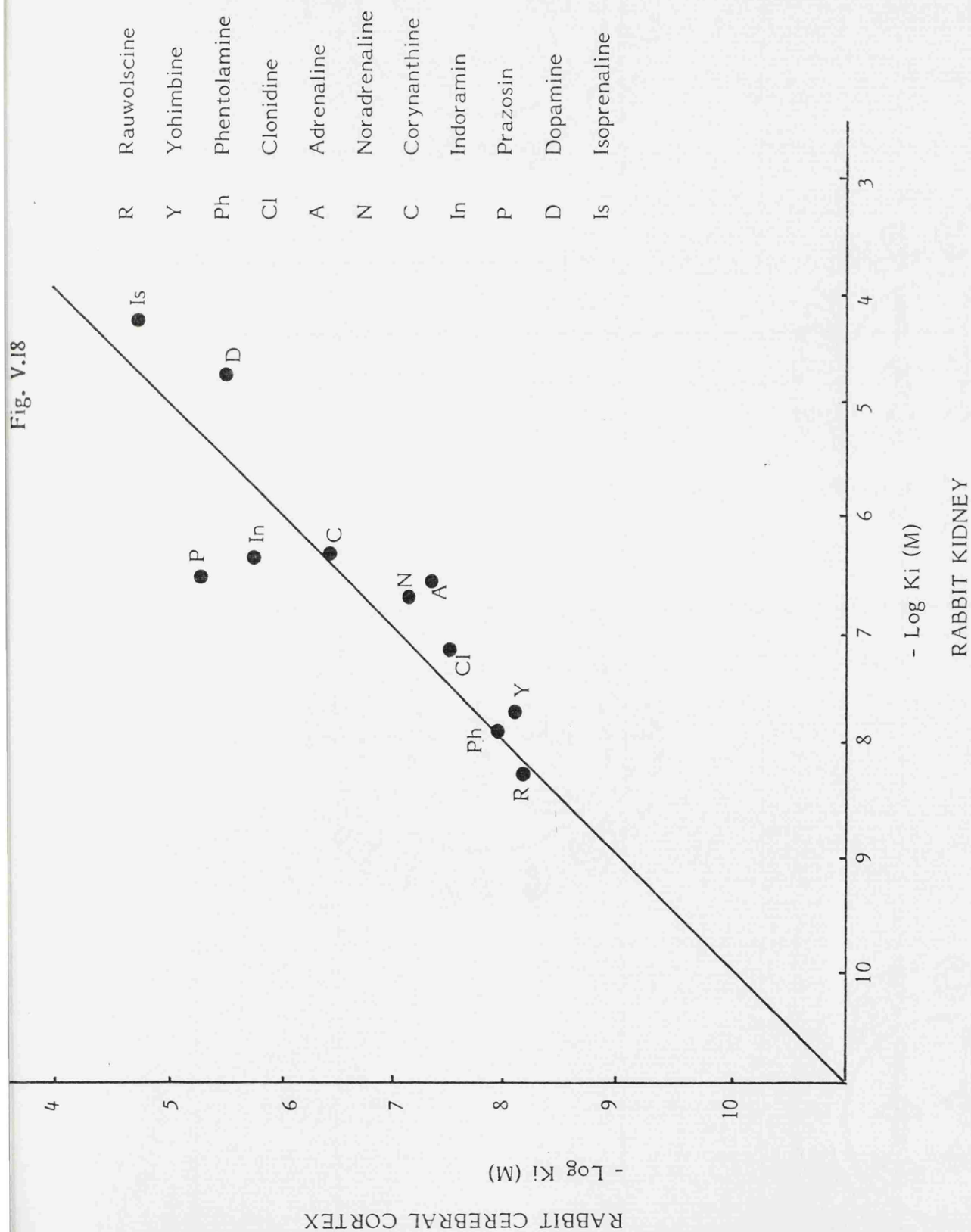
Figure V.17 Comparison of drug affinities at α_2 -adrenoceptors in the human platelet and rat kidney membranes.

Figure V.18 Comparison of drug affinities at α_2 -adrenoceptors in rabbit cerebral and renal cortical membranes.

K_i values, determined from competition with ^3H -rauwolscine binding, were compared between tissues. The line drawn through the intersection of the X- and Y-axes is the line of identity, on which drug affinities are equal between tissues.

Fig. V.17





and the rat kidney membranes, it is clear that this α_1 -antagonist has, like prazosin, a significantly higher relative potency in the rat kidney than in the human platelet.

However, the pharmacological differences observed do not appear to be merely a reflection of species difference. Evidence for inter-tissue differences of α_2 -adrenoceptors within a given species has come not only from the equilibrium and kinetics studies with ^3H -rauwolscine and ^3H -yohimbine, but also from competition studies (tables V.1,3). The comparison of drug affinities between the rabbit brain and kidney is described in fig. V.18. Yohimbine, clonidine, adrenaline and noradrenaline are significantly weaker in the rabbit kidney than in the rabbit brain. In contrast, the α_1 -antagonists prazosin and indoramin are significantly more potent in the rabbit kidney than in rabbit brain. Therefore, pharmacologically, the α_2 -adrenoceptor in the rabbit kidney appears more similar to that in the rat brain or rat kidney. The rabbit cerebral α_2 -adrenoceptor, on the other hand, more resembles that in the human platelet or human brain.

The possibility that ^3H -rauwolscine might label in part the α_1 -adrenoceptors in the rat brain, rat kidney or rabbit kidney where prazosin displayed a low overall inhibition constant can be ruled out, as almost all the antagonists, including prazosin, generated competition curves with slope close to unity in all the tissues examined. As shown in table V.5, either rauwolscine or yohimbine exhibited very low affinity for α_1 -adrenoceptor sites labelled with high affinity by ^3H -prazosin in the rat cerebral and renal membranes. Considering the low concentrations (2-4 nM) of ^3H -rauwolscine or ^3H -yohimbine used for the drug displacement studies with α_2 -adrenoceptors it is hardly possible that any α_1 -adrenoceptors could have been labelled. Furthermore, the overall pharmacological profiles of the ^3H -rauwolscine labelled sites in all the tissues also indicate that it is most unlikely (at least under the conditions of the present assays) that there is any significant interaction of the labelled ligands with other receptor systems.

	Rat Cerebral Cortex	Rat Renal Cortex
	Ki (nM)	Ki (nM)
Prazosin	0.31 ± 0.03 (0.97)	0.265 ± 0.005 (0.97)
Indoramin	25 ± 3.3 (1.02)	35.7 ± 4.45 (1.0)
Corynanthine	262.5 ± 30.6 (1.01)	293 ± 41 (1.08)
Rauwolscine	2147 ± 53.8 (0.98)	2343 ± 63.2 (0.99)
Yohimbine	1015 ± 69.5 (1.03)	1123 ± 50.3 (1.07)

Table V.5

Inhibition constants of adrenergic agents in competition with specific ^3H -prazosin binding to rat cerebral and kidney membranes. ^3H -prazosin binding assays were performed at 1-1.5 nM under conditions similar to those with ^3H -rauwolscine or ^3H -yohimbine binding assays described in Methods in Chapter III. Equilibrium binding constant (K_d) and binding capacity (B_{max}) of ^3H -prazosin were 0.21 nM and 110 fmol/mg.p. respectively in cerebral membranes and 0.29 nM and 35.2 fmol/mg.p. respectively in kidney membranes (N=2). The k_i values presented above are means \pm S.E.M. of three separate experiments performed in duplicate. Numbers in parentheses are mean slope factors of individual displacement curves.

The affinity of agonists and antagonists for α_2 -adrenoceptors appeared to be modulated by mono- and divalent cations (Daiguiji et al., 1981; Barnett et al., 1982; Cheung et al., 1982; also see chapter VI). In this connection, it was found in the present studies that 10 mM Mg^{++} could increase the IC₅₀ values of prazosin (in competition for 3H -rauwolscine binding) by 1.5-2 fold in both human platelets and rat kidney. In contrast, Na^+ at 100 mM reduced the IC₅₀ values of prazosin by 2-3 fold in both of these tissues. However, it is highly unlikely that such concentrations of residual cations could be present in the membrane preparations, considering that membranes prepared in these studies were extensively washed in the presence of EDTA. Furthermore, as the small effects of cations of prazosin also appear quantitatively similar between tissues, it is clear that the marked inter-tissue difference in prazosin affinity for sites labelled by 3H -rauwolscine cannot be adequately accounted for by quantitative difference in ionic effects.

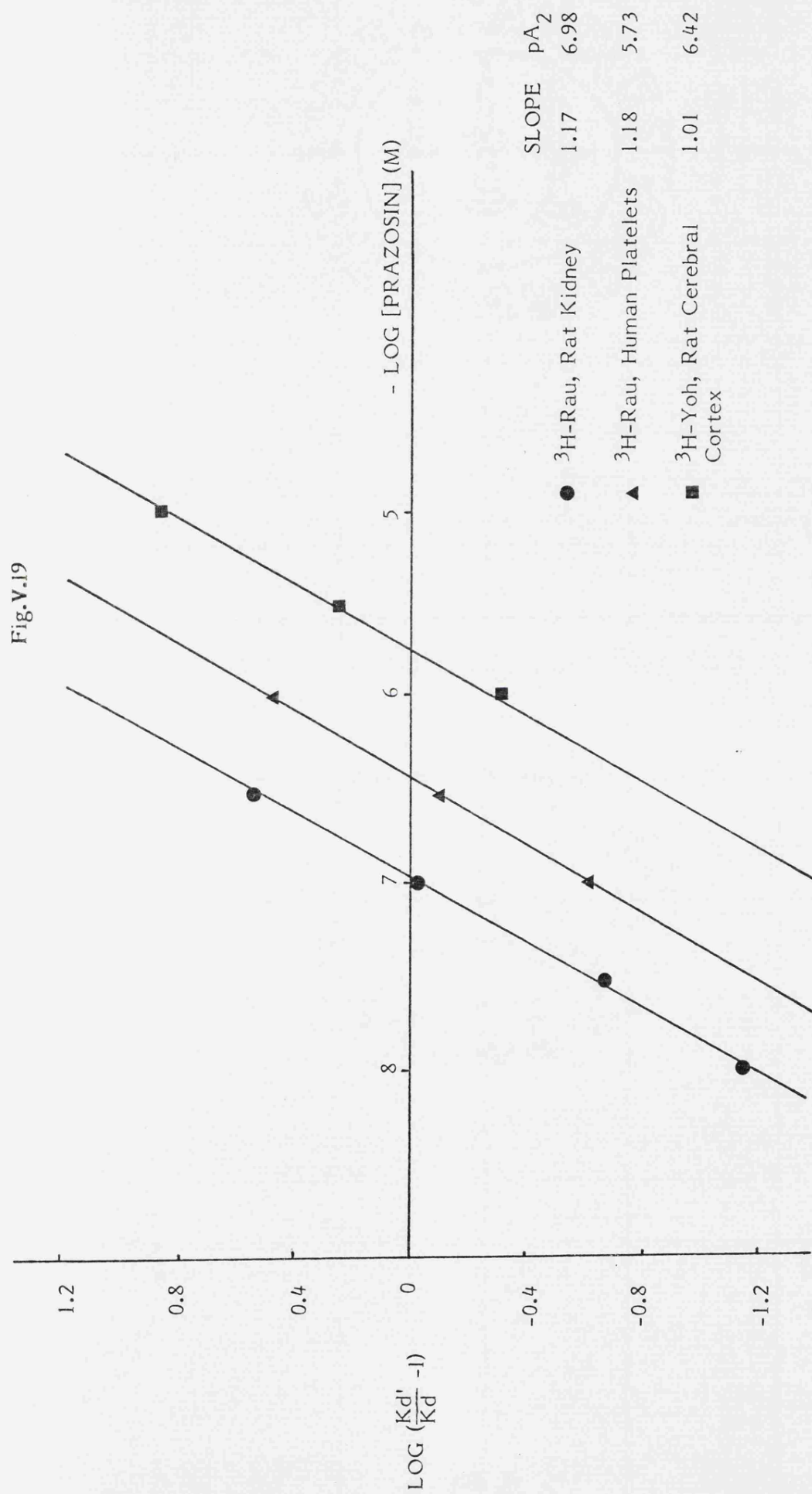
Recently, it was demonstrated that the neuromuscular blocker gallamine can allosterically modulate the binding of agonists and antagonists to muscarinic receptors in some, though not all tissues (Stockton et al., 1983). Similarly, allosteric regulation of the binding of nimodipine (Ca^{++} -antagonist) by d-cis-diltiazem to Ca^{++} -channel binding sites in some tissues has also been described. (Ferry & Glossmann, 1983_a). Therefore, it seems necessary to ascertain if the higher affinity of prazosin for 3H -rauwolscine or 3H -yohimbine labelled sites in certain tissues might actually reflect an allosteric interaction rather than a true receptor heterogeneity. In this connection, it was found that increasing concentrations of prazosin merely increased the K_d values of 3H -rauwolscine or 3H -yohimbine in the human platelet, rat brain or rat kidney without altering the B_{max}. As illustrated in fig. V.19, the Schild plot for each tissue is linear, with a slope not distant from the theoretical value of 1.0, thus suggesting a competitive nature of the antagonism between

Legend to figure V.19

Figure V.19 Competitive interaction of prazosin with α_2 -adrenoceptors.

Saturation experiments were carried out with ^3H -rauwolscine or ^3H -yohimbine as described in Chapter III, in the absence and presence of different concentrations of prazosin. Schild plots were constructed by plotting $\log(\frac{K_d'}{K_d} - 1)$ against log concentration of prazosin, where K_d' and K_d are dissociation constants of the radioligand in the presence and absence of prazosin respectively. The X-intercepts yield the pA_2 values, which are negative logarithms of the dissociation constants for prazosin.

Data shown are from single experiments, which were repeated twice, and had almost identical results.



prazosin and the labelled ligand. Moreover, it is obvious that the affinities of prazosin (apparent pA_2 values) are distinctly different between the three tissues, and in good agreement with the results from simple competition experiments. Non-competitive allosteric interaction between prazosin and 3H -rauwolscine or 3H -yohimbine might also be reflected in the alteration of the binding kinetics of the labelled ligand, particularly the dissociation kinetics. However, although prazosin at 30 nM reduced specific 3H -rauwolscine binding by about 30% in the rat kidney, it had no influence on the dissociation rate of the labelled ligand; the K_2 values for 3H -rauwolscine in the absence and presence of prazosin were virtually identical (fig V.20). Similar experiment was performed with 3H -yohimbine in the human platelet membranes. Again, prazosin did not alter the dissociation rate constant for 3H -yohimbine in this tissue (data not shown). Therefore, these results show that the inter-tissue differences in the affinity of prazosin at α_2 -adrenoceptor sites is also not likely attributed to allosteric interaction with the labelled ligand.

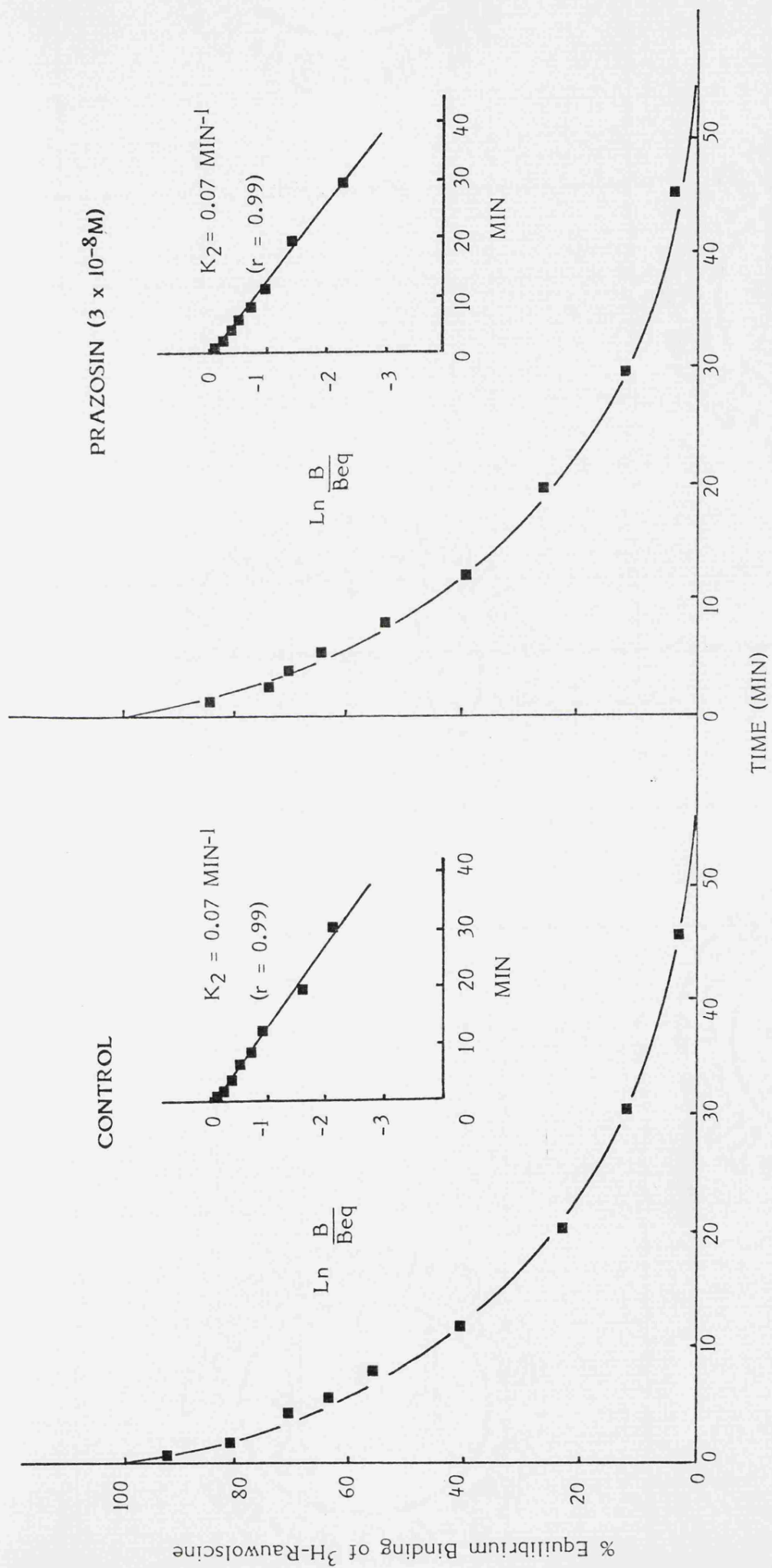
In conclusion, the data presented in these studies is compatible with a suggestion that at least two different molecular types of α_2 -adrenoceptors may be existing in mammalian tissues. α_2 -adrenoceptors with relatively high affinity for the α_1 -antagonists but reduced affinity for the α_2 -antagonists, and in particular, capable of distinguishing the yohimbine and rauwolscine diastereoisomers, appear to possess 'mixed α_1/α_2 ' properties, though the overall pharmacological behaviour is still predominantly of the α_2 -subtype. Indeed, a similar α_2 -adrenoceptor with such strange behaviour has also been described recently in the rat lung (Latifpour et al., 1982). Furthermore, it may be of relevance to note that in classical pharmacological studies, at least three different categories of postsynaptic α -adrenoceptors have been suggested being present among tissues of the rat, on the basis of different affinities for yohimbine, and clonidine (presumably, one category is the classical α_1 -

Legend to figure V.20

Figure V.20 Effect of prazosin on the dissociation of ^3H -rauwolscine binding to rat kidney membranes.

Rat renal cortical membranes were incubated to equilibrium at R.T. with approximately 3 nM ^3H -rauwolscine in the absence and presence of 30 nM prazosin. Specific binding of ^3H -rauwolscine was reduced by about 30% in the presence of 30 nM prazosin. Dissociation experiment was performed and data analysed as described in Chapter III. Insets: first-order rate plots for the dissociation of binding. Data shown is from one of two separate experiments which yielded identical results.

Fig. V.20



adrenoceptor), (Ruffolo et al., 1980, 1981). With a similar approach, three distinct classes of post synaptic alpha-adrenoceptors have also been identified among the aortas of different species (Ruffolo et al., 1982). In these studies, alpha-adrenoceptors exhibiting partial α_1/α_2 properties were described.

Of course, evidence for possible heterogeneity of α_2 -adrenoceptors presented in this chapter is primarily based on binding assays with membranous receptors. Differences in tissue/membrane properties and membrane constraint might contribute to apparent differences in drug affinity between tissues. It is therefore of importance also to assess the pharmacological properties of soluble α_2 -adrenoceptors, and to ascertain if the pharmacological differences observed between the membranous receptors are still present between the solubilized receptors which are presumed to be relatively free of membrane constraint and in a more 'uniform' environment. The characterisation of soluble α_2 -adrenoceptors from the human platelet, rat kidney, and rabbit kidney will be described in chapter VIII.

Chapter VI

AGONIST/ANTAGONIST INTERACTIONS WITH ALPHA₂-ADRENOCEPTORS: EFFECTS OF GUANINE NUCLEOTIDES, MONO- AND DIVALENT CATIONS, AND TEMPERATURE

VI.1. Introduction

Over the recent years, a great deal of work has focused on examining the molecular characteristics of drug-receptor interactions. Consequently, considerably progress has now been made toward understanding the molecular mechanisms by which catecholamine receptor occupancy is coupled to adenylate cyclase.

There is substantial evidence indicating that α_2 -receptors are coupled negatively to adenylate cyclase (Jakobs et. al., 1976; Aktories et. al., 1979; Sabol & Nirenberg, 1979_{a,b}). It is also clear that receptor coupled enzyme stimulation and inhibition both have an obligatory requirement for guanine nucleotides, such as GTP or Gpp (NH)p. The modulatory action of these guanine nucleotides is mediated through a regulatory protein (N-protein) (Rodbell, 1980; Limbird, 1981), which serves as a 'transducer' between the receptor and the enzyme. However, it appears that separate N-proteins (N_s and N_i respectively) are involved in the enzyme stimulation and inhibition. Evidence in support of this has come from various indications, including for examples, the differential quantitative requirement for GTP for receptor-coupled enzyme stimulation and inhibition, the differential responses of enzyme modulation to high and low Mn^{++} concentrations, and the differential changes in the enzyme's affinity for Mg^{++} upon stimulation by N_s-protein and inhibition by stable GTP analogs (see Chapter II). Results from genetic manipulation and reconstitution studies have provided further support for the existence of separate N-proteins.

The S49 lymphoma cell is a specially cultured cell line amenable to genetic dissection of the hormone-sensitive adenylate cyclase system. The 'wild-type' clone can synthesize high concentrations of C'AMP in response to beta-agonists or prostaglandins. The cyc⁻ clone is a genetic variant of the wild-type. These cells apparently contain intact beta-receptors and catalytic sub-units of the cyclase system, but the N_s-proteins are at least partially missing. However, enzyme stimulation by hormones, fluoride, and guanine nucleotides could be reconstituted upon mixing the

N_s -protein extract from wild-type lymphoma cells with cyc^- cells membranes (Howlett et. al., 1979). Interestingly, in the cyc^- cells, adenylate cyclase stimulated by the diterpene forskolin, or reconstituted by purified pre-activated N_s -protein could still be inhibited by stable GTP analogs (Jakobs et. al., 1983_b). Likewise, platelet adenylate cyclase activity stimulated by purified pre-activated N_s -protein could also be inhibited by adrenaline and stable GTP analogs. The observed inhibition was found to be non-competitive with respect to the N_s -protein concentrations, clearly suggesting that it was mediated through a separate N-protein interacting non-competitively with N_s -protein at the catalytic sub-unit of the enzyme system (Jakobs et. al., 1983_a).

At present, there are still some controversies over the exact step-wise mechanisms of receptor-adenylate cyclase coupling. Various models have been developed on the basis of experimental findings in different systems. For example, in the 'floating receptor' model (Jacobs & Cuatrecasas, 1976) it is considered that the receptor and adenylate cyclase are independently mobile entities in membranes. The coupling between the receptor and the enzyme appears to be a two-step process and is facilitated by an initial hormone-receptor interaction. The activated hormone-receptor-enzyme (H-R-E*) complex will accumulate and exhibit higher affinity for the hormone than the uncomplexed receptor. In the 'collision coupling' model (Levitzki, 1978; Tolkovsky & Levitzki, 1978) on the other hand, it is postulated that the enzyme modulation process is a bimolecular reaction and occurs as a result of a brief collision of the freely floating hormone-bound receptor with the enzyme. Recently, an 'allozyme' model has also been developed (Macfarlane, 1982), which is a modified version of the 'collision coupling' model. The 'allozyme' model assumes that the catalytic unit of the enzyme is activated by a brief collision with an hormone-occupied 'activating' receptor and remains active until it is deactivated either by another brief

collision with an agonist-occupied 'deactivating' receptor or with a postulated basal deactivating system. In essence, this 'allozyme' model considers the receptor as a catalyst which modifies the state of adenylate cyclase, and is subject to regulation by an external stimulus, the agonist. Both of the 'collision coupling' and 'allozyme' models assume that there is no accumulation of the H-R-E, H-R-E*, or R-E complexes. The contact between the hormone-receptor (H-R) complex with the enzyme (E) is thought to be so brief that the hormone-receptor binding characteristic is not altered (ie hormone binding to the receptor is of a homogeneous affinity).

There is probably no single model that can be adequately applied to all the systems. The 'collision coupling' or 'allozyme' models, for example, may be more applicable to the intact cell environment (eg intact platelet), in which agonist modulation of cyclase activity does often proceed with the receptors existing predominantly in one affinity state for the agonist (Macfarlane & Stump, 1982). However, results from the studies of most of the particulate α_2 -receptors suggest that the receptor-cyclase coupling mechanism appears to closely resemble that of the beta-receptor system, according to a 'unifying' model reviewed in detail by Swillens & Dumont (1980) (also see Lefkowitz & Hoffman, 1980_a; Kent et. al., 1980).

The coupling of α_2 -receptor to adenylate cyclase inhibition appears to require as a pre-requisite, the formation of an agonist high-affinity state or 'ternary' complex, involving the agonist, the receptor, and the Ni-protein (H-R-Ni) in a way similar to that described for the beta-receptor (Stadel et. al., 1979; DeLean et. al., 1980). Indeed, the existence of such a ternary complex was reflected by an apparent increase in the molecular size of the solubilized platelet α_2 -receptor subsequent to receptor occupancy by agonist, but not antagonist (Smith & Limbird, 1981). The presence of GTP or its analogues appears to cause dissociation

of the Ni-protein from the complex which then exhibits low affinity for the agonist (see diagram in Chapter II) The 'activated' Ni-GTP complex proceeds to act on the catalytic subunit of the enzyme to effect inhibition. The hydrolysis of guanine nucleotides by a GTP ase appears to be a 'turn-off' mechanism, since the non-hydrolysable analogs of GTP produce persistent inhibition of enzyme activity (Jakobs et. al., 1983_a).

The modulation of adenylate cyclase activity by GTP and its analogs is associated with, or apparently preceded by a reduction in the affinity of the receptor for the agonists, as mentioned above. This is well demonstrated in the binding studies in which agonist binding to α_2 -receptors is examined in the absence and presence of high concentrations of guanine nucleotides. Typically, agonists interact with α_2 -receptors in a different manner from the antagonists. In the absence of guanine nucleotides the agonists generate shallow displacement curves, indicating deviation from the law of mass action. Curve-fitting analysis would reveal agonist interactions with at least two affinity states of the receptor (Michel et. al., 1980). The presence of high concentrations of GTP and its non-hydrolysable analogs produces a characteristic shift of the agonist displacement curve to the right, and steepens its slope toward unity suggesting interaction with a class of homogeneous affinity sites. The overall agonist affinity in the presence of guanine nucleotide can be shown to be indistinguishable from that of the low-affinity sites in the absence of the modulator. Therefore, these findings are indeed well consistent with the postulation that the guanine nucleotide-sensitive ternary complex (or agonist high affinity state) represents the 'active' receptor pre-requisite to enzyme inhibition.

The interaction of agonists with α_2 -receptors, and in many cases, the agonist high-affinity states in particular, can be modulated by a variety of factors. For example, besides the guanine nucleotides, mono- and divalent cations have been demonstrated to produce 'agonist-specific' modulation of agonist affinities at α_2 receptors (Tsai & Lefkowitz, 1978; Michel

et. al., 1980; Rouot et. al., 1980; U'Prichard & Snyder, 1980; Glossmann et. al., 1980). These influences are of potential physiological significance, since modulation of the quantity of 'active' receptors being stimulated by agonists might lead to altered magnitude or sensitivity of the effector response. Another factor, temperature, can also be an important modulator of agonist interactions with α_2 -receptors. Indeed, the molecular mechanisms of agonist and antagonist interactions with the beta-receptors have been expounded on the basis of thermodynamic differences (Weiland et. al., 1979).

This work was undertaken with the aim of re-evaluating the influences of cations and guanine nucleotides on agonist/antagonist interactions with α_2 -receptors sites, using for the first time the selective antagonist ligands ^3H -yohimbine and ^3H -rauwolscine as receptor probes. The human platelet α_2 -receptor was used as a primary study model because of the relatively homogeneous platelet membrane preparations that can be derived from human blood. However, some attention has also been given to interactions at ^3H -yohimbine/ ^3H -rauwolscine labelled sites in the heterogeneous cell membrane preparations such as those from the rat cerebral and renal cortices. Furthermore, attempts have also been made to examine the thermodynamic differences of agonist and antagonist binding to platelet α_2 -receptors. The significance of cation and temperature influences on agonist/ α_2 -receptor interactions is discussed.

VI.2. Results

VI.2.1. Agonist/antagonist interactions with α_2 -adrenoceptors and effect of guanine nucleotides

Alpha-agonists and antagonists exhibited different characteristics interacting with α_2 -receptors labelled by ^3H -yohimbine or ^3H -rauwolscine. Typically, antagonists generated displacement curves with slopes close to unity, suggesting interaction with a homogeneous class of binding sites or affinity states. In contrast, agonists displayed shallow curves which suggests interaction with heterogeneous affinity states of the receptors. Figures VI.1.a, b and c illustrate the differential effects of GTP on the interactions of adrenaline (full agonist) clonidine (partial agonist) and rauwolscine (antagonist) with human platelet α_2 -receptors labelled by ^3H -yohimbine. In the presence of 8mM MgCl_2 , 0.1 mM GTP shifted the shallow displacement curve of adrenaline to the right by 10-fold or more. This was also accompanied by a steepening of the curve toward unity. The displacement curve of clonidine was similarly shifted by GTP, but compared to adrenaline, only to a smaller extent. In contrast to the agonists, the displacement curves of rauwolscine were virtually identical in the absence or presence of GTP. Furthermore, when ^3H -yohimbine saturation experiments were conducted in the absence and presence of GTP, Scatchard analysis indicated that 0.1 mM GTP (in the presence of 8 mM Mg^{++}) neither altered the B_{max} nor K_d for ^3H -yohimbine:

	B_{max} (f.mol/mg. protein)	K_d (nM)
Control	202 ± 25.11	2.05 ± 0.07 (n = 3)
+ 0.1 mM GTP	227 ± 36.52	1.99 ± 0.02 (n = 3)

Taken together, these results strongly indicate that, compared to the agonists, antagonist interaction with α_2 -receptor is not susceptible to modulation by guanine nucleotides.

Legend to figures VI.1.a, b and c

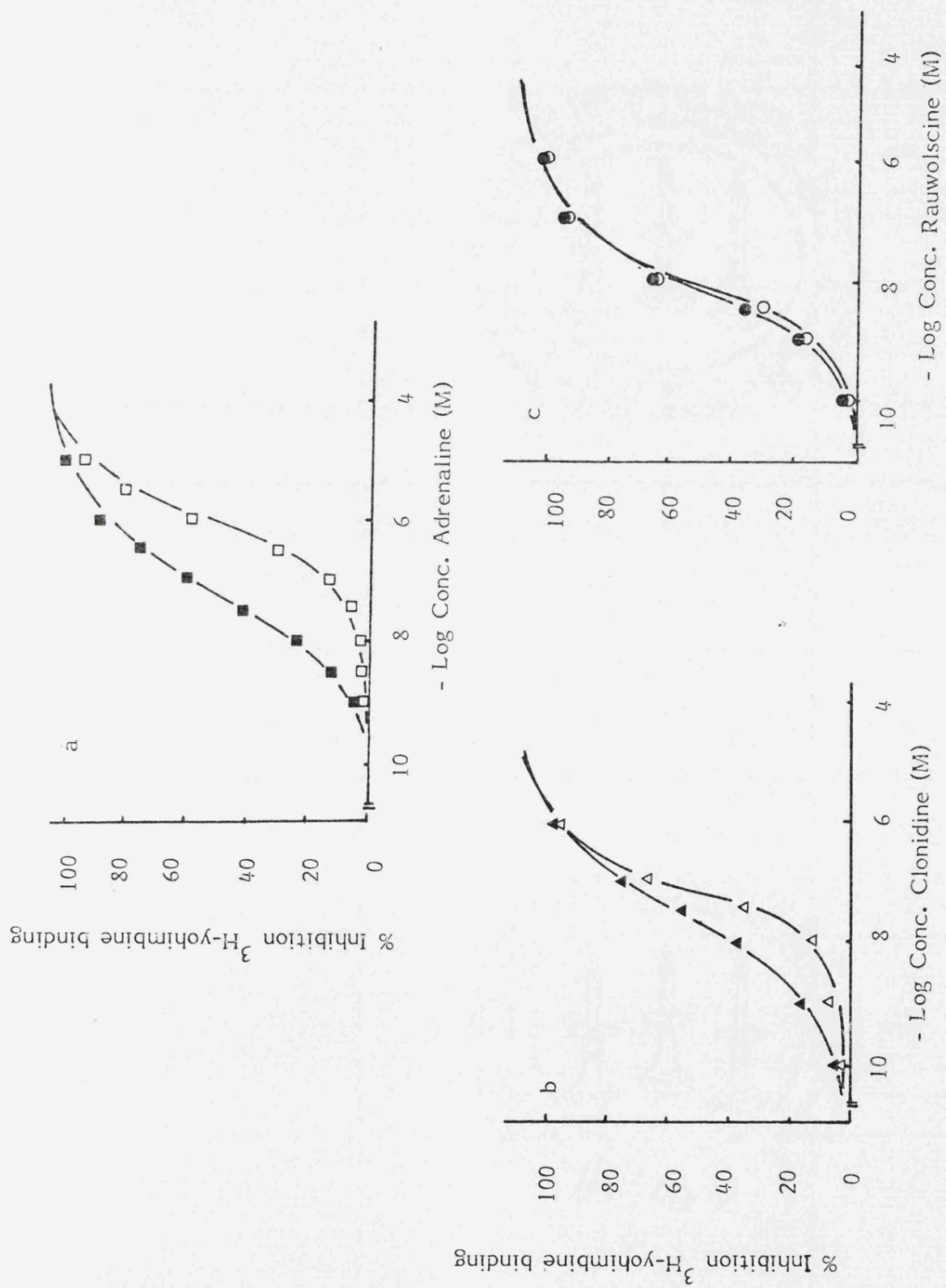
· Effect of GTP on displacement of ^3H -yohimbine binding to human platelet membranes by adrenaline, clonidine and rauwolscine.

- a) Adrenaline
- b) Clonidine
- c) Rauwolscine

Displacement experiments were carried out at R.T. at 2 - 3 nM ^3H -yohimbine in the presence of 8mM MgCl_2 , and run in parallel in the absence and presence of 0.1 mM GTP. Each data point represents the mean of 6 - 12 separate experiments performed in duplicate ($\text{SEM} < \pm 5\%$).

Closed symbols = control; open symbols = + GTP.

Fig. VL.1.



The modulatory effects of GTP and Gpp (nH)p were qualitatively and quantitatively similar, with a maximal shift of the adrenaline displacement curve being reached at around 10 - 100 μ M. The EC₅₀ was about 2 μ M. In earlier studies with human platelet membranes, it was found that the inclusion of EDTA in the membrane washing and/or assay procedures appeared to be essential for the 'GTP-shift' of the agonist displacement curves. This is probably due to the ability of EDTA to remove the inhibitory guanine nucleotides, such as GDP, from the GTP binding sites on the N-protein (Shane et. al., 1981). When assays were performed using platelet membranes previously stored at -70°C, the size of the 'GTP shift' was also somewhat smaller (only about 4-fold, compared to 10-fold or more with the freshly prepared membranes). Such small shift appeared attributed to the more rightward position of the already steep adrenaline displacement curves (slopes were around 0.8 - 0.9) in the absence of GTP, although 8 mM Mg^{++} was present in the assays (see Section VI.3. regarding the effect of cations on agonist/antagonist interaction with α_2 -adrenoceptors). This result suggests that freezing and thawing of platelet membranes may result in a reduced effectiveness of the receptors to form high-affinity states induced or stabilized by agonists.

When adrenaline displacement curves were analysed using computer iterative curve fitting, the results suggested that the agonists interacted with ³H-yohimbine labelled platelet α_2 -receptors in two distinct affinity states. At room temperature (22 - 25°C), and in the presence of 8mM $MgCl_2$, nearly 70% of the receptors were in the high-affinity state (Table VI.1). However, in the presence of 0.1 mM GTP, the adrenaline displacement curves were best fitted to a one-site model. The slope factor was also increased from 0.58 ± 0.016 to 0.97 ± 0.02 . The overall K_i value for adrenaline in the presence of 0.1 mM GTP was indistinguishable from the K_L value in the absence of the modulator (table VI.1). Therefore, these results

		R.T. (22 - 25°C)		37°C	
		High affinity sites	Low affinity sites	High affinity sites	Low affinity sites
O GTP	%	42.8 ± 8.2 ^{*,+}	57.2 ± 8.2	17 ± 1.5 [*]	83 ± 1.5
	K _L or K _H (nM)	22 ± 2.23 ^{*,+}	185 ± 13.7 ⁺	55.8 ± 4.9 [*]	703 ± 188.6
+ 0.1 mM GTP	%	-	100	-	100
	K _L or K _H (nM)	-	162.3 ± 31.5 ⁺	-	866 ± 133
+ 8mM Mg ⁺⁺	%	68 ± 1.6 ⁺	32 ± 1.6	46 ± 5.3	54 ± 5.3
	K _L or K _H (nM)	5.35 ± 0.88 ⁺	170 ± 33 ⁺	24.9 ± 5.5	761 ± 99
8mM Mg ⁺⁺ + 0.1 mM GTP	%	-	100	-	100
	K _L or K _H (nM)	-	212.66 ± 36.7 ⁺	-	897.18 ± 54.8

Table VI.1. Effects of Mg⁺⁺ and temperature on agonist interaction with platelet alpha₂-receptors

Platelet membranes were incubated with about 2nM ³H-yohimbine and 12 - 15 increasing concentrations of adrenaline in assay buffer. Cation and GTP were added to assays as appropriate. Displacement experiments were performed as described in Chapter III and data was analysed using the computer iterative non-linear curve-fitting program LIGAND (Munson & Rodbard, 1980). Results are means ± S.E.M. of 6 - 12 separate experiments performed in duplicate. * indicates statistical significance (p < 0.05) in comparison with corresponding value in the presence of Mg⁺⁺. + indicates statistical significance (p < 0.05) in comparison with corresponding value at 37°C.

clearly suggest that guanine-nucleotides specifically convert the high-affinity agonist state of the α_2 -receptors toward an overall low-affinity state, possibly by destabilizing the formation of the ternary high-affinity complex.

Differential modulatory effects of guanine nucleotides on agonist and antagonist interaction with α_2 -adrenoceptors in other tissues, such as rat cerebral cortex or rat renal cortex appeared similar to those observed in human platelet (Figure VI.2.). However, in those tissues, guanine nucleotides alone were relatively weak in producing the shift of the agonist displacement curves to the right. High concentrations of Na^+ markedly enhanced the modulatory effects of guanine nucleotides, increasing not only the extent of the shift, but also steepening the slopes of the curves close to unity. Either in the presence of 10mM MgCl_2 alone, or together with 200 mM NaCl, 0.1 mM Gpp (NH)p or GTP did not significantly alter the B_{max} or K_d of the labelled antagonist. This is well illustrated from the following data obtained from ^3H -rauwolscine binding to the rat renal cortical membranes:

	B_{max} (f.mol/mg protein)	K_d (nM)
Mg^+ alone	145 ± 23	5.05 ± 0.12 (n = 3)
$\text{Mg}^{++} + \text{Gpp (nH)p}$	156 ± 24.7	4.59 ± 0.16 (n = 3)
$\text{Mg}^{++} + \text{Na}^+$ alone	155 ± 23.5	4.92 ± 0.22 (n = 3)
$\text{Mg}^{++} + \text{Na}^+ + \text{Gpp(nH)p}$	159.6 ± 34	5.17 ± 0.17 (n = 3)

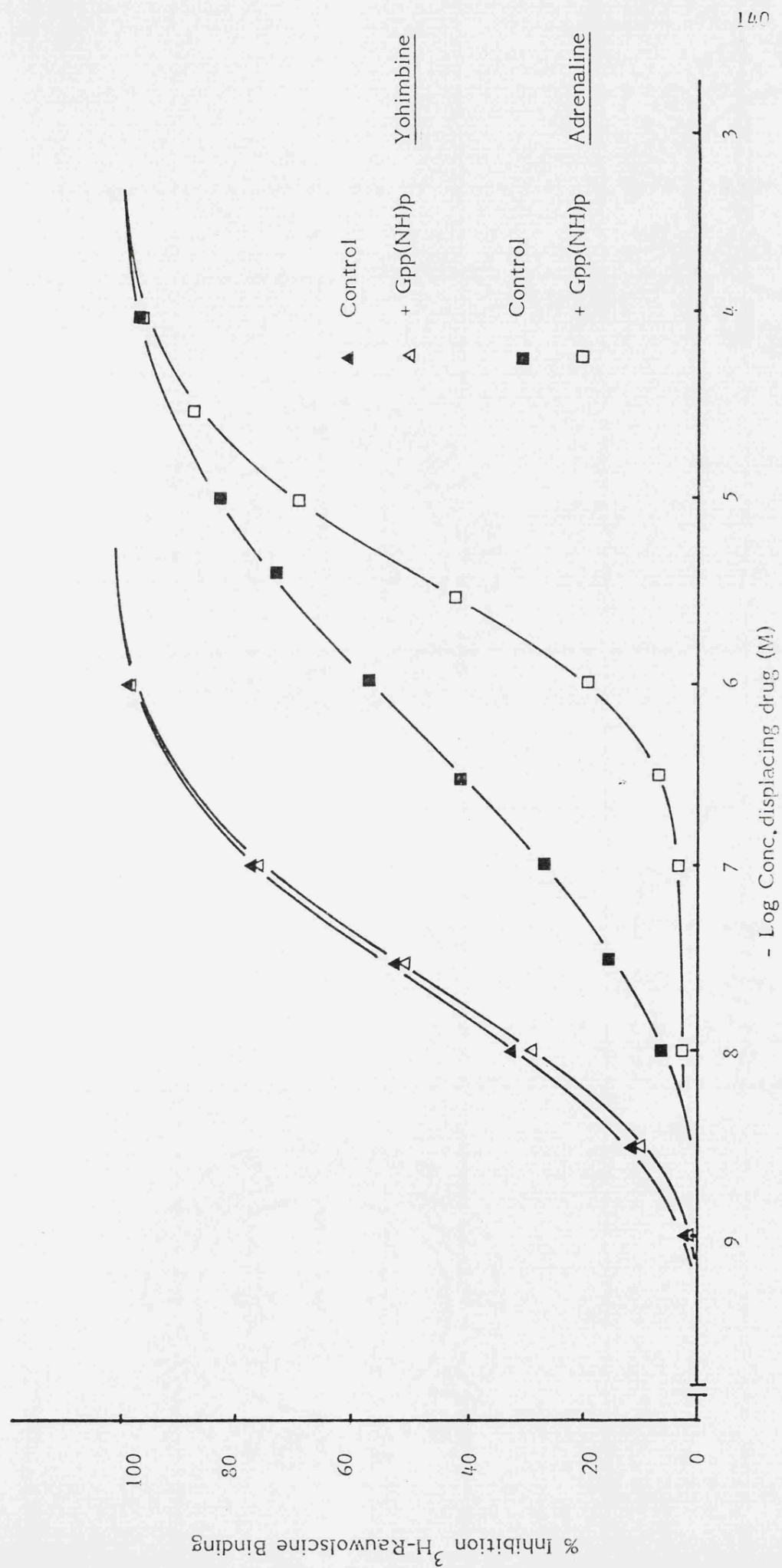
In short, the above data once again indicate that the modulatory effect of guanine nucleotides is selective for the agonist interaction with α_2 -receptors. More specifically, such effect appears limited to the high-affinity states of the α_2 -adrenoceptors, primarily leading to a shift of equilibrium in favour of the formation of low-affinity states.

Legend to figure VI.2.

Effect of Gpp(NH)p on displacement of ^3H -Rauwolscine binding to rat cerebral cortical membranes by yohimbine and adrenaline.

Displacement experiments were carried out as described in Chapter III at R.T. at 2 - 3 nM ^3H -rauwolscine in the presence of 8 mM MgCl_2 , and 200 mM NaCl. Experiments were run in parallel in the absence and presence of 0.1 mM Gpp(NH)p. Each data point represents the mean of six separate experiments performed in duplicate (S.E.M. $< \pm 5\%$).

Fig. VI.2.



VI.2.2. Effect of temperature on agonist and antagonist interaction with α_2 -adrenoceptors

V.2.2.1. Effect of temperature on agonist and antagonist affinity at platelet α_2 -adrenoceptors

Although the overall pharmacological profile of platelet α_2 -adrenoceptors did not change with temperature, the affinities of drugs did exhibit significant temperature dependency at these receptors. Thus, both agonists and antagonists displayed lower affinities at 37°C than at room temperature (22 - 25°C). However, the affinities of the agonists appeared to be more affected by temperature than the partial agonists or antagonists. In parallel experiments in which 8mM MgCl_2 was present, the K_i value for adrenaline at ^3H -yohimbine labelled sites in human platelet lysates was 27.8 ± 3.0 nM ($n = 12$) at room temperature (R.T.) compared to 172.7 ± 16.7 nM at 37°C, a difference of more than 6-fold. In comparison, the K_i for clonidine was 8 ± 0.6 nM ($n = 6$) at R.T. but only increased to 27.5 ± 1.4 nM at 37°C. The effect of temperature on antagonist binding to platelet α_2 -receptors is shown in the following table:

	Bmax (fmol/mg protein)	Kd (nM)
R.T.	159 ± 22	1.66 ± 0.2 ($n = 3$)
37°C	141.5 ± 19.3	3.21 ± 0.28 ($n = 3$)

(all assays contained 8mM MgCl_2)

It is clear that there was no significant difference in the Bmax for ^3H -yohimbine at R.T. and 37°C, although the Kd was increased by about 2-fold at 37°C. Results from kinetics studies indicate that the association rate constant (K_1) for ^3H -yohimbine increased by about 4-fold at 37°C ($0.205 \text{ nM}^{-1} \text{ min}^{-1}$ at 37°C compared to $0.054 \text{ nM}^{-1} \text{ min}^{-1}$ at R.T.), whereas the dissociation rate constant (K_2) increased by nearly

10-fold (0.35 min^{-1} at 37°C , compared to 0.04 min^{-1} at R.T.).

The decreases in drug affinities do not appear to be attributed to a change in the assay pH. The assay pH of 7.5 at R.T. was only reduced to 7.32 at 37°C . Over these two pH points, there was very little change in the specific binding of ^3H -yohimbine. Neither was there any apparent change in the K_i values for adrenaline (data not shown). Therefore, the observed changes in drug affinities appear to reflect a true temperature influence.

Table VI.1. shows the results of computer iterative curve fitting analysis of detailed adrenaline displacement curves both at R.T. and 37°C . It is clear that at 37°C there was a significantly smaller proportion of the platelet α_2 -receptors in the high affinity state, compared to R.T. The agonist affinities for both the high- and low-affinity states were also reduced at 37°C . Moreover, in the presence of 0.1 mM GTP, the overall K_i value for adrenaline was increased by about 5-fold from R.T. to 37°C . These values are not significantly different from the corresponding K_L values derived from curve-fitting analysis, thus further confirming that the temperature effect is not, as assessed in preliminary studies (Barnett et. al., 1982), only limited to the high-affinity agonist interaction with α_2 -adrenoceptors.

VI.2.2.2. Differences in thermodynamics of agonist and antagonist binding to α_2 -adrenoceptors

The standard Gibb's free energy change (ΔG°) for the binding reaction could be calculated from the equation:

$$\Delta G^{\circ} = - RT \ln K_A \quad (1)$$

Where R is the gas constant ($1.99 \text{ cal mol}^{-1} \text{ deg}^{-1}$), K_A is the equilibrium association constant ($1/kd$), and T is the temperature in degree Kelvin.

ΔG° is also related to changes in enthalpy (ΔH°) and entropy (ΔS°) according to the following equation:

$$\Delta G^{\circ} = \Delta H^{\circ} - T\Delta S^{\circ} \quad (2)$$

The combination of equations (1) and (2) gives rise to the van't Hoff equation:

$$\ln K_A = -\Delta H^{\circ}/RT + \Delta S^{\circ}/R \quad (3)$$

ΔH° can be determined from the plot of $\ln K_A$ against $1/T$, the slope of which is $-\Delta H^{\circ}/R$. After ΔH° has been determined, ΔS° can be calculated from equation (2).

Table VI.2. shows the thermodynamic parameters for the binding of adrenaline, clonidine and yohimbine to α_2 -adrenoceptors in the platelet membranes at 37°C. The data suggests that the binding of both agonists and antagonists to α_2 -adrenoceptors is significantly dependent on an enthalpy component, thus explaining the dependency of the equilibrium constants on temperature. However, in contrast to the agonists, whose binding is entirely enthalpy-driven, the interaction of the antagonists with the α_2 -adrenoceptors appears to be motivated by both the enthalpy and entropy components. The binding of the full agonist adrenaline was associated with a large net reduction in entropy, compared to the antagonist yohimbine whose binding had a net gain in entropy. The marked decrease in entropy associated with agonist binding is mainly counter-balanced by a large negative enthalpy, without which the reaction seemed quite impossible to proceed. Compared to adrenaline, the binding of the partial agonist clonidine to platelet α_2 -adrenoceptors had a smaller net decrease in entropy, accompanied with a less negative enthalpy. This reflects the less enthalpy dependency for the interaction of clonidine with the receptor, and may account for the quantitatively smaller reduction in its affinity by increased temperature. In the presence of 0.1 mM GTP, in which adrenaline appeared to bind to a homogeneous low-affinity state of the receptors, the enthalpy and entropy changes were also smaller than those in the absence of GTP. These quantitative differences, particularly in regard to the entropy changes, may reflect the relatively larger entropy reduction

Drug	ΔG° (Kcal mol ⁻¹)	ΔH° (Kcal mol ⁻¹)	ΔS° (entropy units)
Adrenaline only	-9.55	-30.57	-67.80
Adrenaline + 0.1 mM GTP	-8.51	-19.9	-36.74
Clonidine	-10.73	-15.42	-15.13
Yohimbine	-12.06	-8.33	+12.03

Table VI.2.

Thermodynamic parameters for agonist and antagonist binding to platelet α_2 -adrenoceptors at 37°C. Data was determined from equations (1), (2) and (3) as described in text. K_A values for adrenaline and clonidine were calculated as $1/K_i$, where K_i values were determined from IC_{50} as described in Chapter III. The K_d values for 3H -yohimbine were 1.66 nM and 3.21 nM at 22°C and 37°C respectively.

associated with specific agonist-receptor interaction, which appears to involve conformational changes in the receptor and coupling with the N-protein to form the ternary high-affinity complex pre-requisite for modulation of effector activity.

It must be emphasized that the thermodynamic parameters presented here are only rough estimates, since the ΔH^0 , and hence ΔS^0 , were calculated based on affinity differences between two experimental temperatures. Nevertheless, the data suffices to illustrate the basic differences in thermodynamics between agonist and antagonist binding to α_2 -adrenoceptors.

VI.2.3. Cation effects on agonist/antagonist interactions with α_2 -receptors

VI.2.3.1. Cation effects on agonist affinity

The divalent cations, such as Mn^{++} and Mg^{++} , not only modulate adenylylate cyclase activity, but can influence interaction of agonist/antagonist with α_2 -receptors. Hence, in agreement with earlier studies of α_2 -receptors, in which 3H -DHE was used as the radiolabel, (Michel et. al., 1980) agonist interactions with 3H -yohimbine or 3H -rauwolscine labelled sites were modulated by divalent cations.

In the platelet membranes, the affinity of adrenaline at 3H -yohimbine binding sites was increased in a dose-dependant manner by Mg^{++} , with about 4-fold decrease of the IC_{50} at 8 - 10 mM (Fig. VI.3.a.). However, the corrected K_i values for adrenaline (47.2 ± 4.16 nM and 22.8 ± 2.84 nM respectively in the absence and presence of Mg^{++}) was in fact only decreased by about 2 - 3 fold, because of the temperature-induced changes in the K_d for 3H -yohimbine (see Section VI.2.2.1.). Similar effect of Mg^{++} was observed at R.T. and $37^\circ C$. Although Ca^{++} could also enhance the affinity of adrenaline at platelet α_2 -receptors, such an effect appeared less consistent than Mg^{++} , and was usually less pronounced (data not shown).

Computer iterative curve fitting analysis of adrenaline displacement curves shows that Mg^{++} promoted the proportion of high-affinity states, but also increased the affinity (K_H) of such sites. The effect was similar at R.T. and $37^\circ C$ (Table VI.I.). However, Mg^{++} did not appear to produce any significant effect on the affinity (K_L) of the low-affinity sites. These results are consistent with earlier findings which showed that Mg^{++} increased both the B_{max} and affinity of 3H -agonist (3H -clonidine) binding to human platelet (Shattil et. al., 1981).

In contrast to the divalent cations, monovalent cations, such as Na^+ and K^+ were found to markedly reduce the affinity of agonist at α_2 receptors. For instance, adrenaline affinity at 3H -yohimbine labelled sites in the human platelet membranes was reduced by more than 10 -

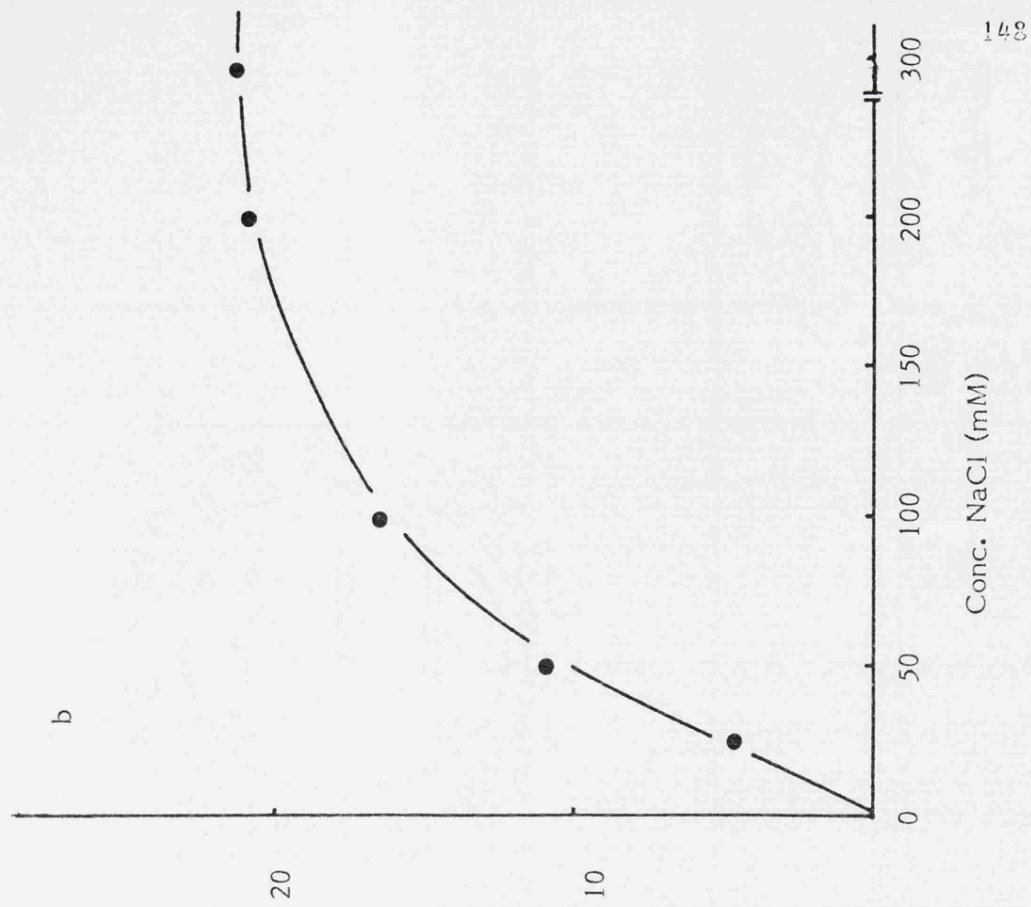
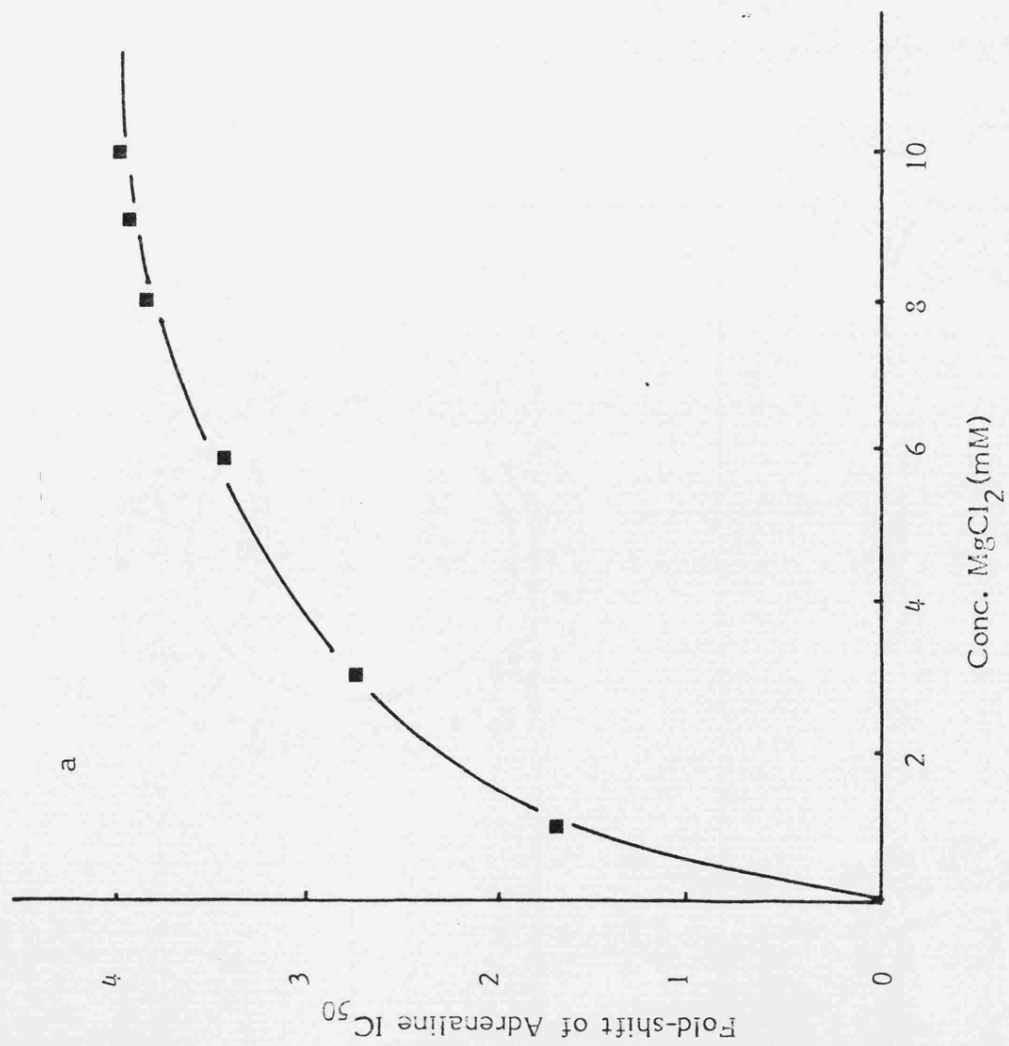
Legend to figures VI.3.a and b

Effects of Mg^{++} and Na^+ on adrenaline affinity at 3H -yohimbine labelled sites on human platelet membranes

- a) Mg^{++} effect
- b) Na^+ effect

Platelet membranes were incubated for 30 - 40 minutes at R.T. in standard assay buffer (Chapter III) with about 2 nM 3H -yohimbine and with increasing concentrations of adrenaline, and in the presence of various concentrations of $MgCl_2$ or $NaCl$. 'Fold-shifts' were calculated by comparing the IC_{50} values of adrenaline in the absence and presence of cations. A one-fold shift means no change in the IC_{50} . Data presented is mean of two similar experiments.

Fig. VI.3.



20 fold in the presence of 100 - 200 mM Na^+ . The effect was dose-dependent, reaching the maximum at around 200 mM Na^+ (Fig. VI.3.b). K^+ appeared to produce qualitatively similar effect, although it was generally much less potent (data not shown).

The modulatory effect of Na^+ was most pronounced on the affinity of the full agonist, and the extent of such effect appeared to be related to the intrinsic activity of the drugs. Hence, the affinity of the partial agonist clonidine was reduced to a smaller extent than the full agonist adrenaline (Fig. VI.4.). The affinity of the antagonist yohimbine was either totally unaffected, or very slightly reduced, depending on the tissue under investigation (see below).

In general, the reduction of agonist affinity by Na^+ was accompanied by steepening of the displacement curves, particularly when Mg^{++} was absent. Thus, in the human platelet membranes, the adrenaline displacement curves against ^3H -yohimbine binding were steepened from a slope of about 0.78 to 0.95 in the presence of 100 - 200 mM Na^+ alone. This suggests that the presence of Na^+ might tend to produce either a reduction of the proportion of the high-affinity states, or a decrease of the ratio of K_L/K_H , or both. Indeed, although an earlier report suggested that Na^+ only produced an overall reduction in agonist affinity and also a decrease in K_L/K_H ratio (Michel et. al., 1980), a more recent report indicated that Na^+ could produce a reduction of both the binding capacity and affinity of ^3H -agonist binding to human platelet membranes (Mooney et. al., 1982).

VI.3.2. Enhancement of guanine nucleotide modulation of agonist affinity by cations

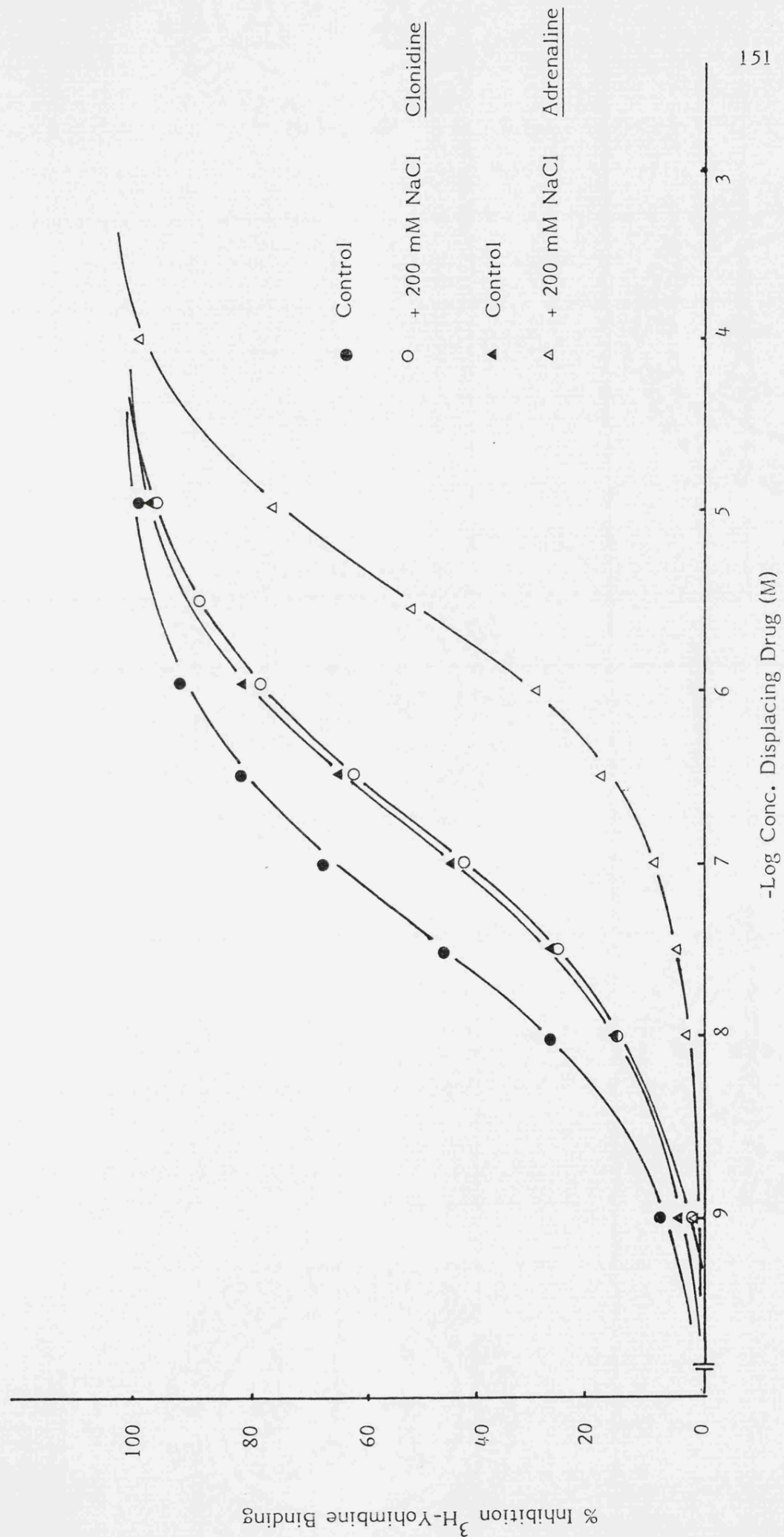
It was suggested that Mg^{++} was essential for the modulatory action of guanine nucleotides in agonist affinity at α_2 -receptors (Tsai & Lefkowitz, 1979). However, it was found in the present studies that Mg^{++} did not appear to produce much significant enhancement of the reduction of agonist affinity by guanine nucleotides (Table VI.1.). At R.T. the presence of

Legend to figure VI.4.

Comparative effect of Na^+ on affinities of full agonist and partial agonist at ^3H -Yohimbine labelled sites on human platelet membranes.

Platelet membranes were incubated in standard assay buffer with about 2 nM ^3H -yohimbine and increasing concentrations of adrenaline or clonidine at R.T. as described in Chapter III. Experiments were run in parallel in the absence and presence of 200 mM NaCl. Data points are means of 3 - 4 separate experiments performed in duplicate

Fig. VI.4.



8 mM Mg^{++} only slightly altered the overall K_i of adrenaline at human platelet α_2 -receptors in the presence of 0.1 mM GTP, but did appear to increase the slope factor from 0.92 ± 0.04 to 0.97 ± 0.02 ($p < 0.05$). At $37^\circ C$, there was virtually no apparent Mg^{++} enhancement of the reduction of agonist affinity by GTP. As illustrated in Fig. VI.5., the larger 'GTP-shift' observed in the presence of Mg^{++} seemed mainly attributed to the ability of Mg^{++} to shift the adrenaline displacement curves to the left. There is indication however that at R.T. the presence of Mg^{++} could indeed more consistently steepen the agonist displacement curves closer to unity, compared to its absence, suggesting that after all, Mg^{++} might have some degree of facilitating action on the full expression of the guanine nucleotide effect - the complete conversion of the high-affinity states to the low-affinity states. It is also interesting to note that in earlier studies in which frozen platelet membranes were used, Mg^{++} did not enhance the shift of the adrenaline displacement curve to the left, but produced a slight enhancement of the 'GTP-shift' to the right, implying that the promotion of agonist affinity by Mg^{++} , and its facilitating effect on GTP modulation of agonist affinity may be mediated through two different allosteric sites.

The effects of Na^+ and guanine nucleotides on agonist interaction with α_2 -receptor may be described as either additive or synergistic. In the human platelet membranes, the presence of 100 mM Na^+ (in addition to 8 mM Mg^{++}) shifted the adrenaline displacement curve to the right by about as much as that produced by 0.1 mM GTP or Gpp (NH)p. The concomitant presence of 100 - 200 mM Na^+ and 0.1 mM Gpp (NH)p produced a further right-ward shift of the adrenaline displacement curve, the extent of which was similar to that produced by 0.1 mM Gpp (NH)p in the absence of Na^+ . Therefore, the effect of Na^+ and guanine nucleotide appeared to be an additive one in this system (Fig. VI.6).

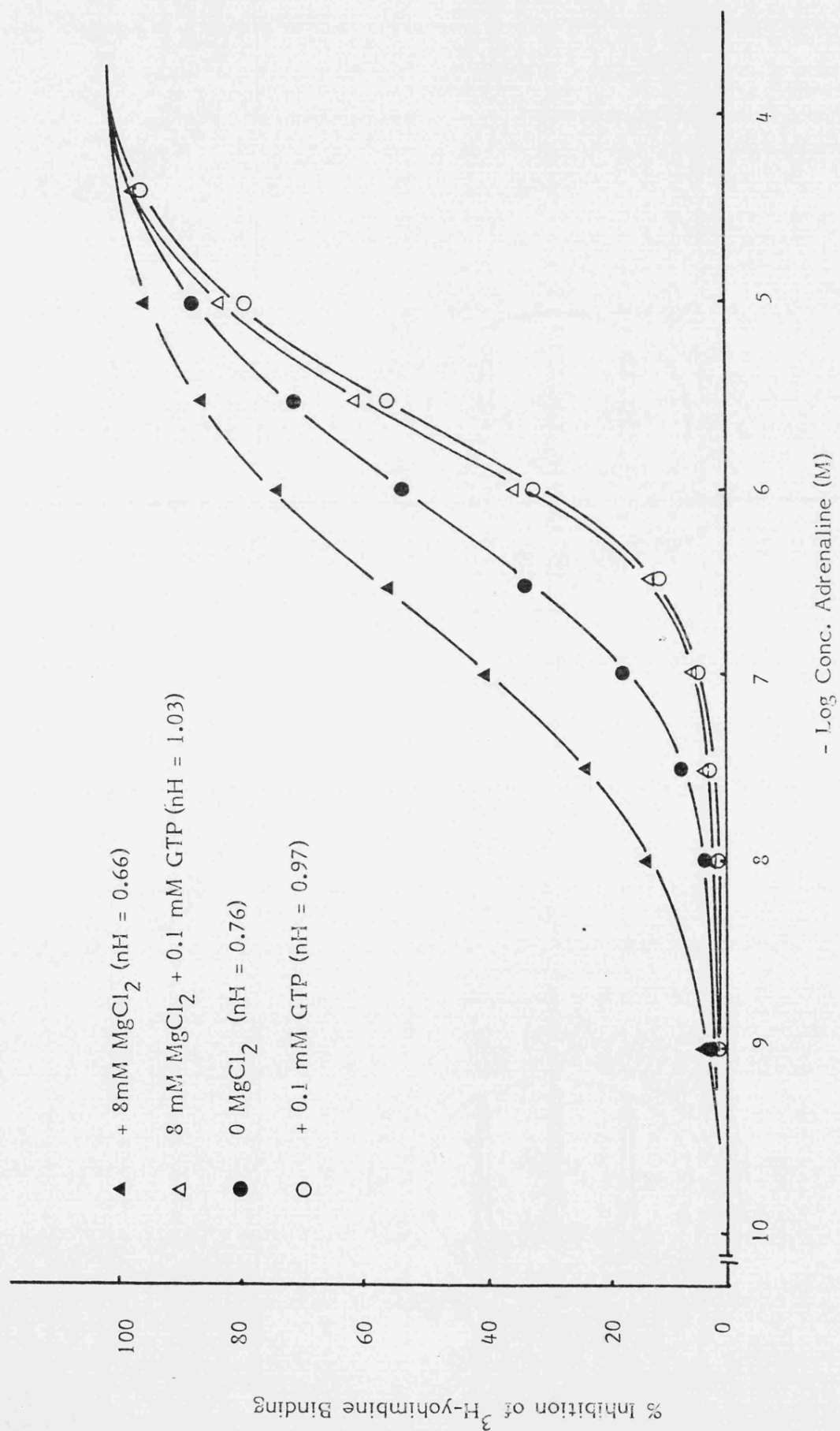
Compared to the human platelet, in tissues such as the rat kidney and rat brain, Na^+ was found to have a much more pronounced effect

Legend to figure VI.5.

Effect of Mg^{++} and guanine nucleotide on adrenaline displacement of ^3H -yohimbine binding to human platelet membranes.

Human platelet membranes were incubated with 2 nM ^3H -yohimbine at 37°C with increasing concentrations of adrenaline in the absence or presence of MgCl_2 (8 mM) or 0.1 mM GTP, or both. Datapoints are means of six separate parallel experiments performed in duplicate. The nH values are mean slope factors.

Fig. VI.5.



Legend to figures VI.6. and 7.

Fig. VI.6

Effects of Na^+ and guanine nucleotides on adrenaline displacement of ^3H -rauwolscine binding to human platelet membranes.

Fig. VI.7.

Effects of Na^+ and guanine nucleotides on adrenaline displacement of ^3H -rauwolscine binding to rat and renal cortical membranes.

Adrenaline displacement experiments of ^3H -rauwolscine binding (2 - 3 nM) to human platelet and rat renal cortical membranes were carried out at R.T. as described in Chapter III. Parallel assays were performed in the absence and presence of 0.1 mM $\text{Gpp}(\text{NH})\text{p}$, or 200 mM NaCl or both. 8 mM MgCl_2 was present in all assays. Data points are means of 4 - 6 separate experiments performed in duplicate. The S.E.M. of each data point is generally less than \pm 5-6%

Fig. VI.6.

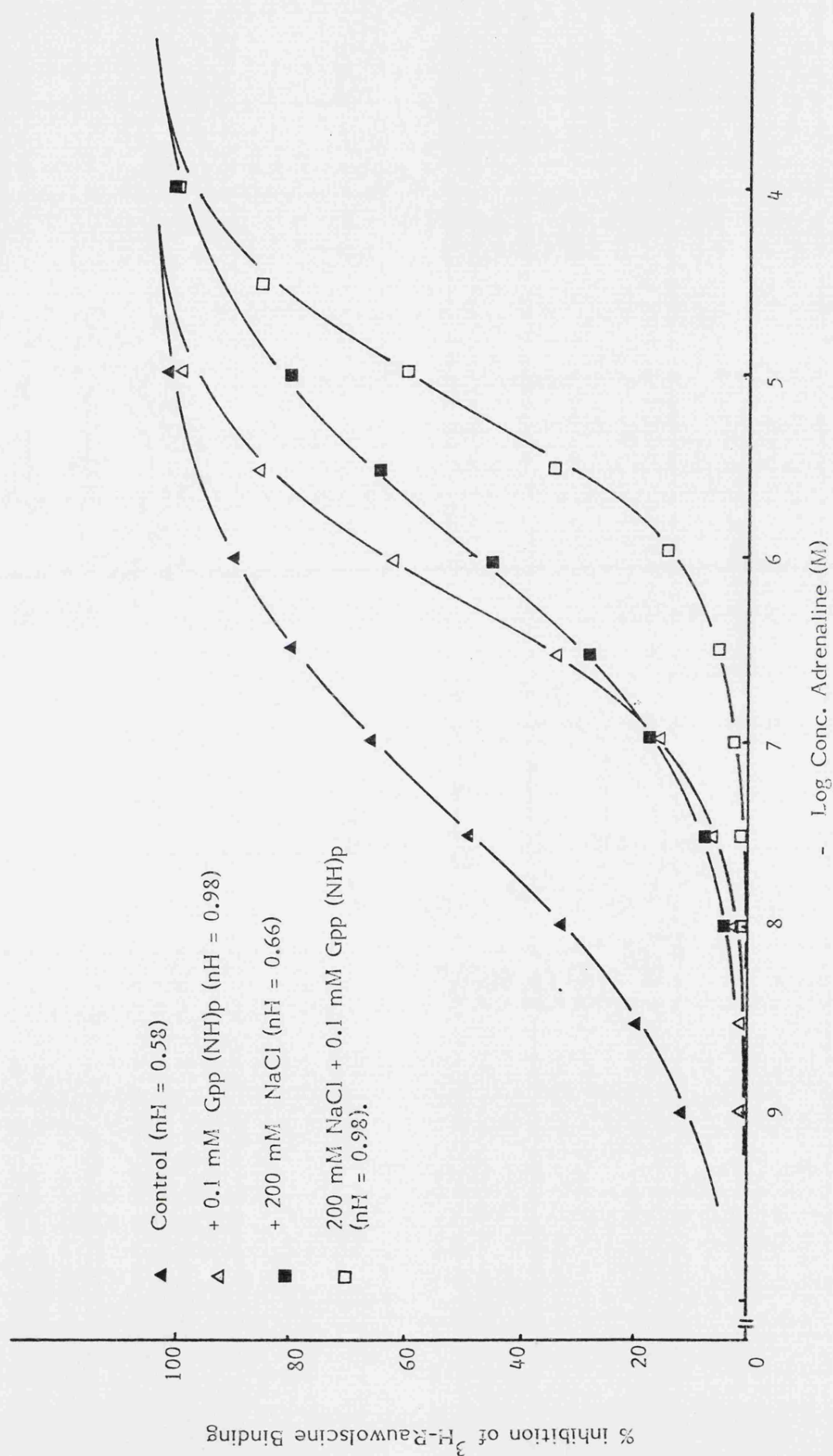
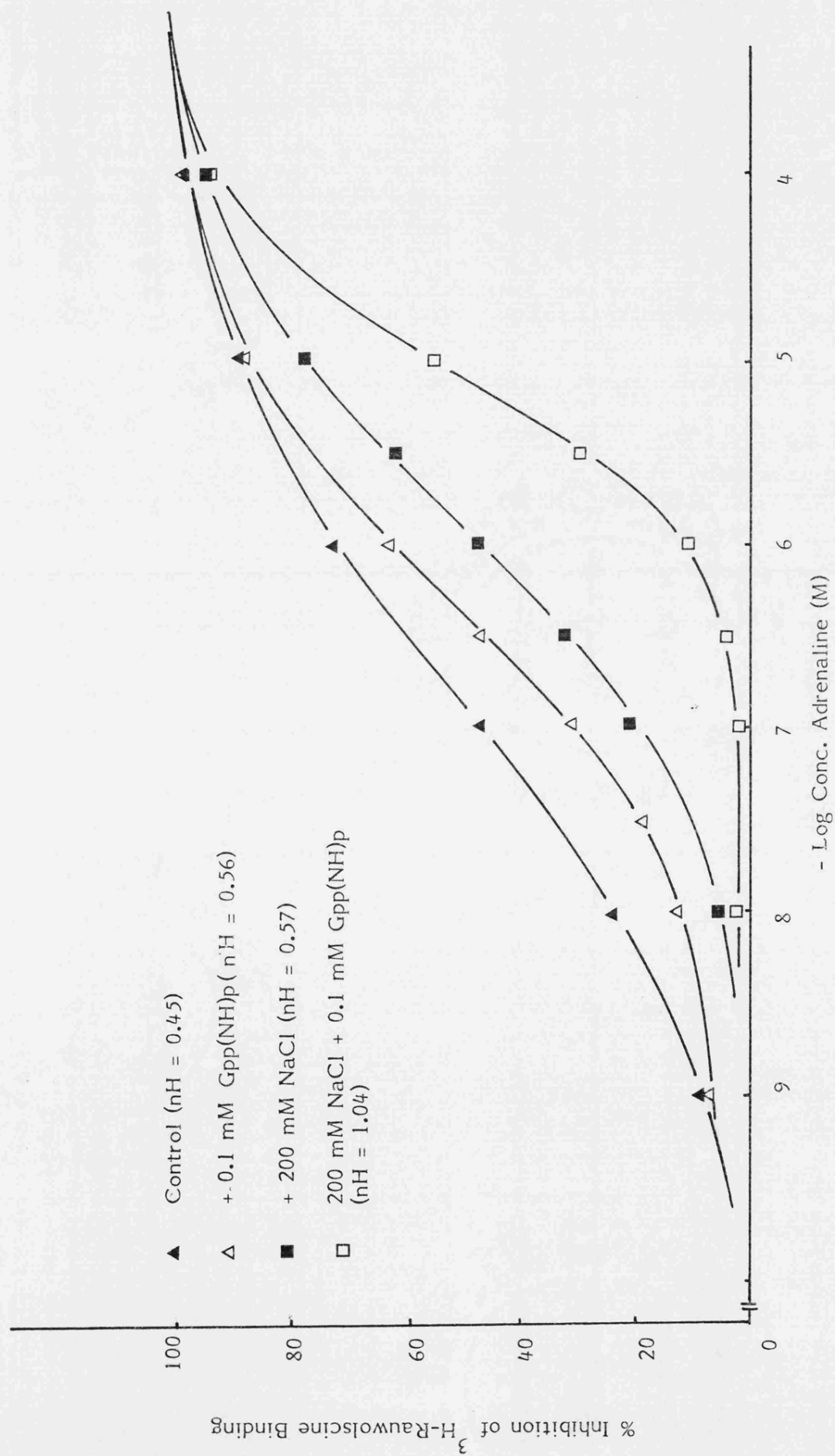


Fig. VI.7.



on the modulatory action of guanine nucleotide. For example, in the rat renal cortical membranes 0.1 mM Gpp (NH)p alone (plus 8 mM Mg^{++}) was only able to produce a small shift of the adrenaline displacement curve to the right (Fig. VI.7). The slope of the curve was increased slightly, but still significantly distant from unity. The presence of 200 mM Na^+ alone markedly reduced the affinity of adrenaline, and in the presence of this ion, 0.1 mM Gpp (NH)p produced a significantly larger 'shift' of the adrenaline displacement curve than that in the absence of Na^+ . Moreover, it is only when high concentrations of Na^+ were present that the guanine nucleotide-shifted curve was steepened close to unity.

VI.2.3.3. Cation effects on antagonist affinity

Although earlier studies did not reveal any influence of cations on antagonist affinity (Tsai & Lefkowitz, 1978), it was noticed in the present studies that divalent cations such as Mg^{++} could significantly reduce the affinity of antagonists at α_2 -receptors. For example, in the human platelet membranes, 8 mM Mg^{++} was found to increase the K_d of 3H -yohimbine from 0.88 ± 0.16 nM to 1.66 ± 0.2 nM at R.T. without altering the B_{max} (Fig. VI.8.a). This effect was dose-dependent, reaching a maximum at around 20 - 30 mM of Mg^{++} , with an ' IC_{50} ' of about 4 mM (Fig. VI.8.b.). Among the divalent cations tested, Mn^{++} appeared to be the most potent modulator and Ca^{++} was slightly less potent than Mg^{++} (data not shown). Similar divalent cation modulatory effect on antagonist binding was observed in all the tissues examined. Table VI.3. shows the effect of 20 mM Mg^{++} on the binding characteristics of 3H -rauwolscine on the various tissues. It is clear that in all cases, no significant changes in the apparent B_{max} were observed but the apparent K_d values were increased by about 2 - 3 fold. As suggested by Salama et. al., (1982) different drugs might exhibit different degrees of sensitivity to the divalent cation effect. The affinity of phentolamine, for example, appeared to be relatively unaffected by divalent cations. It is interesting to note that the affinity of the α_1 -

Legend to figures VI.8.a. and b.

Effect of Mg^{++} on 3H -yohimbine binding to human platelet membranes.

a) Scatchard plots of 3H -yohimbine binding data in the absence and presence of 8 mM $MgCl_2$.

Closed symbols = Control

$B_{max} = 198 \text{ f.mol/mg.p.}; K_d = 0.69 \text{ nM}$

Open symbols = + 8 mM $MgCl_2$

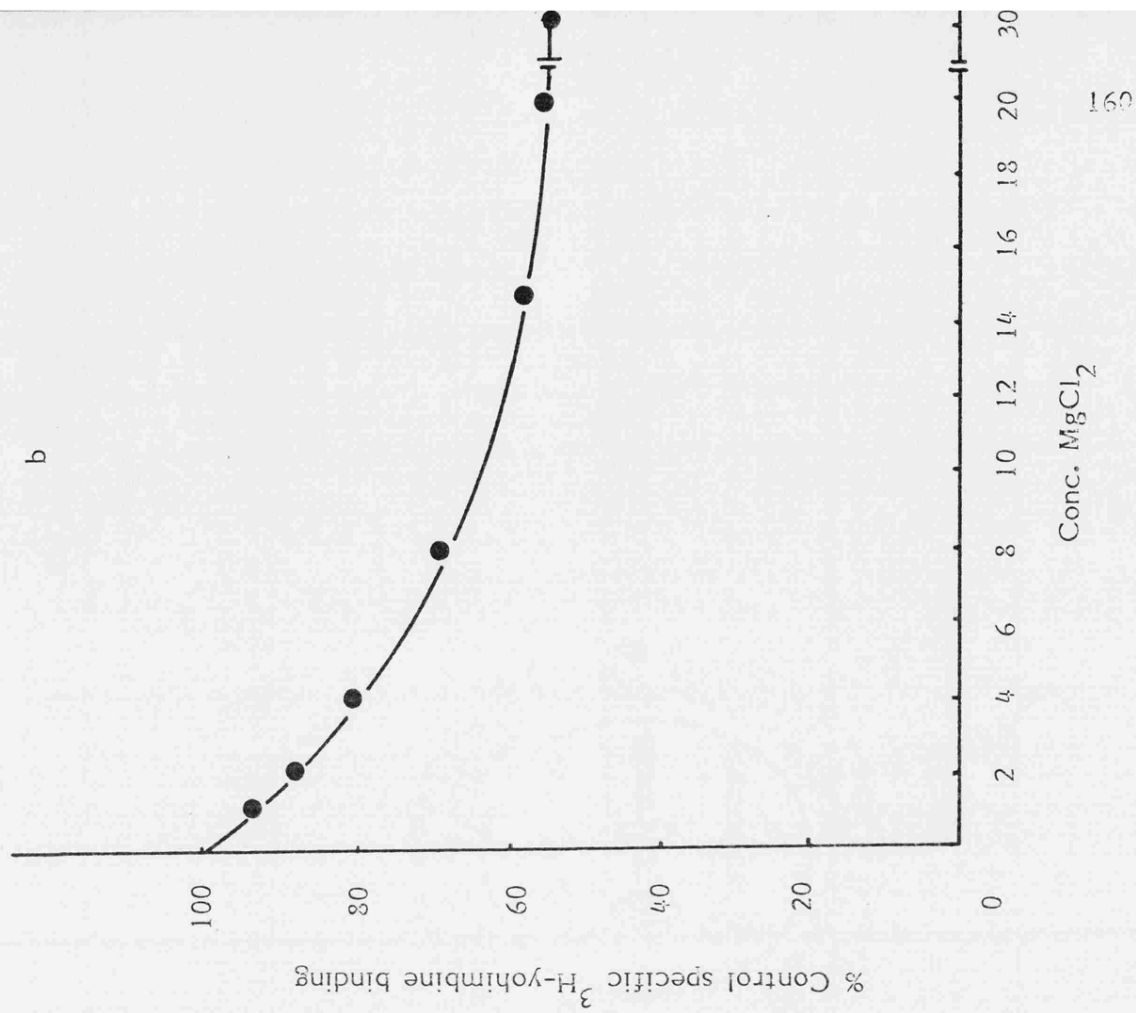
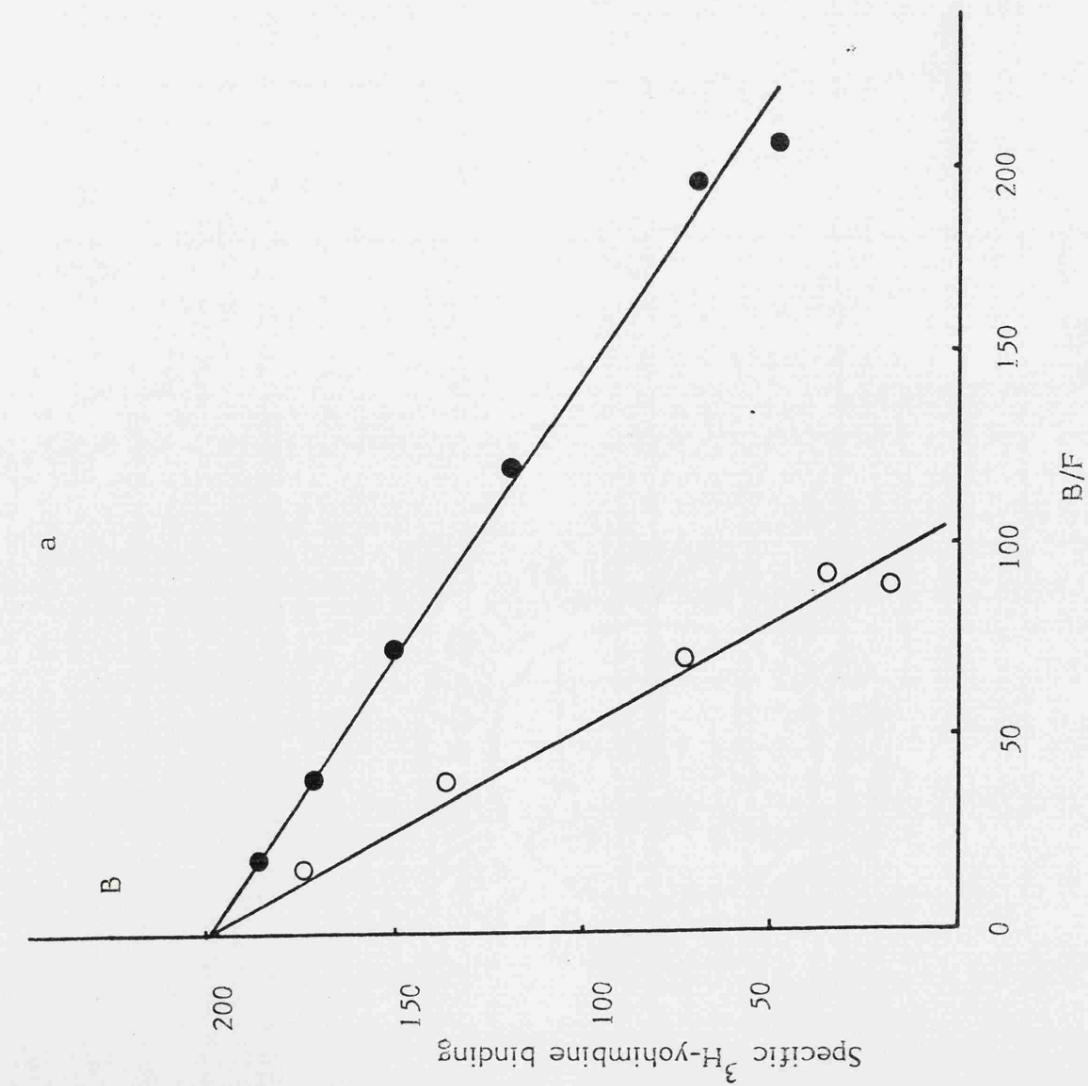
$B_{max} = 199 \text{ f.mol/mg.p.}; K_d = 1.97 \text{ nM}$

Saturation experiments were performed in parallel at 3H -yohimbine concentrations of 0 - 20 nM in the absence and presence of 8 mM $MgCl_2$. The data illustrated is from one experiment representative of three similar experiments performed in duplicate.

b) Dose - response relationship between Mg^{++} concentration and reduction of control 3H -yohimbine specific binding to platelet membranes.

Data is the mean of 2 similar experiments.

Fig. VI.8.



antagonist prazosin was also slightly reduced, although at 8 mM Mg^{++} , the IC_{50} of prazosin (against 3H -yohimbine or 3H -rauwolscine binding) was increased no more than 2-fold in all the tissues examined in these studies (data not shown).

As with the divalent cations, the monovalent cation modulation of drug interactions with α_2 -receptors is obviously not agonist-specific, as previously suggested (Tsai & Lefkowitz, 1978). Thus, Na^+ reduced the binding of 3H -yohimbine or 3H -rauwolscine to the human platelet membranes in a dose-dependent manner (Fig. VI.9), with a maximal effect being reached at 150 - 200 mM. The effects of 200 mM Na^+ on 3H -rauwolscine binding to the various tissues are summarized in Table VI.3. Similar to the effect of Mg^{++} , Na^+ did not alter the B_{max} of 3H -rauwolscine but it either decreased or did not alter the K_d , depending on the tissue under investigation. For instance, in the platelet membranes, 200 mM Na^+ reduced the affinity of 3H -yohimbine or 3H -rauwolscine in a similar manner to Mg^{++} . In the rabbit brain membranes, the affinity of 3H -rauwolscine was reduced to a smaller extent by 200 mM Na^+ than by 20 mM Mg^{++} . However, in rat brain, rat kidney, as well as rabbit kidney, the K_d for 3H -rauwolscine was virtually unaltered by Na^+ , despite of an approximately 2-fold increase produced by Mg^{++} . These effects of Na^+ on 3H -rauwolscine binding were qualitatively similar on 3H -yohimbine binding (data not shown). The interactions of α_1 -antagonists with α_2 -receptors were also influenced by Na^+ , although the results were variable. For example, the IC_{50} values for prazosin in competition with 3H -rauwolscine binding to both human platelet and rat kidney membranes were decreased by about 2 - 3-fold in the presence of 100-200 mM Na^+ . In contrast, the IC_{50} values for indoramin were increased by about 3-fold, and those for corynanthine were totally unchanged. Finally, it must be stressed that these effects of Na^+ and Mg^{++} on antagonist affinities by no means altered the rank orders of drug potency or pharmacological profiles exhibited by α_2 -receptors in the various tissues.

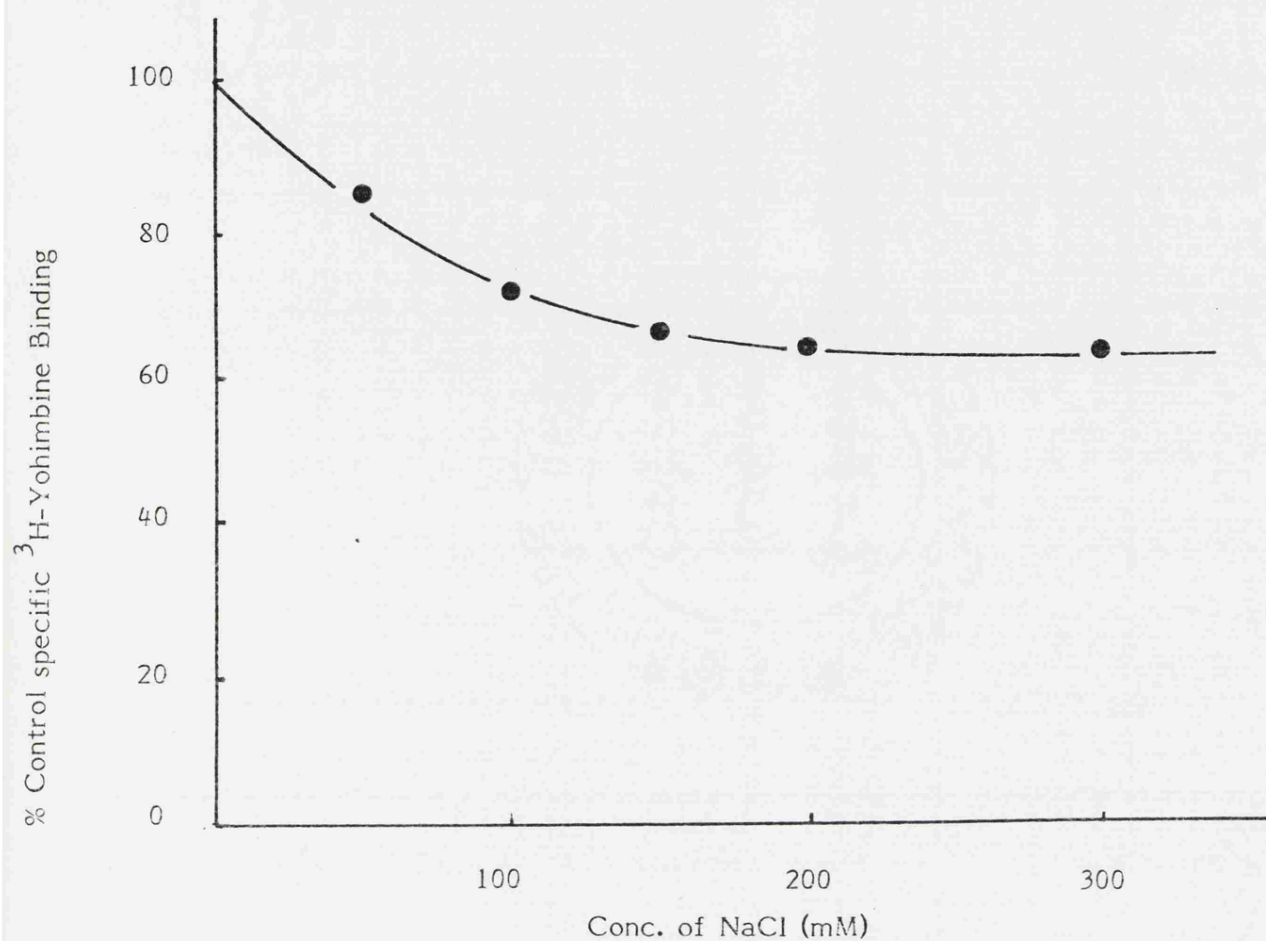


Fig. VI.9.

Effect of Na^+ on ^3H -yohimbine binding to human platelet membranes. Platelet membranes were incubated with approximately 2 nM ^3H -yohimbine at R.T. for 30 - 40 minutes in the absence and presence of increasing concentrations of NaCl. The data presented is the mean of two similar experiments performed in duplicate.

Table VI.3. Effects of Na⁺ and Mg⁺⁺ on specific ³H-rauwolscine binding

	Human Platelet	Rat Cerebral Cortex	Rat Renal Cortex	Rabbit Cerebral Cortex	Rabbit Renal Cortex
Control					
Bmax (f.mol/mg.p.)	192 ± 13	138 ± 13.4	158 ± 22	134 ± 8.6	76.8 ± 6.4
Kd (nM)	0.93 ± 0.05	2.98 ± 0.16	3.1 ± 0.45	6.31 ± 0.48	5.6 ± 0.55
+ 20 mM MgCl ₂					
Bmax (f.mol/mg.p.)	227 ± 24	128 ± 9.3	148 ± 13	143.5 ± 10.1	78.9 ± 5.96
Kd (nM)	2.83 ± 0.07*	7.9 ± 0.61*	9.7 ± 0.92*	17.5 ± 2.3*	10.5 ± 0.5*
+ 200 mM NaCl					
Bmax (f.mol/mg.p.)	225 ± 23	131 ± 15	157 ± 19.1	155 ± 14.5	66.4 ± 5.9
Kd (nM)	2.9 ± 0.06*	3.37 ± 0.4	2.78 ± 0.41	11.7 ± 0.66*	5.4 ± 0.5

The ³H-rauwolscine saturation experiments were performed in parallel at R.T. as described in Chapter III at 0-25nM. MgCl₂ and NaCl were dissolved in assay buffer and added directly to assay. The results presented are from Scatchard analysis of binding data of 3 - 4 separate experiments performed in duplicate. * indicates statistical significance (p < 0.05) in comparison with the 'control' Kd in individual tissue.

VI.3. Discussion

Earlier studies using ^3H -dihydroergocryptine have provided evidence that, like other catecholamine receptors linked to adenylate cyclase, α_2 -receptors interact with agonists, though not antagonists in distinct affinity states interconvertible under the influence of guanine nucleotides (Michel et. al., 1980). In the present studies in which the α_2 -selective antagonists ^3H -yohimbine and ^3H -rauwolscine were used as receptor probes, similar characteristics of agonist interaction with α_2 -receptors have been observed.

In agreement with results of earlier reports (Tsai & Lefkowitz, 1979; Hoffman et. al., 1982) guanine nucleotides exhibited specific modulatory effect on agonist affinities at α_2 -receptors. The extent of reduction of agonist affinity by guanine nucleotides appeared to relate to the intrinsic activity of the individual agonists. Thus, full agonists were more influenced by guanine nucleotides than partial agonists, and the affinities of antagonists were not affected at all.

The modulatory effect of guanine nucleotides on adenylate cyclase activity and agonist affinity is now well recognised to be mediated through a guanine nucleotide binding protein (N-protein) (Rodbell, 1980; Limbird, 1981). A ternary complex composed of the agonist, receptor and N-protein appears to represent the agonist high-affinity state pre-requisite for modifying the activity of the enzyme. The presence of guanine nucleotides, such as GTP or Gpp(NH)p, dissociates the N-protein from the ternary complex, and converts the receptor to a low-affinity state (see Chapter II). Results from the present studies have indeed demonstrated such an effect on agonist interaction with platelet α_2 -receptors labelled by ^3H -yohimbine or ^3H -rauwolscine.

Although it was suggested that the modulatory effect of guanine nucleotides on agonist affinity requires the presence of divalent cations

such as Mg^{++} (Tsai & Lefkowitz, 1979), the present results indicated that Mg^{++} ion seemed to exert a more pronounced effect on promoting the formation of the high-affinity states, than on facilitating the destabilizing action of guanine nucleotides. Mg^{++} was found to increase not only the proportion of the high-affinity agonist states, but also the affinity of such sites. This finding is also supported by results from direct 3H -agonist binding to α_2 -receptors both in the peripheral tissue (Shattil et. al., 1981) and in the CNS (Glossmann et. al., 1980). It was noticed that the presence of Mg^{++} did seem to consistently generate steeper adrenaline displacement curves at R.T. in the presence of GTP, possibly suggesting that Mg^{++} may be able to facilitate the full expression of the guanine nucleotide effect. However, such a possible allosteric action of Mg^{++} could be mimicked by increased experimental temperature, as a higher temperature seems to favor the reduction of the high-affinity states (see below), and might possibly also catalyse the exchange of inhibitory guanine nucleotides for GTP on the N-protein.

In contrast to the divalent cations, monovalent ions such as Na^+ could markedly reduce the affinity of agonists at α_2 -receptors. Results from 3H -agonist binding to platelet membranes suggested that Na^+ could reduce not only the affinity, but also the apparent B_{max} of high affinity 3H -agonist binding (Mooney et. al., 1982). Thus, it appears that the divalent and monovalent cations have opposite effects on controlling the formation of agonist high-affinity states of α_2 -receptors. This further implies that physiological Mg^{++} and Na^+ may serve to regulate the functional responses of catecholamines mediated through α_2 -receptors.

With respect to the effect on agonist interaction with α_2 -receptors, Na^+ and guanine nucleotides share similar properties. Both modulators can reduce the agonist affinities, although they do not seem to act through similar mechanisms. Indeed, the effect of Na^+ on agonist affinity appears

to be less specific than the guanine nucleotides. Both low- and high-affinity states of the α_2 -receptors can be influenced by Na^+ (Michel et. al., 1980), but only the high-affinity states are sensitive to the guanine nucleotides. In the platelet membranes, the effects of Na^+ and guanine nucleotides (GTP or Gpp (NH)p) appear to be only additive. However, interestingly, in tissues such as the rat brain and rat kidney, the two modulators exhibit synergistic influence on the agonist interaction with α_2 -receptors. In these tissues, agonist displacement curves (against ^3H -yohimbine or ^3H -rauwolscine binding) were relatively shallow, particularly when Mg^{2+} was present. High concentrations of guanine nucleotides alone were unable to produce a large shift of the curve to the right. The presence of high concentrations of Na^+ (200 mM) was required to facilitate a larger shift of the agonist displacement curve by guanine nucleotides. Furthermore it was only at such a high concentration of Na^+ that a significant steepening of the shifted curve was observed. The reason for such Na^+ requirement for the full expression of the guanine nucleotide effect is not clear at the moment. The presence of high concentrations of enzymes capable of hydrolysing guanine nucleotides is not a likely cause, since the non-hydrolysable guanine nucleotide Gpp (NH)p was found to be of similar potency as GTP. The second possibility is that there may be inhibitory substances which could inactivate the N-protein or the guanine nucleotide binding sites, and that Na^+ could counteract such inactivation. However, after membranes had been deliberately washed for 7 - 8 times, similar lack of sensitivity of agonist affinity toward guanine nucleotide modulation was still observed except in the presence of 200 mM Na^+ (data not shown). Therefore, one likely possibility is that there may be basic differences in the coupling mechanisms for α_2 -receptors in the platelet and other tissues. It is possible, though purely speculative that in contrast to the platelet α_2 -receptor, those in the heterogeneous cell preparations such as the rat brain and kidney membranes may be 'coupled' to other kinds

of regulatory membrane components, or N-proteins, besides the Ni-protein. Thus, by some unknown mechanisms, Na^+ may favor a more selective coupling of the α_2 -receptors with the Ni-protein. In view of the apparent ability of Na^+ to reduce the formation of agonist high-affinity states (Mooney et. al., 1982), it is also possible that the larger effect of guanine nucleotides on agonist affinity in the presence of Na^+ might be due to a direct allosteric action of this ion on the Ni-protein, perhaps facilitating the dissociation of GDP in exchange for GTP. Afterall, at least in the beta-receptor systems, the rates of agonist-induced release of ^3H -GDP were found to be quite different between tissues (Pike & Lefkowitz, 1981). Similarly, the effect of Na^+ on the Ni-protein in facilitating GDP/GTP exchange might also differ between tissues. Furthermore, it is possible that Na^+ might simply influence the availability of the responsible Ni-protein subunits with which GTP interacts. In this regard, differences in the structure or stoichiometry of the Ni-protein subunits between tissues may relate to the extent of the effect of Na^+ . It may be of relevance to notice that in the rat kidney, α_2 -receptor mediated cyclase inhibition appeared to require a relatively high concentration of NaCl for achieving a maximal effect (Woodcock & Johnston, 1982).

Agonist interaction with α_2 -receptors, similar to that described for the beta-receptors (Weiland et. al., 1979), also appears to be modulated by temperature. The binding of agonists to α_2 -receptors seems to be primary enthalpy-driven, compared to the antagonist, whose binding is much dependent on an entropy component. Similar to that observed at the beta-receptors, agonist binding to α_2 -receptors is characteristically associated with a large net reduction in entropy. It has been suggested that this may result partly from a conformation change in the receptor upon agonist occupancy (Weiland et. al., 1979). This interpretation seems quite compatible with the possible molecular events involved in the formation

of the agonist high-affinity states at the α_2 -receptors. However, the analysis of adrenaline displacement data in the human platelet membranes suggests that there is a significant loss of the high-affinity sites at 37°C compared to R.T. This may imply that the formation of the agonist high-affinity states is an enthalpy-driven mechanism itself.

From the physiological point of view, temperature may be important in controlling the α_2 -mediated responses through regulating the proportion of the α_2 -receptors in the high-affinity states. For example, platelet aggregation may be more sensitive to catecholamines at lower temperatures because of the presence of a higher proportion of the 'active' high-affinity states of the α_2 -receptors. In this connection, it may be of interest to mention that in contrast, the prostaglandins, which stimulate adenylate cyclase, were found to exhibit reduced capacity to inhibit platelet aggregation at lower temperatures (Mikhailidis et. al., 1981).

While divalent cations promote high-affinity agonist binding, these ions also exert an opposite effect on the interaction of antagonists with α_2 -receptors. The divalent cations generally reduced the affinities of antagonists by 2 - 3 fold without altering the receptor density of α_2 -receptors. Na^+ , on the other hand, appeared to have a rather variable effect on the antagonist interactions at α_2 -receptors. A recent report suggested that Na^+ could slightly increase both the B_{max} and affinity of ^3H -yohimbine in the human platelet membranes (Limbird et. al., 1982). However, the present results indicated that Na^+ did not significantly alter the B_{max} for ^3H -yohimbine in the same tissue. In fact, it was noticed that Na^+ reduced the affinity of ^3H -yohimbine in a dose-dependent manner. In tissues such as rat brain and rat kidney, Na^+ neither altered the B_{max} nor K_d of ^3H -rauwolscine or ^3H -yohimbine. In further studies, it was found that the α_1 -antagonists exhibit variable responses to Na^+ . For example, the affinity of prazosin at ^3H -rauwolscine labelled sites could be increased 2 - 3 fold by Na^+ in all the tissues studied. In contrast,

the affinity of indoramin was reduced by about 3-fold, and corynanthine was totally unaffected. The discrepancies between the results of Limbird et. al. (1982) and the present work cannot be fully explained. It is likely that differences in assay conditions may be important. For instance, these workers routinely included in their assays Mg^{++} ions, the presence of which can already reduce the affinities of antagonists for α_2 -receptors. It is also interesting to note that the 'control' K_d values for 3H -yohimbine in the platelet membranes reported by these workers were for some unknown reason significantly higher than those observed in this and other laboratories (Motulsky et. al., 1981; Daiguji et. al., 1981).

Very little is yet known regarding the mechanisms and sites of action for the cation effects on the agonist and antagonist interactions with α_2 -receptors. The cations can alter the affinity of the receptors for the agonist and/or antagonists in a relatively non-specific manner. Influences of this kind may therefore be exerted via cation sites situated on or closely adjacent to the receptor protein itself. In view of the potential of the cations also to modulate the formation of the agonist high-affinity sites, it is likely that other specific cation allosteric sites on the Ni-protein may be responsible for such kind of influences.

That cations and guanine nucleotides could modulate the agonist and antagonist interactions with α_2 -receptors in an opposite manner is a tempting thought. Indeed, Woodcock and Murley (1982) recently described the ability of Na^+ and guanine nucleotides to increase the apparent binding capacities of 3H -yohimbine in the rat cerebral cortex. Such finding has been re-evaluated in the present work. As will be discussed in the next Chapter, there is strong evidence suggesting that increases of 3H -antagonist binding sites in the presence of Na^+ and guanine nucleotides may merely relate to a reversal of the interference of antagonist binding by retained agonist.

Nevertheless, the view that agonists and antagonists can interact with α_2 -receptors in a 'reciprocal' manner has recently been carried further by U'Prichard and co-workers (U'Prichard, 1983; Bylund & U'Prichard, 1983). A hypothesis has been developed which suggests that antagonists also exhibit different affinities for the agonist high- and low-affinity states of the α_2 -receptors. Accordingly, antagonist interactions with α_2 -receptors would be expected being modulated by cations and guanine nucleotides in a manner opposite to the agonists. This view obviously receives no support from results of the present work, which revealed neither heterogeneous antagonist binding to α_2 -receptors nor its modulation by guanine nucleotides. It is perhaps not improbable that apparent modulation of antagonist binding by cations and guanine nucleotides observed by U'Prichard and co-workers may in part also relate to influences of retained agonist.

Chapter VII

INTERACTIONS OF EXOGENOUS AND ENDOGENOUS AGONIST WITH CEREBRAL CORTICAL ALPHA₂-ADRENOCEPTORS LABELLED BY ³H-ANTAGONIST

VII.1 Introduction

In chapters IV and V, it has already been demonstrated that both ^3H -yohimbine and ^3H -rauwolscine are useful and specific radiolabelled probes for studying α_2 -adrenoceptors. Indeed, these two ligands have now been widely used for studying α_2 -adrenoceptors in a variety of tissues (Daiguji et al., 1981; Cheung et al., 1982; Hoffman et al., 1981; Tharp et al., 1981; Schmitz et al., 1981; Perry & U'Prichard, 1981). Using these ligands, it has also been able to demonstrate that agonists interact with α_2 -adrenoceptors in a manner qualitatively similar to that observed with other receptors linked to adenylate cyclase (see Chapter VI). Thus, the α_2 -adrenoceptors appears to exist in two distinct agonist affinity states modulated by the presence of guanine nucleotides and divalent cations and the overall agonist affinity can be markedly reduced by monovalent ions, such as Na^+ . In contrast, antagonist binding to the α_2 -adrenoceptor is relatively little affected by these modulators (Tsai & Lefkowitz, 1978; Tsai & Lefkowitz, 1979; Michel et al., 1980; Limbird et al., 1982; also see Chapter VI). However, as discussed in the last Chapter, there have been recent suggestions that antagonists may also display different affinities for the agonist high and low affinity states of the α_2 -adrenoceptor. According to such a model antagonist binding might be modulated by guanine nucleotides and monovalent ions in a manner opposite to that with the agonists (Bylund & U'Prichard, 1983).

In a recent study using rat cerebral cortical membranes, Woodcock and Murley (1982) reported a rather low maximal binding capacity of ^3H -yohimbine, which could be markedly increased when incubations were performed in the presence of Na^+ and guanine nucleotides. However, no change in the dissociation constant of ^3H -yohimbine by these modulators was observed. In view of the quite different binding capacities reported by these workers compared to our own studies (Cheung et al., 1982) in the same tissue, it remained possible that differences in membrane preparations could be

particularly significant. Woodcock and Murley (1982) used a hypertonic sucrose containing buffer for cortical membrane preparation, and it seemed possible that in this medium, intact synaptosomes containing norepinephrine (noradrenaline) could significantly interfere with subsequent α_2 -adrenoceptor binding assays with the ^3H -antagonist, and that these effects could be reversed by Na^+ ions and guanine nucleotides. The present study aimed at examining these possibilities in detail and has provided evidence for a pseudo non-competitive interaction of α_2 -adrenoceptors with endogenous norepinephrine. These results could have wide implications in the interpretation of apparent altered receptor site density in many systems, and might also be of relevance to observations regarding apparent enhancement of antagonist binding by Na^+ and guanine nucleotides.

VII.2 Methods

VII.2.1 Preparation of Rat Cerebral Cortical Membranes

Adult male Sprague-Dawley rats (200-300 g) were decapitated and cerebral cortical membranes were prepared by one of the following methods:

A Cerebral cortex was homogenised in 20 volumes of sucrose buffer (50 mM Tris-HCl, 0.25 M sucrose, 10 mM MgSO_4 , pH 7.7) using a motor-driven glass-teflon homogenizer as described by Woodcock and Murley (1982). Homogenates were filtered through a double-layer of cheesecloth and centrifuged at 27,000 g for fifteen minutes. Pellets were subsequently washed three times with the same sucrose buffer, and finally resuspended in assay buffer (50 mM Tris-HCl, 10 mM MgSO_4 , 0.5 mM EDTA, 1 mM mercaptoethanol pH 7.7) at a protein concentration of 4-6 mg/ml.

B Cerebral cortex was homogenised and washed in hypotonic buffer (5 mM Tris, 5 mM EDTA pH 7.7) as previously described in Chapter III. Final pellets were resuspended in the same assay buffer as in A.

C Cerebral cortex was initially homogenised and washed in sucrose buffer as in A, but after the third washing, pellets were homogenised once more in the buffer (5 mM Tris, 5 mM EDTA) used in B before being resuspended in the assay buffer as in A.

VII.2.2 Receptor Binding Assays

All binding assays were performed in assay buffer (50 mM Tris-HCl, 10 mM MgSO_4 , 0.5 mM EDTA, 1 mM mercaptoethanol pH 7.7) in a final volume of 250 μl . Assays procedures and analysis of results were similar to those described in Chapter III.

VII.2.3 Norepinephrine (Noradrenaline) Assays

Membranes prepared as above were homogenised with 0.1 N perchloric acid and following centrifugation, catecholamines were extracted with alumina and separated on a reverse phase-ion pair HPLC column (Ultrasphere 5 μ ODS) and assayed by electrochemical detection (Mefford, 1981). The

limit of norepinephrine detection was 0.2 pmol/mg membrane protein.
(These assays were performed by Mr J Strupish).

VII.3 Results

VII.3.1 Effect of Methods of Cortical Membrane Preparation on Binding Capacities of ^3H -yohimbine

Cerebral cortical tissue was pooled and divided into three portions. Membranes were prepared simultaneously by methods A, B and C and assayed for ^3H -yohimbine binding. Significant differences in the binding capacities in these preparations were observed, even under identical assay conditions (Table VII.1 Figs. VII.1a and b). B_{max} values for ^3H -yohimbine were generally low in membranes prepared by methods A, which was similar to that adopted by Woodcock and Murley (1982). In contrast, the B_{max} of ^3H -yohimbine was significantly higher in membranes prepared by method B, in which the hypotonic buffer instead of sucrose buffer was used in the homogenisation and washing processes. When membranes prepared by method C, (initial preparation by method A, followed by an additional homogenisation and washing in hypotonic buffer) were used for assays, ^3H -yohimbine B_{max} was also found to be higher than membranes prepared by method A. The apparent K_d of ^3H -yohimbine binding, however, remained unchanged regardless of the method of membrane preparation.

VII.3.2 Effect of NaCl and Guanine Nucleotide on ^3H -yohimbine Binding Capacities in Cortical Membranes

In agreement with the results reported by Woodcock and Murley (1982), the apparent densities of α_2 -adrenoceptors determined by ^3H -yohimbine binding in cortical membranes prepared by method A (sucrose containing buffer) could be increased more than two fold if assays were performed in the presence of NaCl (200 mM) and the hydrolysis-resistant GTP analogue Gpp (NH)p (10 μM) (Table VII.1). However, there were no significant differences in the apparent B_{max} values of ^3H -yohimbine binding in membranes prepared by method B, (hypotonic buffer) in the absence or presence of NaCl and Gpp (NH)p.

In all cortical preparations the apparent K_d of ^3H -yohimbine

Table VII.1 Effect of Methods of Cortical Membrane Preparation on Binding Capacities of ³H-yohimbine

Membrane Preparation* Method	Norepinephrine Content (pmol/mg protein)	Assay Buffer		Assay Buffer plus NaCl (200 mM), Gpp(NH)p (10 μM)	
		Bmax (fmole/mg protein)	K _d (nM)	Bmax (fmole/mg protein)	K _d (nM)
A	1.98 ± 0.24	49 ± 5.94**	11.6 ± 1.28	125.7 ± 14.6 ⁺	11.22 ± 1.03
B	< 0.2	124.3 ± 9.84	12.18 ± 0.99	138.3 ± 16.2	11.97 ± 0.95
C	< 0.2	105.4 ± 15.4	11.80 ± 0.89	141.08 ± 17.06	11.84 ± 1.17

* Membranes were parpared by methods A, B and C and saturation binding assays were carried out in parallel experiments as described in the text.

Values shown were obtained from scatchard plots and are means ± SEM of at least six separate experiments. Comparisons were made by Student's 't' test.

** Indicates significantly different from membrane preparations B and C is assay buffer (P < 0.05).

+ Indicates significantly different from membrane preparation A in assay buffer (P < 0.05).

All other comparisons were not significantly different.

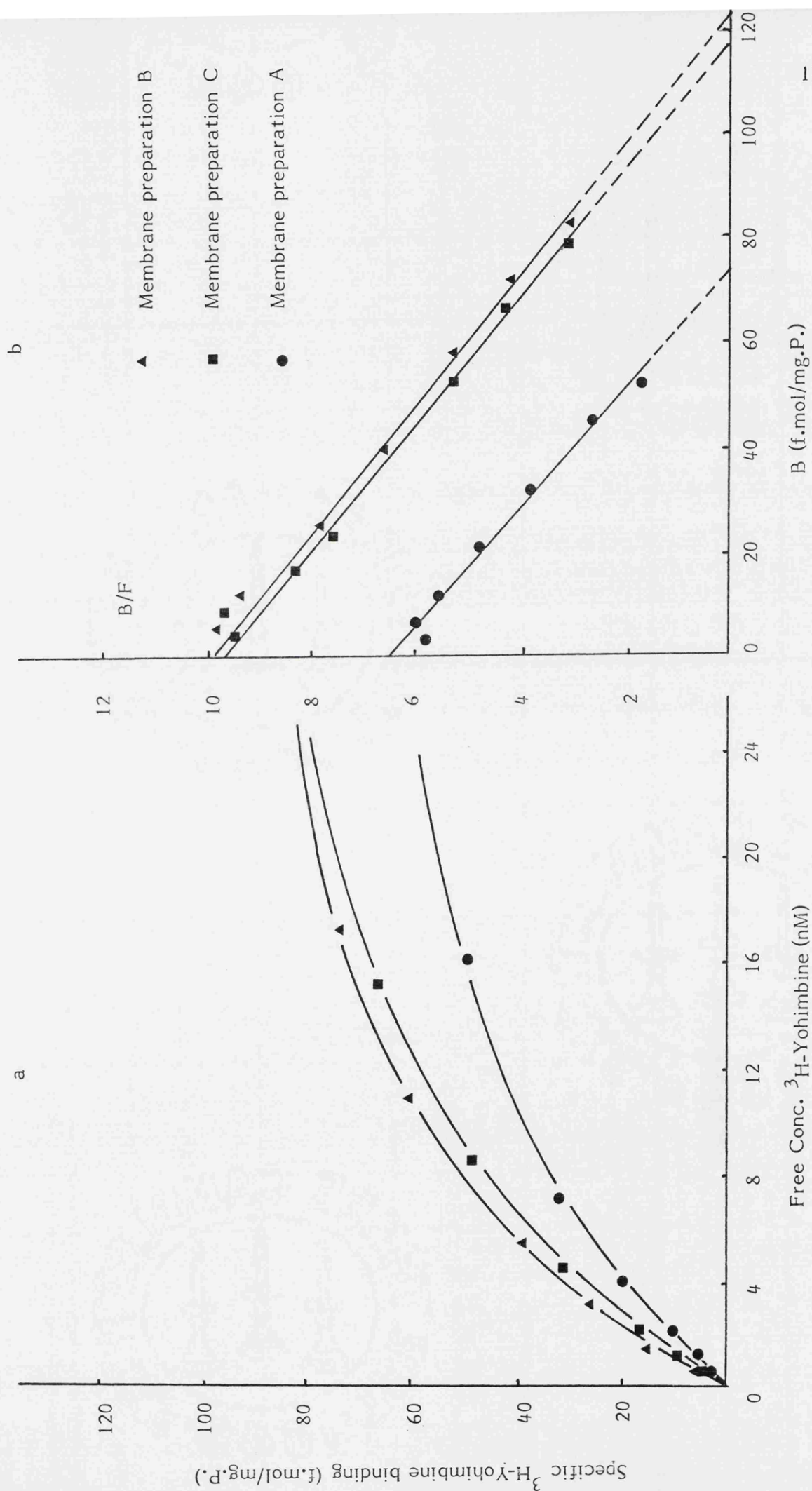
Legend to Figure VII.1.**Specific binding of ^3H -Yohimbine binding to rat cerebral cortical membranes prepared to different methods**

Membranes were prepared as described in the text using hypertonic sucrose buffer (Method A); hypotonic Tris buffer (Method B); hypertonic sucrose buffer followed by one final wash in hypotonic Tris buffer (Method C). Saturation experiments of ^3H -Yohimbine binding to the above membranes were performed in parallel at approximately 0 - 30 nM at R.T. as described in Methods section of this chapter.

- a) Saturation isotherms
- b) Scatchard plots of binding data

Data presented is from one experiment representative of six similar experiments performed in duplicate. The Bmax (f.mol/mg protein) are: 73, 124, 117 respectively for membrane preparation A, B and C. The Kd (nM) are: 10.23, 11.1 and 10.95 respectively for membrane preparation A,B, and C.

Fig. VII.1.



was not altered when incubations were carried out in the presence of NaCl and Gpp(NH)p. Furthermore, it should be noted that in the presence of these modulators the B_{max} was not significantly different between the three preparations.

VII.3.3 Norepinephrine Contamination of Cortical Membranes

Samples of membranes prepared by methods A, B and C were analysed using HPLC/electrochemical detection for possible contamination with endogenous norepinephrine with the following results: membranes A 1.98 ± 0.24 pmol/mg protein (mean \pm SEM, $n = 3$); membranes B < 0.2 pmol/mg protein; membranes C < 0.2 pmol/mg protein.

In the final binding assay (assuming that all the catecholamine is free) the overall concentration of norepinephrine in assays containing membranes A would be 5 ± 0.4 nM ($n = 3$) whereas in membranes B or C this concentration is < 0.5 nM.

VII.3.4. Effect of Exogenous Norepinephrine on ^3H -yohimbine Binding Capacities

In view of the possibility that the enhancement of the apparent binding capacity of ^3H -yohimbine to cerebral membranes by Na^+ and Gpp(NH)p could relate to retained endogenous norepinephrine, experiments were performed in which exogenous norepinephrine was added to membranes prepared in hypotonic buffer. The results are shown in Table VII.2 and Figs. VII.2a and b.

When saturation experiments were performed using membranes prepared in the hypotonic buffer, (very low residual endogenous norepinephrine), the addition of 10 nM norepinephrine was found to cause an apparent large decrease in ^3H -yohimbine binding capacity. A further loss in sites was observed when a higher concentration (100 nM) of norepinephrine was added. However, these changes in B_{max} values were not accompanied by any change in the apparent affinity of ^3H -yohimbine. Furthermore, in the presence of 200 mM NaCl and 10 μM Gpp(NH)p, the 'lost' binding sites

Table VII.2 **Effect of Exogenous Norepinephrine on ^3H -yohimbine Binding Capacities in Cortical Membranes prepared in Hypotonic Buffer**

	Assay Buffer		Assay Buffer plus NaCl (200 mM), Gpp (NH)p (10 μM)	
	Bmax (fmole/mg protein)	K _d (nM)	Bmax (fmole/mg protein)	K _d (nM)
Control*	135.8 \pm 29.9	11.5 \pm 0.6	193.4 \pm 25.3	11.6 \pm 1.2
+ 10 ⁻⁸ M norepinephrine	70.3 \pm 4.8**	11.7 \pm 1.6	178.1 \pm 36.5 ⁺	12.8 \pm 1.9
+ 10 ⁻⁷ M norepinephrine	49.2 \pm 5.4**	10.9 \pm 2.4	162.6 \pm 32.3 ⁺	13.7 \pm 1.8

* Membranes were prepared in hypotonic buffer (method B) and assayed as described in the text. Saturation binding assays were carried out in parallel experiments without addition of agonist (control) or in the presence of norepinephrine in final concentrations of 10⁻⁸ M or 10⁻⁷ M as listed, and with or without addition of NaCl (200 mM) and Gpp(NH)p (10 μM).

Values shown were obtained by Scatchard analysis and are means \pm SEM of at least three separate experiments. Comparisons were made using Student's 't' test.

** Indicates significantly different from 'control' in assay buffer (P < 0.05).

+ Indicates significantly different from results in the absence of NaCl and Gpp (NH)p (P < 0.05).

All other comparisons were not significantly different.

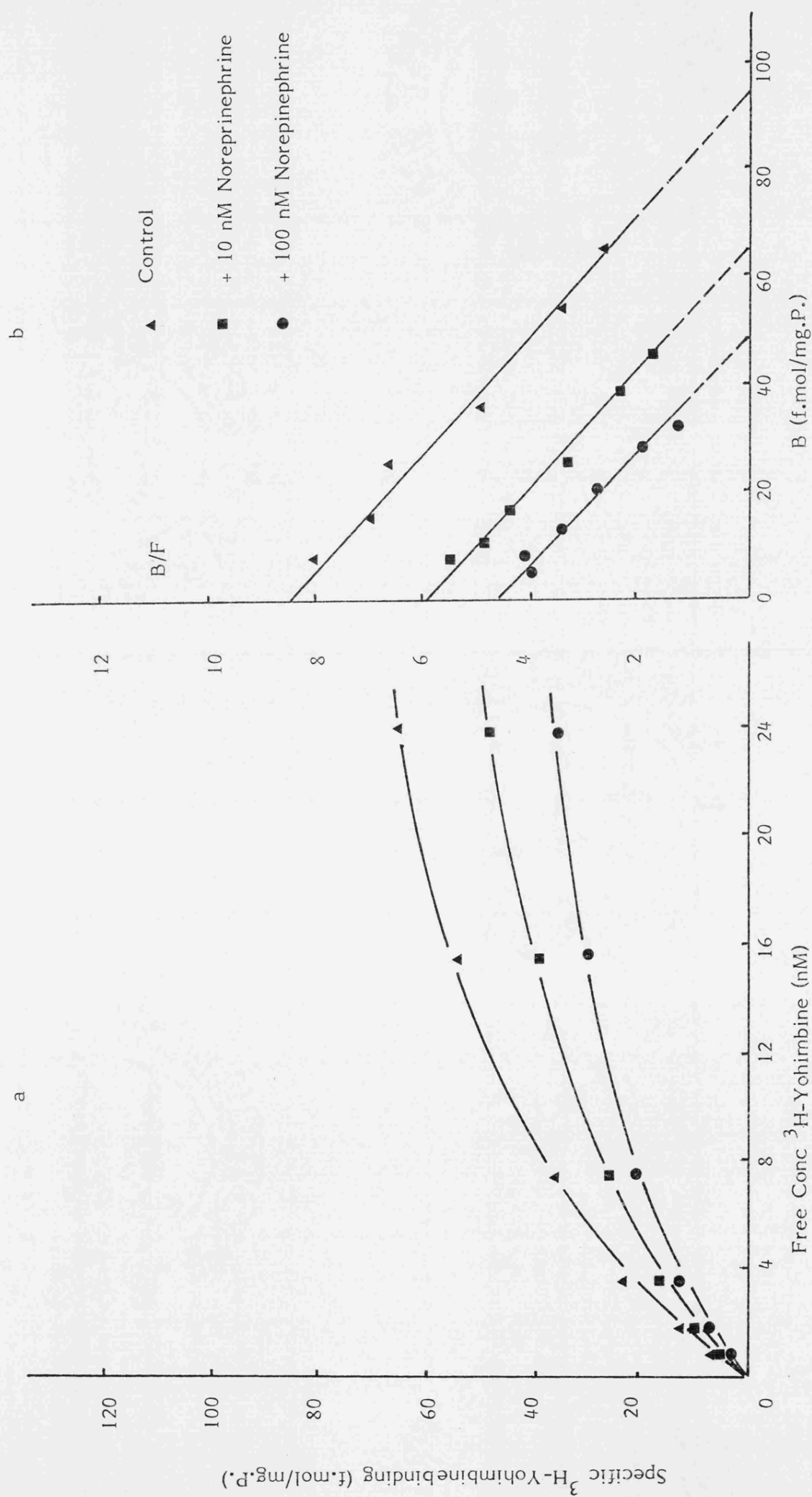
Legend to Figure VII.2.**Effect of exogenous norepinephrine (noradrenaline) on ^3H -yohimbine binding to rat cerebral cortical membranes.**

Membranes were prepared as described in the text by method B (ie in hypotonic Tris buffer only). ^3H -yohimbine saturation experiments were performed in parallel in the absence and in the presence of final concentration of 10^{-8}M or 10^{-7}M of norepinephrine.

- a) Saturation isotherms
- b) Scatchard analysis of binding data

Data illustrated is from one experiment representative of three similar experiments performed in duplicate. The B_{max} (f.mol/mg protein) = 97, 60.38, 42.8 and K_d (nM) = 11.1, 10.52 and 9.07, both for control and in the presence of 10^{-8}M and 10^{-7}M norepinephrine respectively.

Fig. VIII.2.



could be recovered. Thus, in the presence of NaCl and Gpp(NH)p a similar binding capacity was observed regardless of the presence or absence of exogenous norepinephrine.

VII.3.5 Effect of NaCl and Guanine Nucleotide on Norepinephrine Displacement of ^3H -yohimbine Binding to Cortical Membranes

The data in Fig. VII.3 demonstrate the effect of NaCl and Gpp(NH)p alone or in combination on the ability of norepinephrine to displace specific ^3H -yohimbine binding to α_2 -adrenoceptors in rat cerebral cortical membranes, prepared in hypotonic buffer. Norepinephrine characteristically displaces the labelled antagonist with a curve of low Hill slope ($n\text{H} = 0.58$). In presence of Gpp(NH)p there is a small shift of the curve to the right with some steepening of the slope ($n\text{H} = 0.74$). NaCl, however, more effectively reduced the overall affinity of norepinephrine, and the effect of NaCl and Gpp(NH)p together appeared to be synergistic. In the presence of both of these modulators, norepinephrine displaced ^3H -yohimbine with low affinity and with a steep displacement curve ($n\text{H} = 0.91$).

Note that NaCl and Gpp(NH)p did not influence the capacity of ^3H -yohimbine binding in these membranes (Table VII.I).

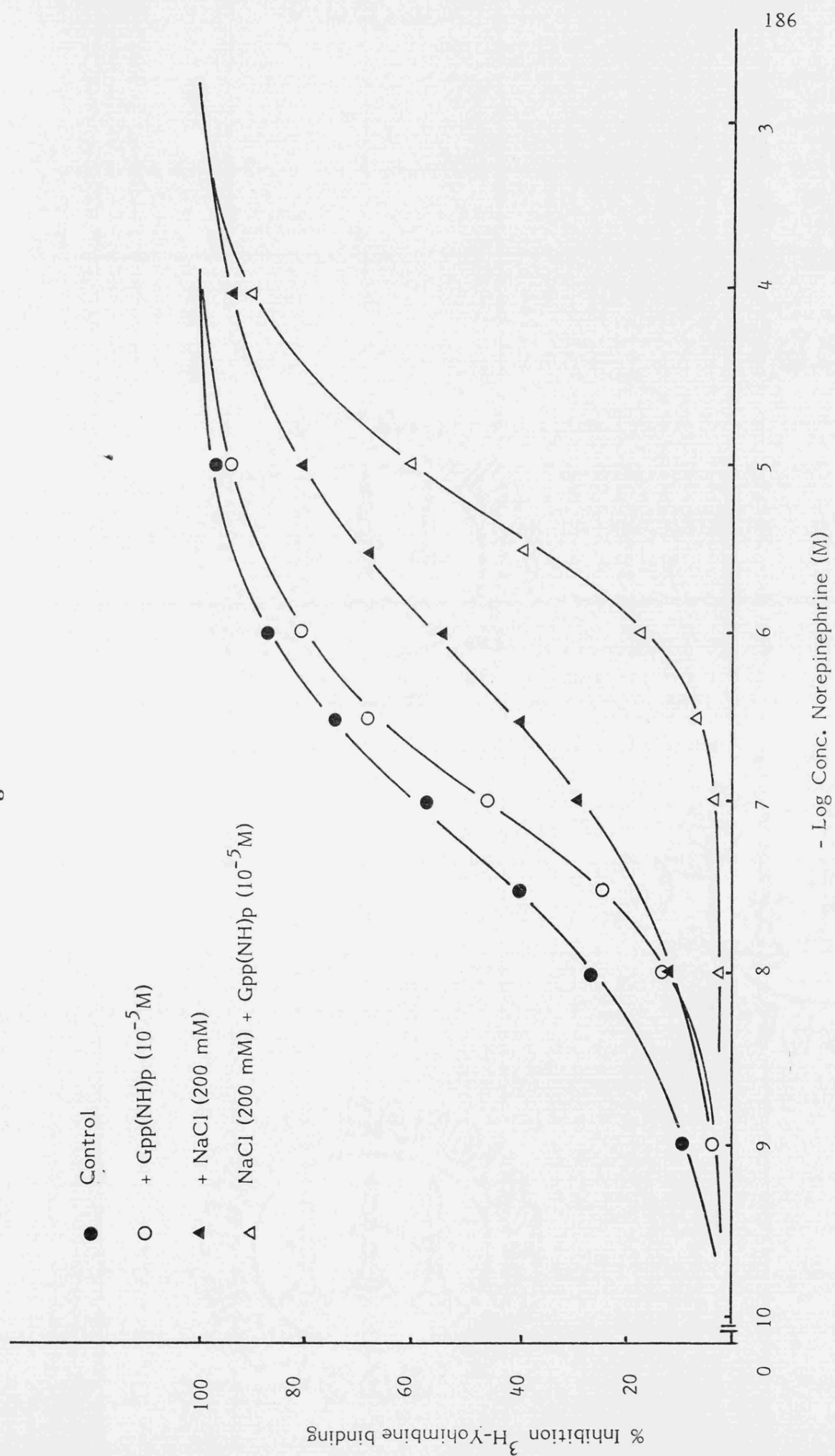
Legend to Figure VII.3.**Effect of Na^+ and Gpp(NH)p on norepinephrine displacement of ^3H -Yohimbine binding to cerebral cortical membranes**

Rat cerebral cortical membranes were prepared in hypotonic Tris buffer (Method B) as described in the text. Incubations were carried out at about 4 nM ^3H -yohimbine with increasing concentrations of norepinephrine in the absence or presence of 10^{-5} M Gpp (NH)p, 200 mM NaCl or both.

Data represents the means of four experiments performed in duplicate.

S.E.M. for each point is less than $\pm 5\%$.

Fig. VII.3.



VII.4 Discussion

The differential interactions of agonists and antagonists with α_2 -adrenoceptors have been discussed in Chapters II and VI. Thus, agonists, but not antagonists, appear to induce or stabilise high affinity guanine nucleotide sensitive states at catecholamine receptors coupled either positively (Kent et al., 1980; DeLean et al., 1980) or negatively (Michel et al., 1980; Limbird et al., 1982; Tsai & Lefkowitz, 1979; Barnett et al., 1982) to adenylate cyclase. In addition, divalent cations, such as Mn^{++} or Mg^{++} have also been shown to promote the formation of high affinity agonist states (Rouot et al., 1980; U'Prichard & Snyder, 1980; Glossmann et al., 1980; also see Chapter VI). Therefore, the presence of divalent cations and retained endogenous agonists in membrane preparations could potentially result in problems with radiolabelled ligand binding assays to receptors linked to adenylate cyclase. In view of this, it was felt that the interesting finding by Woodcock and Murley (1982) that Na^+ ions and guanine nucleotides increase the apparent density of cerebral α_2 -adrenoceptors labelled by 3H -yohimbine, could in fact relate to the interaction of such modulators with retained norepinephrine. The results of the present experiments provide very strong evidence for this interpretation.

Preparation of cerebral homogenates in hypertonic sucrose solutions is known classically to produce a high proportion of 'pinched off' nerve endings or synaptosomes (Gray & Whittaker, 1962). As expected, such preparations contained a relatively high content of retained norepinephrine when compared to preparations that had been washed thoroughly in hypotonic buffers. In the present study, an inverse relationship has been demonstrated between the quantity of endogenous or added exogenous norepinephrine and the apparent binding capacity of 3H -yohimbine to cerebral cortical α_2 -adrenoceptors.

The presence of retained agonist in membrane preparations might merely be expected to reduce the affinity of the radiolabelled ligand for

the receptor sites. In a recent study with intact platelet α_2 -adrenoceptors, an agonist-induced apparent reduction of ^3H -yohimbine binding (determined at a single sub-saturating ligand concentration) was described (Karliner et al., 1982). From their results, the authors indeed suggested that high concentrations of retained agonist could simply increase the apparent K_d of ^3H -yohimbine by shifting the binding isotherm to the right, though producing no true reduction of the maximal binding capacity (i.e. receptor 'down-regulation'). However, an apparent reduction in the binding capacity of labelled antagonist by retained agonist can be predicted in view of the differential molecular behaviour of the agonists and antagonists at adenylylate cyclase linked receptors. In membranes (though not necessarily in intact cells) (see Chapter IV), catecholamine agonists, even present at low concentrations, can preferentially occupy receptor sites by inducing or stabilising the formation of high affinity state of the α_2 -adrenoceptor (Tsai & Lefkowitz, 1979; Michel et al, 1980; Limbird et al., 1982; Barnett et al., 1982). As discussed before, the formation of such high affinity states, which are regulated by ions and guanine nucleotides, appears to involve interactions between the receptor and a guanine nucleotide binding protein (Ni) (Smith & Limbird, 1981) to form a 'ternary complex' in a manner similar to that described for the beta adrenoceptor agonist-receptor interaction (DeLean et al., 1980). In contrast, the α_2 -adrenergic antagonists appear to bind to the receptors with homogeneous affinity without the involvement of guanine nucleotide binding proteins. This is well supported by the finding that only agonist, though not antagonist binding to α_2 -adrenoceptors could be significantly regulated by guanine nucleotides (Tsai & Lefkowitz, 1979; Michel et al., 1980; also see Chapter VI). Furthermore, since yohimbine and other antagonists all generate displacement curves with slopes close to unity, it is likely that antagonists only interact with α_2 -adrenoceptors in a single homogeneous affinity state. Therefore, in membranes, low but significant concentrations of residual endogenous agonist could preferentially

occupy a proportion of the receptor sites in the high affinity state and inhibit the binding of the ^3H -antagonist in an apparent non-competitive fashion. Only unoccupied sites, which would predominantly be of the low agonist affinity state, could be labelled by the antagonist. In other words, retained or added agonist could be expected to preferentially increase the K_d for only a proportion of the ^3H -antagonist binding sites. Consequently, unless the concentration range of ^3H -antagonist was deliberately extended, Scatchard analysis would reveal an apparent fall in its B_{max} (see fig. VII.4.) Of course, an alternative interpretation is that agonists competitively displace ^3H -antagonist from two sites, one of high and the other of low agonist affinity. It should be noted that this 'Pseudo non-competitive' interaction of agonists has also been observed at dopamine D_2 receptors that like the α_2 -adrenoceptors, are negatively coupled to adenylate cyclase at least in the pituitary (Sibley & Creese, 1980).

Although low concentrations of agonists might only block the binding of ^3H -antagonist to high affinity agonist sites, sufficiently high concentrations of agonists could inevitably inhibit both the high and low affinity sites. Under such circumstances, the inhibition might be surmounted only by increasing the concentration of the labelled antagonist to a much higher level, though overall increases in the apparent K_d would also be expected.

The modulatory effects of Na^+ and guanine nucleotides on agonist affinity at α_2 -adrenoceptors have now been well recognised (Tsai & Lefkowitz, 1978; Glossmann & Presek, 1979; Michel et al., 1980; Limbird et al., 1982; Snively & Insel, 1982; also see Chapter VI).

The results in this study have further illustrated the ability of these two modulators to restore the binding capacities of ^3H -yohimbine in cortical membranes previously depressed by the presence of endogenous or exogenous norepinephrine. In the presence of high concentrations of Na^+ and guanine nucleotides, agonist affinity can be markedly decreased as shown in the experiments illustrated in Fig. VII.3. Consequently, the entire population

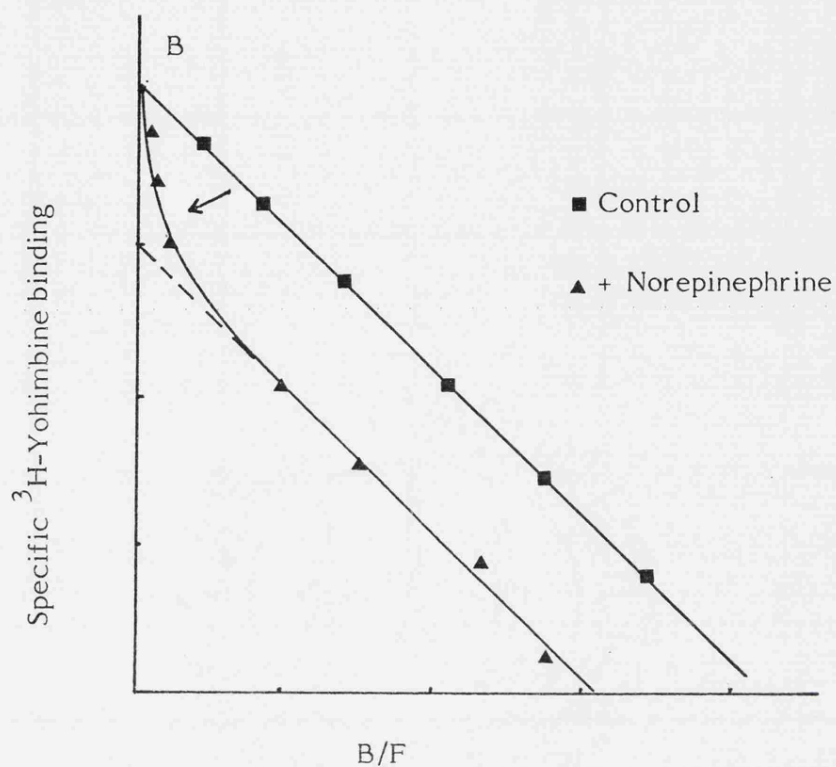


Fig. VII.4.

Diagrammatic interpretation of pseudo non-competitive inhibition of ^3H -Yohimbine binding by exogenous norepinephrine

Low concentrations of norepinephrine (noradrenaline) preferentially occupy and raise the K_d for only a proportion of the ^3H -yohimbine sites. Scatchard plot of binding data would therefore reveal an apparent fall in the B_{max} unless ligand concentrations were much extended.

of receptors is free from agonist occupancy and available for labelling by the ^3H -antagonist. Therefore, a more consistent range of receptor concentrations can be obtained in the presence of high concentrations of both Na^+ and guanine nucleotides, regardless of membrane preparation methods or the presence of low concentrations of agonist.

In this study, exogenous norepinephrine apparently competes for ^3H -yohimbine binding less potently than retained endogenous agonist. Thus, in competition experiments (Fig VII.3) 5 nM norepinephrine displaces approximately 25% of the binding, compared to the apparent 50% reduction in binding capacity in the synaptosomal cerebral membranes A. This may relate to difference of access to the receptor site between exogenous and endogenous agonist and requires further investigation.

Nevertheless, results from this study have indeed demonstrated the inverse relationship between the binding capacity of ^3H -yohimbine to α_2 -adrenoceptors and the norepinephrine content in cerebral cortical membrane preparations. The inhibition of ^3H -yohimbine labelled sites by endogenous or exogenous norepinephrine can be readily reversed by high concentrations of NaCl and guanine nucleotide. The increases in binding capacities of ^3H -yohimbine in rat cerebral cortex by these modulators, as previously reported (1982), might not necessarily represent a unique molecular behaviour of the cortical α_2 -adrenoceptors compared with other α_2 -adrenoceptors. Such a phenomenon probably reflects an interaction between these modulators and the residual endogenous agonist at the receptors in cerebral membrane preparations.

These observations have significant wide implications since they could clearly influence the interpretation of apparent receptor 'down regulation' by agonists. Thus, unless membranes are fully cleared of agonist, an apparent reduction in B_{max} for a labelled antagonist without a change in apparent K_d could be interpreted as receptor 'down regulation'. It would seem likely that this may be avoided by preparing well washed membranes in

hypotonic buffer and/or performing assays in the presence of Na^+ and guanine nucleotides.

Chapter VIII
CHARACTERISATION OF SOLUBLE ALPHA₂-ADRENOCEPTORS

VIII.1. Introduction

Solubilization is a process by which a relatively water insoluble compound is 'dissolved' in an aqueous medium by association with the surface active agent (or detergent) micelles. It occurs by virtue of the ability of the detergent to reduce the interfacial free energy between the water and hydrophobic solubilize molecules allowing a dispersed system of association colloids 'emcasing' the solubilize to exist in solution. Since solubilization depends on the presence of micelles, it only occurs above the C.M.C. - the concentration of the detergent exceeding the surface monolayer formation (Reber & Schott. 1975).

The solubilization of receptor proteins is based on the same principles. Most of the membrane proteins, including the neurotransmitter receptor proteins, are probably intrinsic proteins (Singer 1974). Therefore, extraction or solubilization of such proteins depends on replacing the hydrophobic lipid-protein interactions in membrane with detergent-protein interactions (Helenius & Simons. 1975; Lindstrom. 1978). Detergents differ in their ability to solubilize a given type of receptor protein from a particular tissue, or from different tissues. Obviously, successful solubilization of a receptor protein by a detergent depends not only on the properties of the detergent and receptor protein, but also on the characteristics of the membrane lipid-protein associations in a particular system.

Generally, solubilization is a rather non-specific process (Reber & Schott 1975). Therefore, a good yield of extracted protein may not necessarily mean a good yield of specific receptor protein. To find a good detergent for solubilizing a particular neurotransmitter receptor is probably a matter of 'trial and error'. A variety of detergents have been tried for different receptors. For instance, the glycoside detergent digitonin has been found to successfully solubilize beta-receptors (Dickinson & Nahorski 1981), dopamine D₂ receptors (Gorissen et al. 1980), muscarinic receptors

(Aronstam et al. 1978), α_2 -receptors (Smith & Limbird 1981) and opiate receptors (Howells et al. 1982). The zwitterionic detergent CHAPS has also been shown to solubilize α_2 -receptors (Nambi et al. 1982), the putative calcium channels (Ferry & Glossmann 1983_b), and dopamine D_2 receptors (Kuno et al. 1983).

At the present time, there have been no reports on the pharmacological characterisation of solubilized α_2 -receptors, except the human platelet (Michel et al. 1981; Smith & Limbird 1981), and recently, the calf cerebral cortex (Sladezek et al. 1984). The present studies were undertaken in an attempt to solubilize and characterize α_2 -receptors in a number of tissues. A major aim was to compare the pharmacological properties of the soluble α_2 -receptors in the light of the previously observed differences among particulate α_2 -receptors (see Chapter V). It is conceivable that even identical receptor protein from different tissues may exhibit apparent differences in drug affinities. This could be attributed to differences in the membrane environment or constraint, thus resulting in conformational differences in the receptor molecules and influencing the relative extent to which the interactions between the drug molecules and the recognition sites, or the receptor groups, can be optimized. Having been solubilized from tissue membranes, soluble α_2 -receptors would be expected to be relatively free from membrane constraint and exist in a more uniform environment. Thus, studying the characteristics of soluble α_2 -receptors would be helpful in ruling out membrane constraint/environment as a possible factor for the apparent observed pharmacological differences between the particulate α_2 -receptors.

Although a number of detergents were used in the present studies, only digitonin appeared to be generally effective in preparing soluble assayable α_2 -receptors. Furthermore, only α_2 -receptors from the human platelet, rat kidney and rabbit kidney have been successfully solubilized and detected. Consequently, in the present studies, digitonin-solubilized

alpha₂-receptors from these three tissues have been characterized using ³H-rauwolscine, coupled with an efficient PEG precipitation - filtration assay method.

The results show that soluble alpha₂-receptors exhibit pharmacological characteristics similar to those in membranes, and still reveal significant differences between tissues. Therefore, these data provide further evidence for the heterogeneity of alpha₂-receptors.

VIII.2. Methods

VIII.2.1. Solubilization of α_2 -adrenoceptors

VIII.2.1.1 General Procedures

Tissue membranes were prepared as described in Methods (Chapter II). After the final washing, membranes were resuspended in assay buffer (50mM Tris HCl, 0.5 mM EDTA, 0.1% ascorbic acid, pH 7.5) at a concentration of 6 - 25 mg/ml of protein (approximately 6 - 8 mg/ml for platelet, 16 - 20 mg/ml for rat kidney and 20 - 24 mg/ml for rabbit kidney). The membranes were then mixed with equal volume of buffer containing digitonin 10 - 26 mM and NaCl 0.2 - 0.4 M (final concentrations for digitonin and NaCl respectively = 5mM and 0.2M for platelet, 10 mM and 0.1 M for rat kidney, 13 mM and 0.1 M for rabbit kidney). The mixtures were thoroughly mixed and left for 15 minutes at 4°C with occasional shaking. Subsequently, the mixtures were homogenized for 1 minute with a Ultra-Turrax homogenizer. The homogenates were then centrifuged at 4°C for 1 hour at 50,000 xg. The yellowish-brown supernatant, representing the solubilized receptor preparation was carefully harvested using transfer pipets, and kept chilled on ice until being used for assays.

VIII.2.1.2. Special Considerations

VIII.2.1.2.A Choice of detergent

A few of the mostly commonly used receptor solubilizing detergents were tested with respect to their ability to solubilize α_2 -adrenoceptors. These included deoxycholate, sodium cholate, CHAPS (3 - [(3-Cholamidopropyl) dimethylamino] - 1-propane sulfonate), and digitonin.

Preliminary studies were carried out using the rat renal cortical membranes. Table VIII.1. summarizes the comparative effectiveness of these detergents in solubilizing the rat kidney α_2 -receptors. Both cholate and deoxycholate were ineffective, as only 5% or less of specific ^3H -rauwolscine binding sites were detectable, although the total protein yields were more than 40%. Of course, it must be pointed out that possible

Table VIII.1. Solubilization of α_2 -receptors from rat renal cortex by detergents

Detergent	% Protein Yield	% Yield of Specific ^3H -Rauwolscine Binding
Cholate (0.4 - 0.8%)	42	4 - 5
Deoxylate (0.4 - 0.8%)	40	4 - 5
10 mM Digitonin	51	19
10 mM Digitonin + 0.1M NaCl	60	38
NaCl (2M)	45	2
10mM CHAPS	63	3
10mM CHAPS + 2 - 4M NaCl	75	28

Membranes of rat renal cortex were solubilized as described in Section VIII.2. and soluble extracts were assayed for ^3H -Rauwolscine binding under identical conditions. Results are mean estimates of 2 separate experiments.

interference with ^3H -rauwolscine binding assays by detergents cannot yet be excluded. The use of 2 M NaCl alone could apparently also extract a large amount of membrane protein, but the receptor yield was almost negligible. CHAPS appeared to have the capacity to solubilize α_2 -receptors. However, a relatively good yield of specific ^3H -rauwolscine binding sites was only possible when exceptionally high concentrations of NaCl (2-4M) were used. The presence of such high concentrations of NaCl would seem undesirable for binding assays, as it might potentially influence the physico-chemical properties of drug-receptor interactions. In fact, there is a possibility that incomplete sedimentation of membranes may occur as the result of increased density of the medium at high salt concentrations (Gorissen et al. 1981; Laduron & Ilgen 1982). Moreover, the presence of high concentrations of NaCl also tended to increase non-specific protein extraction and hence, non-specific ligand binding. In comparison, the glycoside detergent digitonin was not only able to produce a high yield of total membrane protein, but also a relatively high yield of ^3H -rauwolscine binding sites. Furthermore, the presence of 0.1 M NaCl enhanced the receptor yield almost 100%.

Since the main aim of this study was to compare the characteristics of the soluble α_2 -receptors from different tissues, solubilization and assay conditions were intended to be kept as uniform as possible. This also included choosing a single detergent which hopefully could be applied effectively to all the tissues under investigation.

Digitonin was indeed as effective in solubilizing α_2 -receptors from the human platelet as from the rat kidney. The use of 5mM digitonin in the absence of NaCl resulted in a total protein yield of about 45% and a detectable specific ^3H -rauwolscine binding yield of about 20%. The presence of 0.2M NaCl enhanced the protein and receptor yields to about 52% and 37% respectively. CHAPS could also produce relatively high yields of protein and ^3H -rauwolscine binding sites from platelet membranes,

compared to digitonin, but this again required the presence of higher concentrations of NaCl (eg. specific ^3H -rauwolscine binding yield was about 15% at 0.2M NaCl and about 40% at 0.5M).

Digitonin extracted about 43% of protein from the rabbit renal cortical membranes, with about 23% yield of detectable specific ^3H -rauwolscine binding sites, when solubilization was carried out under optimal conditions (13 nM digitonin and 0.1 M NaCl). However, the glycoside detergent has limited capacity in solubilizing α_2 -receptors from rat or rabbit cerebral cortical membranes. Under the optimal conditions, the best yields of detectable receptor sites were no more than 5 - 10%. Moreover, CHAPS was also ineffective in solubilizing assayable α_2 -receptors from these two tissues.

VIII.2.1.2.B. Optimal Solubilizing Conditions

Although protein yields tended to increase with increasing detergent concentrations, the yields of specific ^3H -rauwolscine binding did not necessarily vary in direct proportion to detergent concentrations. In fact, optimal receptor yield appeared to require an appropriate balance between the membrane protein content and detergent concentration, and excessively high detergent concentrations could reduce ^3H -rauwolscine specific binding yields. Figures VIII.1.A, B and C illustrate the relationship between specific ^3H -rauwolscine binding and detergent concentration in the rat kidney, platelet and rabbit kidney respectively, when solubilization was carried out at a constant amount of membrane protein. The optimal detergent concentrations were found to be about 10 mM, 5 mM and 13 mM respectively for the above tissues.

As indicated earlier, the presence of NaCl markedly enhanced the yield of ^3H -rauwolscine binding sites. However, the best yields appeared to occur only at an optimal concentration of NaCl (Figs. VIII.2.A, B and C). Thus, the optimal NaCl concentrations were 0.1M, 0.2M and 0.1M respectively for the rat kidney, human platelet and rabbit kidney membranes

Legend to figures VIII.1.a, b, and c

Relationship between assayable specific ^3H -Rauwolscine binding
and digitonin concentration in solubilization

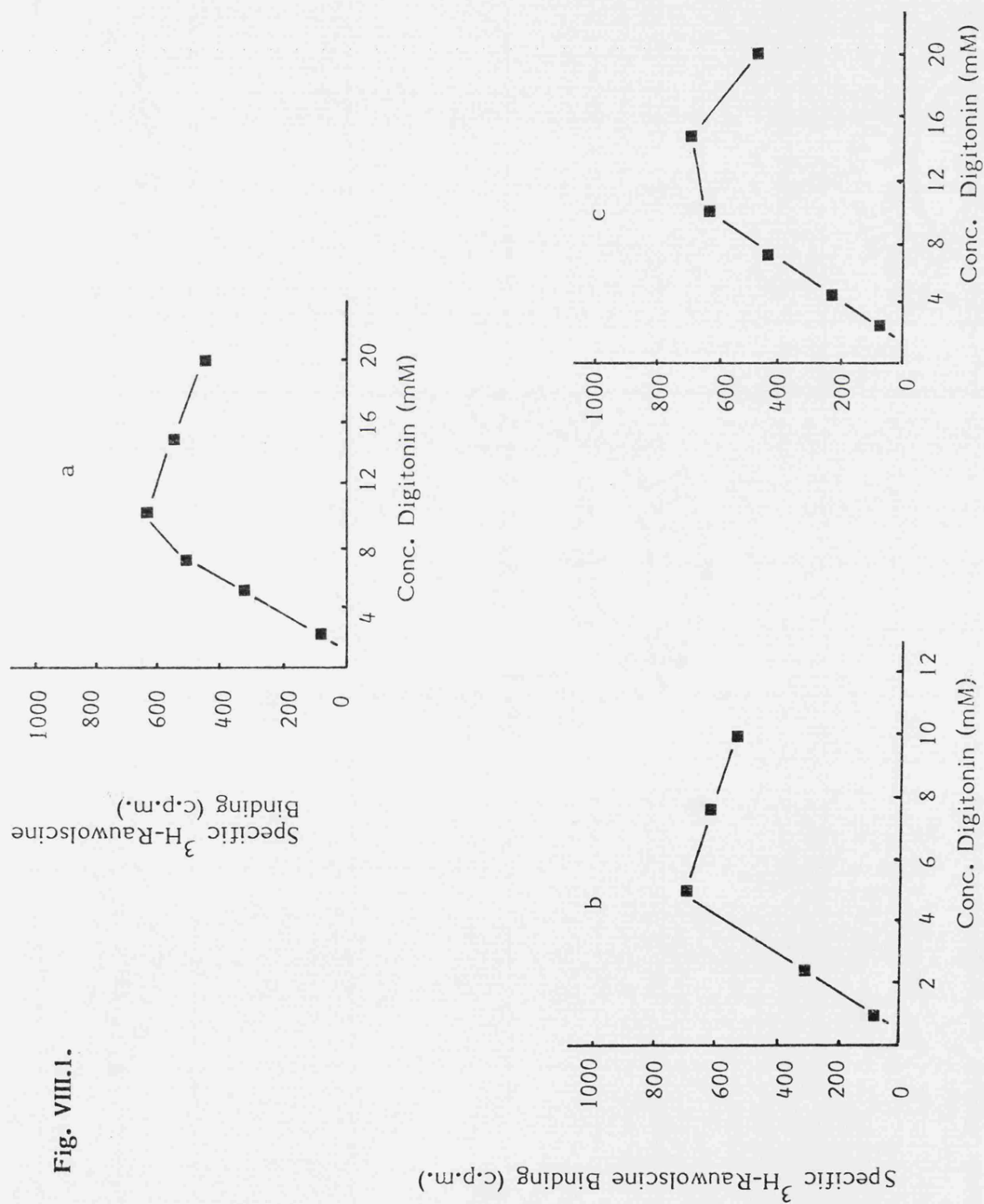
- a) Rat renal cortex
- b) Human platelet
- c) Rabbit renal cortex

Membranes of each tissues were prepared as described in Chapter

III. For each tissue, equal aliquots of membranes were treated in identical manner with an equal volume of digitonin (in Tris-HCl assay buffer) of variable concentration strength as described in Section VIII.2. The mixtures contained a final NaCl concentration of 100 mM. Aliquots of the resultant soluble extracts were assayed for specific ^3H -Rauwolscine binding.

The digitonin concentrations indicated in the figures represent the final concentrations in the membranes and detergent mixtures. Data illustrated is from one of two - three similar experiments performed in duplicate.

Fig. VIII.1.



Legend to Figures VIII.2.a, b and c

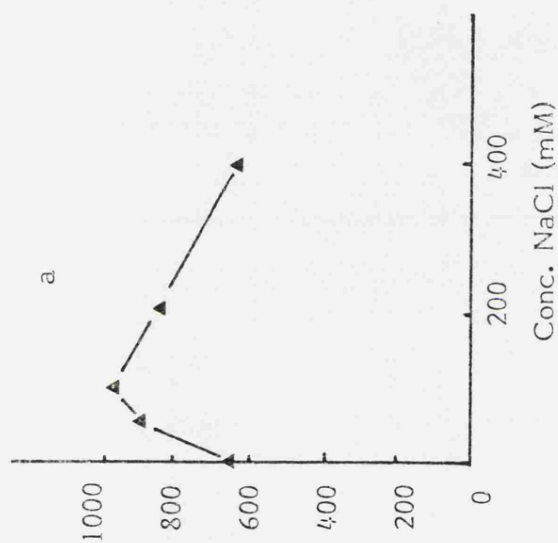
Relationship between assayable specific ^3H -Rauwolscine binding to digitonin-solubilized preparation and NaCl concentration in solubilization.

- a) Rat renal cortex
- b) Human platelet
- c) Rabbit renal cortex

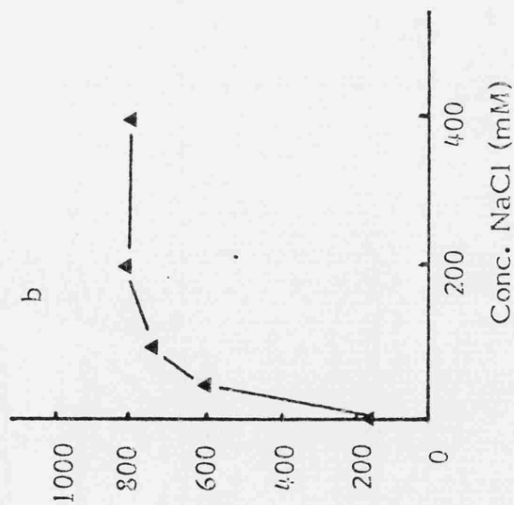
Membranes of human platelet, rat renal cortex and rabbit renal cortex were prepared as described in Chapter III. For each tissue, membrane suspension was divided into equal aliquots. The aliquots were treated in an identical manner by homogenizing with equal volume of digitonin in Tris-HCl assay buffer at an optimal final digitonin concentration (ie 10mM, 5mM and 13mM respectively for rat kidney, human platelet and rabbit kidney) as described in Section VIII.2. The solubilization was carried out in the absence and presence of various concentrations of NaCl. Aliquots of the resultant soluble extracts were assayed for specific ^3H -Rauwolscine binding according to the method described in Section VIII.2.

The NaCl concentrations indicated in the figures represent final concentrations in the membranes and detergent mixture. Data presented is from one of two similar experiments performed in duplicate.

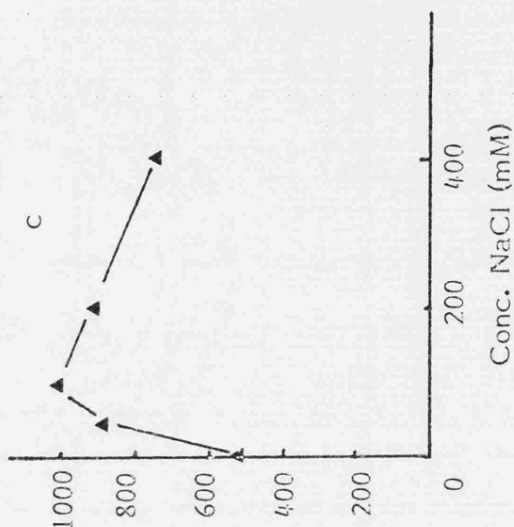
Specific ^3H -Rauwolfscine Binding
(c.p.m.)



Specific ^3H -Rauwolfscine Binding
(c.p.m.)



b



c

Fig. VIII.2.

when solubilization was carried out, using optimal detergent concentrations for the individual systems.

VIII.2.1.2.C. Methods of Solubilization

Rat kidney α_2 -receptors were solubilized with digitonin under identical conditions by the following methods to evaluate possible differences in the yield of specific ^3H -rauwolscine binding:

- A. Membranes were treated by homogenization with digitonin - NaCl (final concentrations = 10 mM and 0.1 M respectively) for 1 minute only before being centrifuged at 4°C for 60 minutes at 50,000 xg.
- B. Membranes were mixed with digitonin - NaCl and stirred in cold for 15 minutes before being subjected to homogenization and centrifugation as in A.
- C. Membranes were only stirred in cold with digitonin - NaCl for 30 minutes before centrifugation. The results indicated that there was little difference in the total protein yield between the three methods, but that method B appeared to produce the highest yield of specific ^3H -rauwolscine binding sites (though it was in fact only slightly higher than the other two methods - 32% compared to 27%). Apparently, solubilization was complete within a fairly short period of extraction time. However, it is quite clear that considerable amount of protein/receptors might have also been extracted during the 1 hour centrifugation process itself.

VIII.2.1.2.D. The 'soluble' nature of digitonin extracts

The routine solubilization procedures in this study involved centrifuging digitonin-treated membrane suspensions for 60 minutes at 50,000 xg. The yellowish-brown supernatant was regarded as the soluble receptor preparation. The true 'soluble' nature of such preparations was evaluated

by subjecting the 50,000 xg supernatant to one further hour of centrifugation at 100,000 xg and to ultrafiltration through 0.2 μ m millipore filter.

The results showed that after ultracentrifugation, there was no apparent visual sediment. Furthermore, specific ^3H -rauwolscine activity in the supernatant and ultrafiltrate was more than 96% of that in the 50,000 xg supernatant. Hence, the 'soluble' nature of the digitonin-solubilized 50,000 xg supernatant was firmly established.

VIII.2.1.2.E. Stability of soluble α_2 -receptors

The stability of soluble α_2 -receptors was assessed using rat kidney soluble preparations.

If kept at -40°C or -70°C , rat kidney soluble preparations appeared reasonably stable for at least 2 - 3 weeks, without significant reduction of ^3H -rauwolscine specific activity.

Less than 15% specific ^3H -rauwolscine binding to soluble rat kidney preparation was lost after 2 hours of storage at 4°C .

If soluble preparation was left at R.T. ($22 - 25^\circ\text{C}$), less than 4 - 5% of specific ^3H -rauwolscine binding was lost in over 1 hour (excluding assay time). The half-life was approximately $4\frac{1}{2}$ hours.

VIII.2.2. Radioligand binding assays of soluble α_2 -receptors

VIII.2.2.1. General Procedures

Assays of soluble receptor preparation were performed under conditions as close to those with particulate preparation as possible.

Assays were performed in a total volume of 250 μ l of Tris-HCl 50 mM, EDTA 0.5 mM, ascorbate 0.1%, pH 7.5 containing 50 μ l radioligand and 100 μ l soluble receptor preparation. Final assay concentration of NaCl was adjusted to 80 mM in all samples.

Incubations were carried out at R.T. ($22 - 25^\circ\text{C}$) for 40 minutes upon the addition of soluble receptor preparation. Reaction was stopped by adding 0.25 ml ice-cold bovine γ -globulin (0.5 mg/0.25 ml), followed

immediately by 1 ml of ice-cold polyethylene glycol 6000 (M.W. 6 - 8000)(15%) in assay buffer. The contents were quickly and thoroughly mixed using a vortex mixer and left in cold (4°C) for 10 minutes before being filtered on GF/B filters under vacuum. The precipitates collected on the filters were counted for radioactivity on a liquid scintillation counter as in the particulate binding assays. Non-specific binding was defined as that binding of ^3H -rauwolscine not displaceable by 5 μM phentolamine.

VIII.2.2.2. Special Considerations

VIII.2.2.2.A. Assay Methods

Soluble receptors can usually be assayed by methods such as gel-filtration, charcoal adsorption and polyethylene glycol(PEG) or ammonium sulphate precipitation.

Although the gel-filtration method is a relatively high-capacity method, it is also more time-consuming, compared to the adsorption or precipitation methods which allow large number of samples to be assayed efficiently.

Preliminary evaluation of the charcoal adsorption method in this study indicated that high concentrations of charcoal (6 - 8%) was needed to adsorb at least 98% of free ^3H -rauwolscine. At such high concentrations of charcoal, separation of bound and free radioligand became a problem. Indeed, the non-specific binding was of high percentage relative to the total binding.

In comparison, the precipitation method coupled with filtration under vacuum appeared to be an efficient way of assaying soluble α_2 -receptor binding. The PEG precipitation in particular produced a relatively low % background (or non-specific) radioligand binding.

VIII.2.2.2.B. Optimal Conditions for PEG Precipitation Assays

In practice, PEG precipitation of soluble proteins requires the presence of a 'carrier' such as γ -globulin which itself quickly precipitates in aqueous

solutions upon the addition of PEG, presumably as a result of competition for water molecules hydrating the proteins (Chard 1980).

Optimal concentrations of γ -globulin and PEG 6000 were determined which would produce a precipitating system best suited for the ^3H -rauwolscine- α_2 -receptor binding assays. It was found that the best combination was bovine γ -globulin at a final concentration of 1 mg/ml together with PEG at a final concentration of 10%. Such combination provided the highest specific ^3H -rauwolscine binding and specific activity.

VIII.2.2.2.C. Washing of filters

This step seemed very essential, since much of the free ligand could be easily 'trapped' on the filters because of the high viscosity of the medium. 15 to 20 ml washing with ice cold 10% PEG could sufficiently reduce the NSB to a minimum, but 3 x 5 ml washes produced the highest specific ^3H -rauwolscine binding activity. The washing procedure only lasted for 15 - 20 seconds. Hence, the dissociation of radiolabel from the filters would be expected to be negligible.

VIII.2.2.2.D. Time interval between PEG addition and filtration

Precipitation of proteins appeared to be complete very quickly subsequent to PEG/ γ -globulin addition, provided the contents were thoroughly mixed. Specific ^3H -rauwolscine binding in fact showed an approximately 5 - 6% reduction over the first 2 minutes between PEG addition and filtration. However, even if samples were left at R.T. for a further 10 - 12 minutes before filtration, there was little change in the ^3H -rauwolscine specific binding or specific activity. However, to avoid possible radioligand dissociation which might occur in some systems but not others, samples were usually left in the cold (4°C) for 10 minutes between PEG addition and filtration.

VIII.2.2.2.E. Effectiveness of PEG precipitation-filtration as a means of separation

A series of dilutions were made from a concentrated rat kidney membrane preparation. Equal aliquot from each dilution was treated in

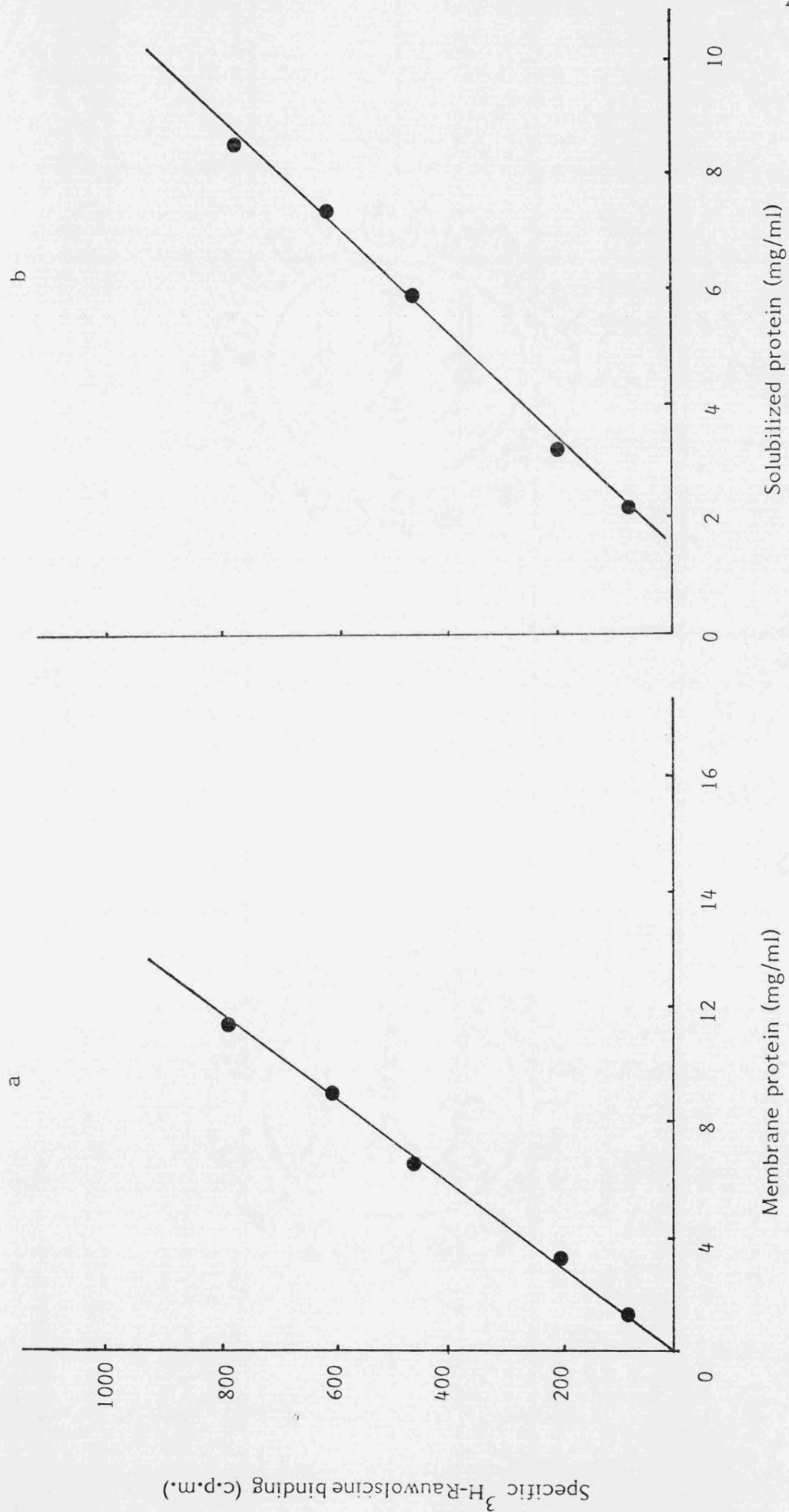
identical manner with an equal quantity of digitonin-NaCl. ^3H -Rauwolscine binding to the solubilized preparations was assayed using the PEG precipitation-filtration method. As shown in Fig. VIII.3.A., there was a good linear relationship between specific ^3H -rauwolscine binding in the resultant soluble preparation and starting membrane protein concentration (at least over the concentration range of 1.5 - 12 mg/ml). Moreover, there was also a good linear relationship between specific ^3H -rauwolscine binding and the extracted soluble protein concentration (Fig. VIII.3.B.), at least over the range of approximately 2 - 9 mg/ml. The soluble protein concentrations used in the present studies generally fell within this range. Therefore, these results confirm that the PEG precipitation-filtration method is an appropriate method for the assays of soluble α_2 -receptors in these studies.

Legend to Figures VIII.3.a., and b

VIII.3.a. Relationship between specific ^3H -rauwolscine binding in soluble preparation and starting membrane concentration. Rat kidney membranes were prepared as described in Chapter III. concentrated kidney membranes were diluted 3/4, 1/2, 1/4 and 1/8. Equal aliquots were solubilized with equal volumes of digitonin (20 mM)/NaCl (0.2M) as described in Methods section of this chapter. Soluble preparations were assayed for specific ^3H -rauwolscine binding by PEG precipitation-filtration method. Results are from one of two similar experiments performed in duplicate.

VIII.3.b. Relationship between specific ^3H -rauwolscine binding and soluble protein concentration. Protein concentrations in the above soluble membrane extracts were determined and plotted against assayable ^3H -rauwolscine specific binding. A linear relationship was obtained between the two parameters at least over the soluble protein range of about 2 - 9 mg/ml. Results presented are from one single experiment.

Fig. III.3.



VIII.3. Results

VIII.3.1. Characterisation of soluble human platelet α_2 -adrenoceptors

VIII.3.1.1 Equilibrium binding and kinetics studies with ^3H -rauwolscine

^3H -rauwolscine bound to digitonin-solubilized receptor preparations of the human platelet with a capacity comparable to that assessed in platelet membranes. The binding was saturable and reversible (Fig. VIII.4,5). Scatchard analysis of binding data suggests that ^3H -rauwolscine bound to a homogeneous population of receptor sites, with an apparent B_{max} of 177 ± 19.64 f.mole/mg protein, and a K_d of 4.59 ± 0.47 nM ($n = 6$) (table VIII.2).

The binding of ^3H -rauwolscine to soluble platelet receptor preparations at R.T. was relatively fast, reaching equilibrium in 30 - 40 minutes. The dissociation occurred at a rather slow rate, with a rate constant (K_2) of 0.03 min^{-1} (Fig. VIII.5) which was identical to that in membranes. However, the association rate constant (K_1) was considerably lower in the soluble receptor preparations than in membranes ($0.011 \text{ nM}^{-1} \text{ min}^{-1}$, compared to $0.073 \text{ nM}^{-1} \text{ min}^{-1}$). Hence, the kinetically derived higher K_d for ^3H -rauwolscine in the soluble platelet receptor preparations, compared to that in the membranes (2.73 nM and 0.4 nM respectively) appeared to be mainly attributed to the slower association rate.

VIII.3.1.2 Pharmacological characteristics

As in the platelet membranes, specific ^3H -rauwolscine binding to platelet soluble receptor preparations could be displaced by various agents to produce a pharmacological profile. As shown in table VIII.3 and Figures VIII.6A and B, the rank order of drug potency in inhibiting ^3H -rauwolscine binding is identical to that observed in platelet membranes, and suggests interaction at α_2 -adrenoceptors. Thus, yohimbine appeared almost equipotent with rauwolscine but both were much more potent than the α_1 -antagonists corynanthine, prazosin, indoramin and labetalol in that order. In addition, the α_2 -agonists, clonidine, adrenaline and

Table VIII.2. Specific ^3H -Rauwolscine binding to particulate and soluble α_2 -receptors

		Human platelet	Rat renal cortex	Rabbit renal cortex
Membrane Preparation	Bmax (f.mol/mg.P.)	144 \pm 5	120.38 \pm 10.2	76.73 \pm 6.32
	Kd (nM)	0.55 \pm 0.06	2.68 \pm 0.29	5.61 \pm 0.35
Soluble Preparation	Bmax (f.mol/mg.P.)	177 \pm 19.64	63.08 \pm 5.17	33.8 \pm 5.32
	Kd (nM)	4.59 \pm 0.47	6.34 \pm 1.12	7.02 \pm 1.43

^3H -Rauwolscine saturation experiments for membranous and soluble receptor preparations were performed at ligand concentrations of approximately 0-35 nM as described in Chapter III and Chapter VIII (Section VIII.2.). Binding data was analysed by Scatchard plots. Results presented are mean \pm S.E.M. of at least 3 - 5 separate experiments performed in duplicate.

Legend to Figure VIII.4.

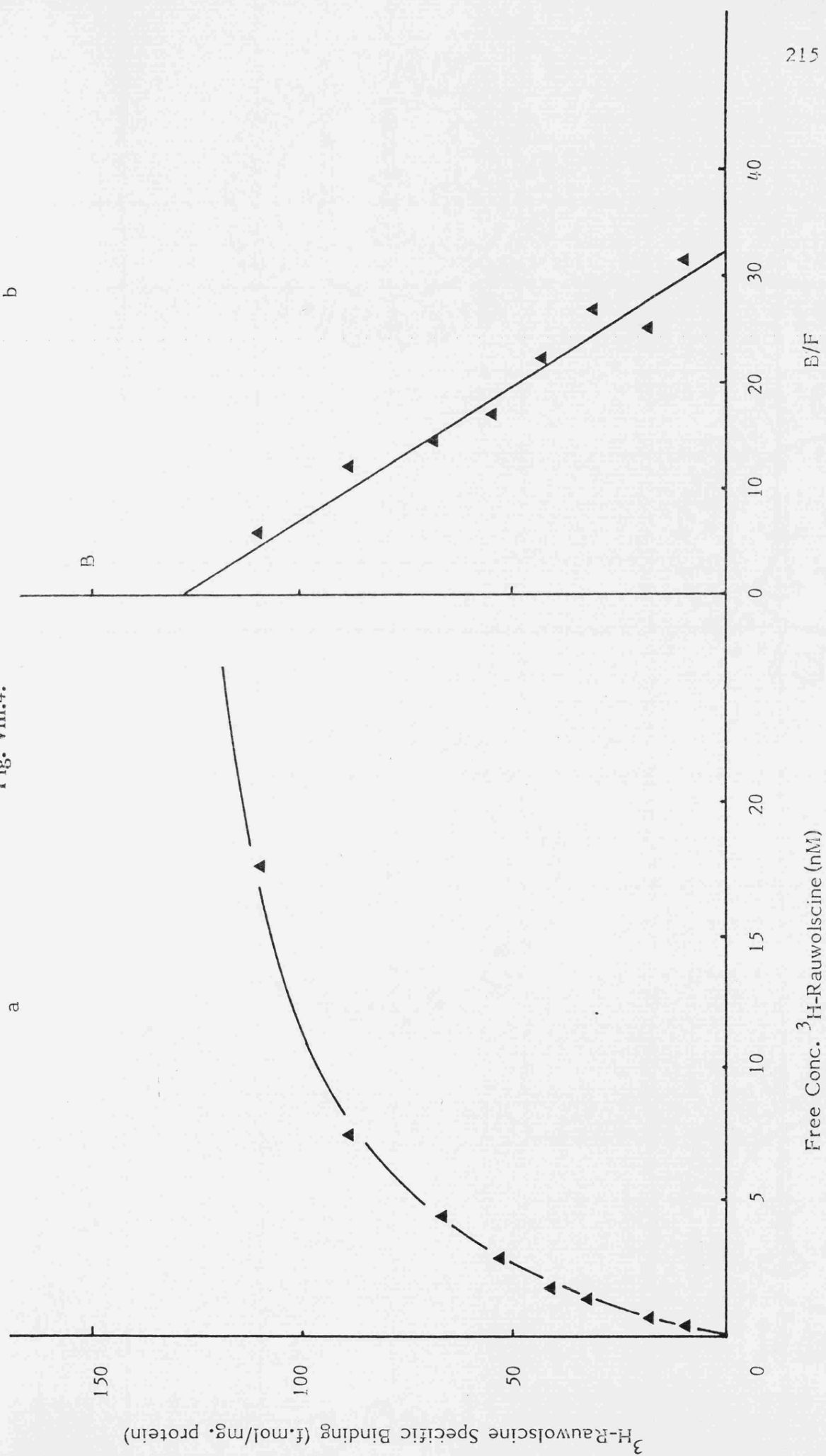
Specific ^3H -rauwolscine binding to soluble platelet preparation.

Platelet membranes were prepared and subjected to solubilization as described in Methods section of this chapter. ^3H -rauwolscine saturation binding assays were performed at R.T. at approximately 0 - 35 nM at a protein concentration of about 2 mg/ml.

- a) Saturation isotherm
- b) Scatchard plot of binding data.

Data presented is from one experiment representative of 4 separate experiments performed in duplicate. $B_{\text{max}} = 126 \text{ f.mol/mg protein}$; $K_d = 3.9 \text{ nM}$.

Fig. VIII.4.



Legend to Figure VIII.5.

Kinetics of ^3H -Rauwolscine binding to soluble human platelet preparation at R.T.

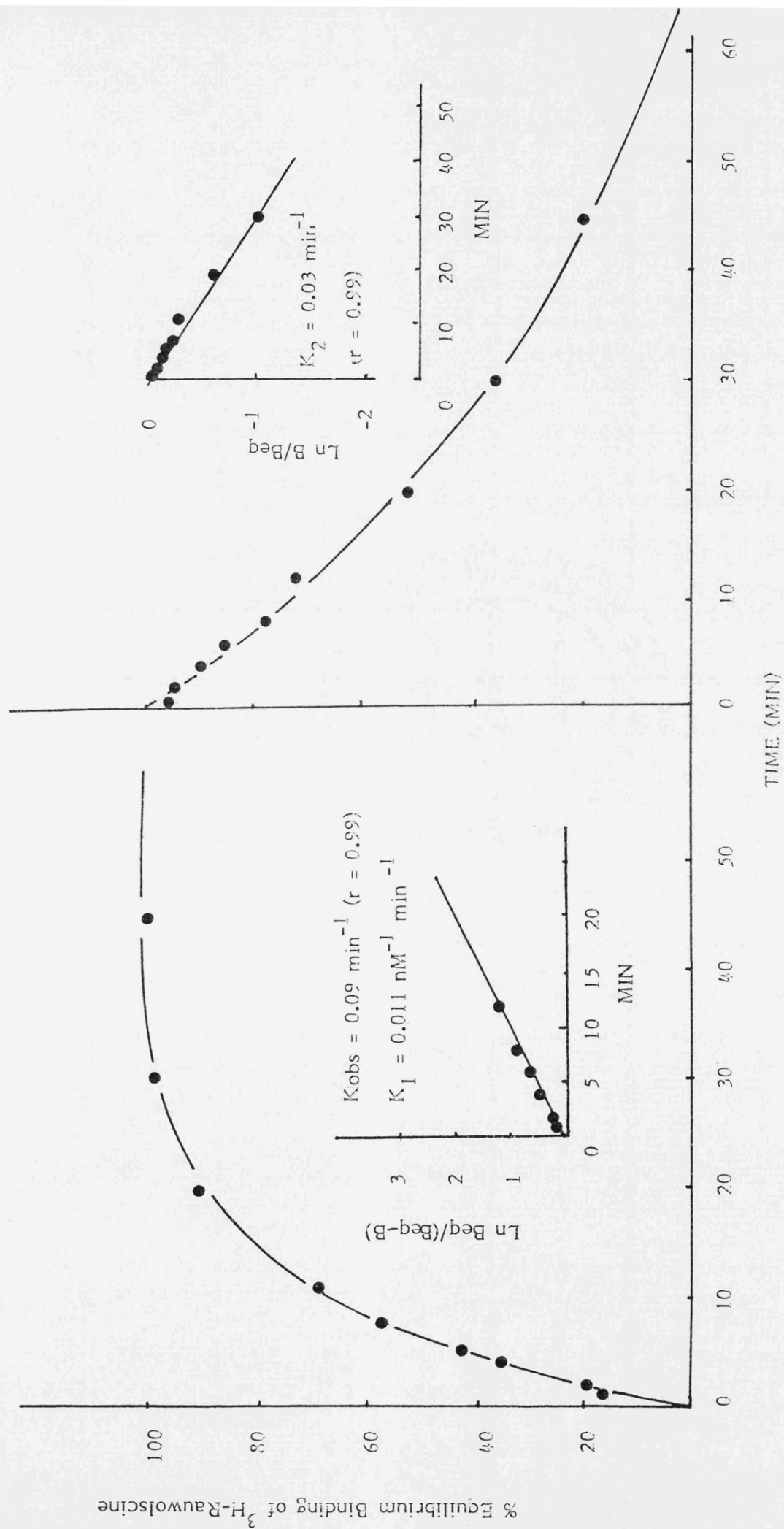
Human platelet soluble α_2 -receptors were prepared as described in Section VIII.2. Kinetics experiments of ^3H -Rauwolscine binding were conducted in a similar manner to that described in Chapter III under assay conditions described in Section VIII.2. (this Chapter).

Left panel = association curve; the inset shows the pseudo first-order rate plot for the association reaction.

Right panel = dissociation of ^3H -Rauwolscine binding; the inset shows the first-order dissociation rate plot.

Data was analysed as described in Chapter III. The data illustrated here is from one of two similar experiments performed in duplicate.

Fig. VIII.5.



Legend to figures VIII.6.a. and b.

Displacement of specific ^3H -rauwolscine binding to soluble platelet preparation by drug agents.

- a) Antagonists
- b) Agonists

Assays were performed at 4 - 6 nM ^3H -rauwolscine with increasing concentrations of displacing drugs under conditions described in Section VIII.2. The data presented are means of 3 - 6 separate experiments performed in duplicate. The S.E.M. are generally less than $\pm 5\%$

Fig. VIII.6.b.

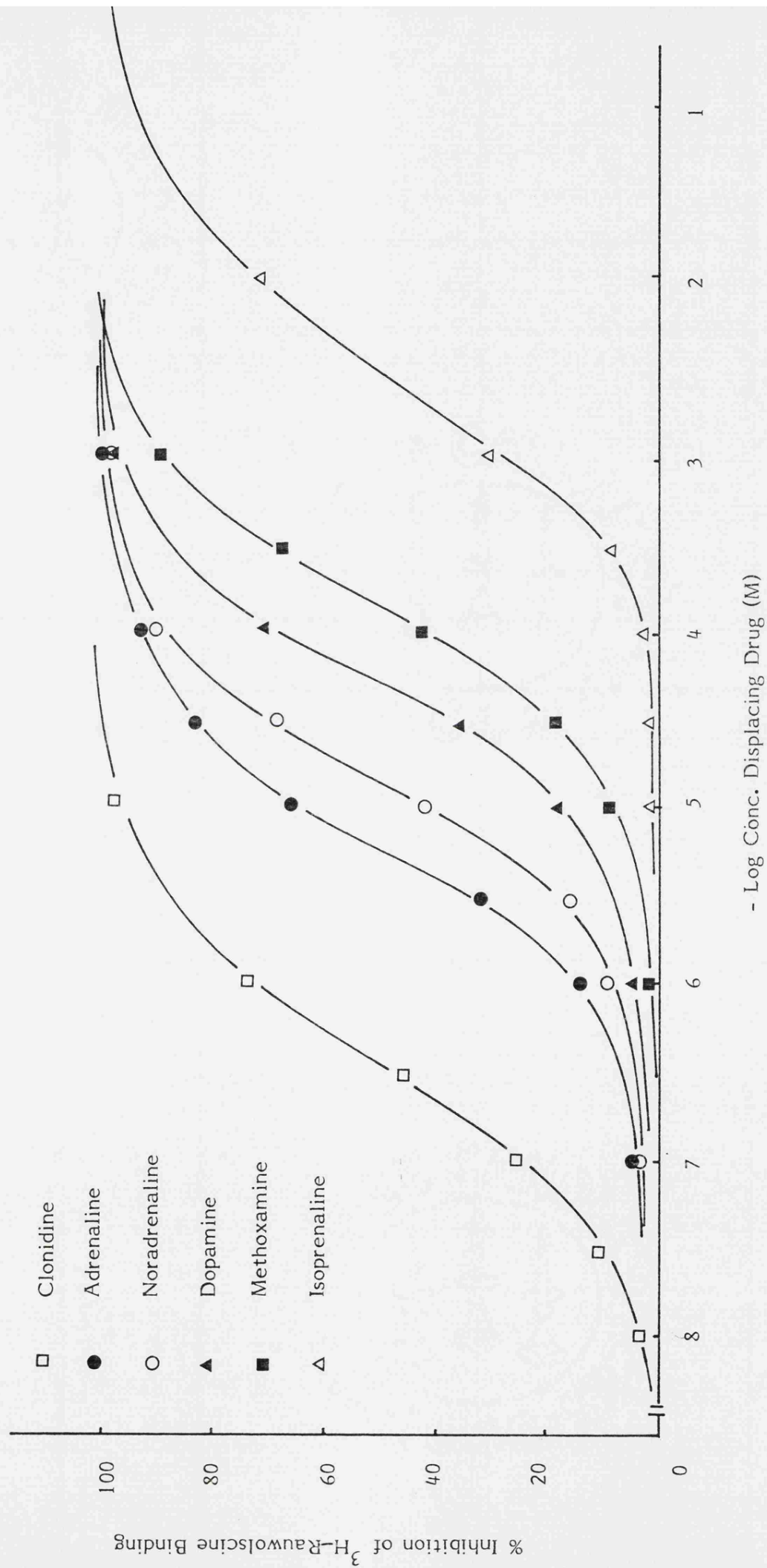


Table VIII.3. Affinities of drugs at soluble α_2 -receptors labelled by ^3H -Rauwolscine

Drug	Ki (nM)			
	Human Platelet	Rat Kidney	Rabbit Kidney	
Rauwolscine	5.15 \pm 0.75 (0.99)	7 \pm 0.24 (1.01)	5.34 \pm 0.68	(1.0)
Yohimbine	4.1 \pm 0.51 (0.95)	15 \pm 1.7 (1.03)	18 \pm 2.8	(1.0)
Phentolamine	21.4 \pm 1.48 (0.97)	28 \pm 4.4 (0.95)	42 \pm 6	(0.96)
Corynanthine	1274 \pm 197 (0.96)	421 \pm 50.7 (1.0)	878 \pm 171	(0.97)
Prazosin	1875 \pm 169 (0.99)	73.6 \pm 6.2 (1.0)	326 \pm 25.7	(0.98)
Indoramin	19000 \pm 2757 (0.99)	2735 \pm 355 (1.02)	1947 \pm 249	(0.97)
Labetalol	10037 \pm 1276 (0.97)	4810 \pm 318 (1.01)	-	
Clonidine	122 \pm 11.8 (0.93)	55.3 \pm 6.6 (0.89)	109 \pm 10.2	(0.74)
Adrenaline	2420 \pm 147 (0.98)	3898 \pm 361 (0.63)	6523 \pm 323	(0.76)
Noradrenaline	6887 \pm 457 (0.97)	6490 \pm 560 (0.63)	6700 \pm 397	(0.85)
Methoxamine	66450 \pm 4702 (0.97)	57500 \pm 4419 (0.99)	49150 \pm 5680	(0.97)
Dopamine	20640 \pm 1824 (0.97)	19833 \pm 1687 (0.9)	123200 \pm 8108	(0.99)
Isoprenaline	1118000 \pm 59254 (0.96)	701000 \pm 52317 (0.95)	748000 \pm 47140	(0.97)

Competition experiments were performed as described in Methods (Section VIII.2.) at 4 - 6 nM ^3H -Rauwolscine. Ki values were determined from IC_{50} as described in Chapter III. Values shown are means \pm S.E.M. of at least 3 - 6 separate experiments performed in duplicate. The numbers in parentheses are mean slope factors of displacement curves.

noradrenaline were more potent than the α_1 -agonist methoxamine. Both dopamine and isoprenaline were relatively weak. It is quite interesting that almost all these drugs exhibited weaker affinities in the soluble receptor preparations than in membranes. Although the presence of 80 mM NaCl in the soluble receptor preparations might potentially cause a reduction in the affinity of drugs, particularly for the agonists, this does not appear to sufficiently account for the overall large discrepancies in drug affinity between the soluble and membranous platelet preparations. Indeed, it was found that the presence of 100 mM NaCl produced considerably less influence on the affinities of the antagonists in the membranes than in the soluble preparations. Finally it is worth noticing that all the drugs, agonists and antagonists alike, generated steep displacement curves with slopes close to unity. This suggests that platelet α_2 -receptors are uncoupled from the N-protein during solubilization, thus interacting with agonists in a homogenous low-affinity state (Smith & Limbird 1981).

VIII.3.2. Characterisation of soluble α_2 -adrenoceptors of the rat renal cortex

VIII.3.2.1. Equilibrium binding and kinetics studies with ^3H -rauwolscine

The apparent maximal binding capacity (B_{max}) for ^3H -rauwolscine was somewhat lower in the rat renal cortical soluble preparation than in membranes (Table VIII.2). However, this probably only reflects a lower yield of detectable specific ^3H -rauwolscine binding sites relative to the total protein yield. The apparent dissociation constant (K_d) of the ligand as derived from equilibrium binding studies also appeared higher in the soluble receptor preparations than in membranes. Nevertheless, the binding of ^3H -rauwolscine was relatively rapid, reaching equilibrium in 30 - 40 minutes at R.T. (Fig. VIII.8). Moreover, the specific binding was saturable and reversible (Fig. VIII.7 and 8) and indicative of an interaction with a homogenous population of receptor sites.

The kinetics of ^3H -rauwolscine binding to soluble receptor preparations of the rat kidney and human platelet were quite similar. For example, the association rate constant was $0.017 \text{ nM}^{-1} \text{ min}^{-1}$ in the rat kidney compared to $0.011 \text{ nM}^{-1} \text{ min}^{-1}$ in the platelet. The dissociation rate constants were 0.30 min^{-1} and 0.04 min^{-1} respectively. Consequently, the calculated K_d (k_2/k_1) values for ^3H -rauwolscine in the soluble platelet and rat kidney receptor preparations, 2.73 nM and 2.27 nM respectively, were quite similar to each other and were in reasonably good agreement with those derived from equilibrium binding studies. However, despite of the similarity of the kinetically derived K_d values for ^3H -rauwolscine between the rat kidney soluble and membranous receptor preparations the apparent association and dissociation rate constants were both somewhat higher in the membranes than in the soluble receptors (k_1 and k_2 were $0.0176 \text{ nM}^{-1} \text{ min}^{-1}$ and 0.04 min^{-1} respectively in the soluble receptor preparations, compared to $0.04 \text{ nM}^{-1} \text{ min}^{-1}$ and 0.08 min^{-1} respectively in the membranes).

Legend to Figure VIII.7.

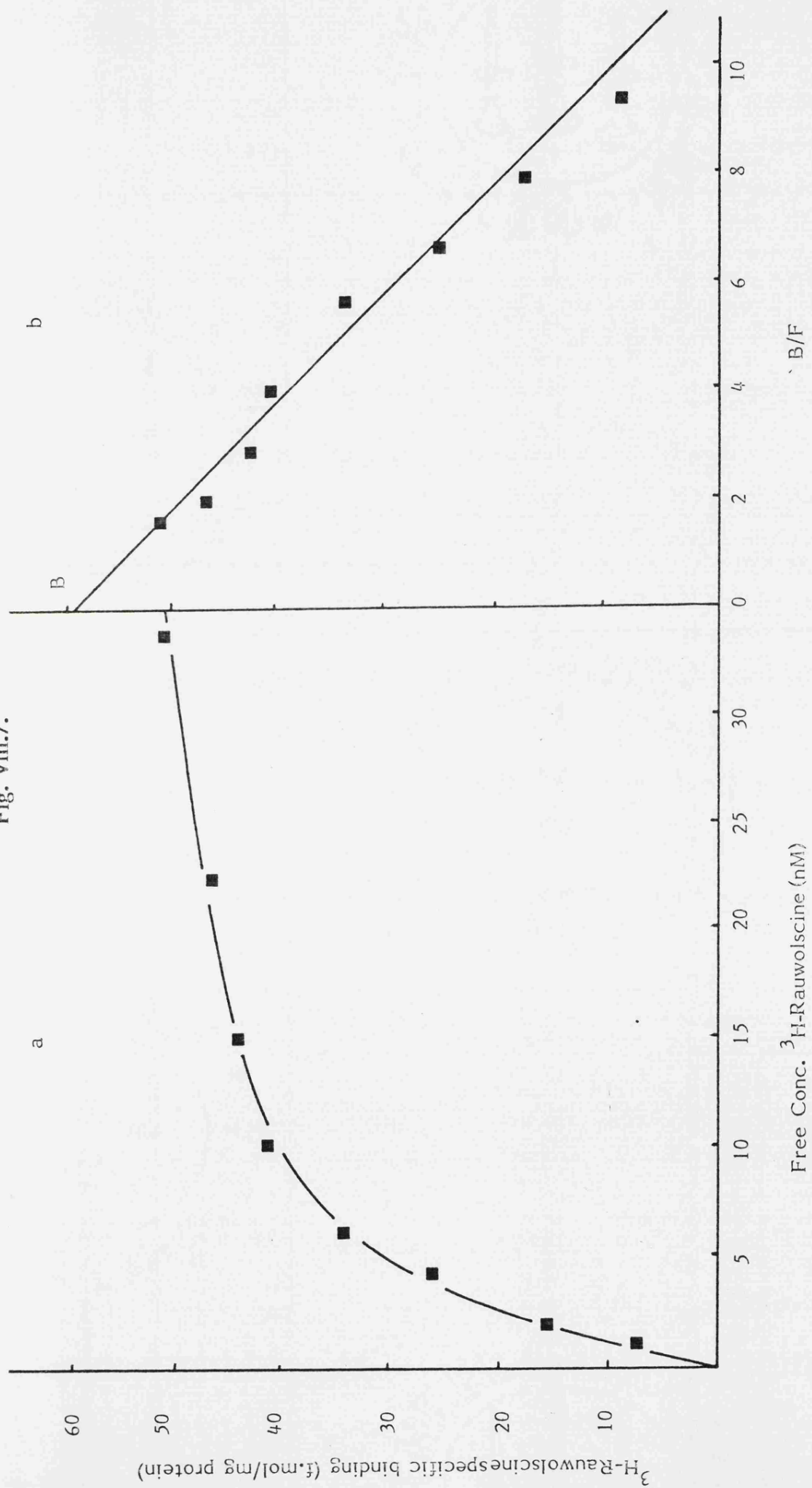
³H-rauwolscine specific binding to soluble rat renal cortical preparation.

Rat renal cortical membranes were treated with digitonin-NaCl as described in the Methods section (VIII.2.). Saturation binding assays of ³H-rauwolscine (approximately 0-35 nM) were performed with soluble rat kidney preparation at a protein concentration of 5 - 6 mg/ml.

- a) Saturation isotherm
- b) Scatchard analysis of binding data

Results illustrated are from one of five similar experiments performed in duplicate. Bmax = 59.4 f.mol/mg protein; Kd = 5.28 nM.

Fig. VIII.7.



Legend to Figure VIII.8.

Kinetics of ^3H -Rauwolscine binding to soluble rat renal cortical preparation at R.T.

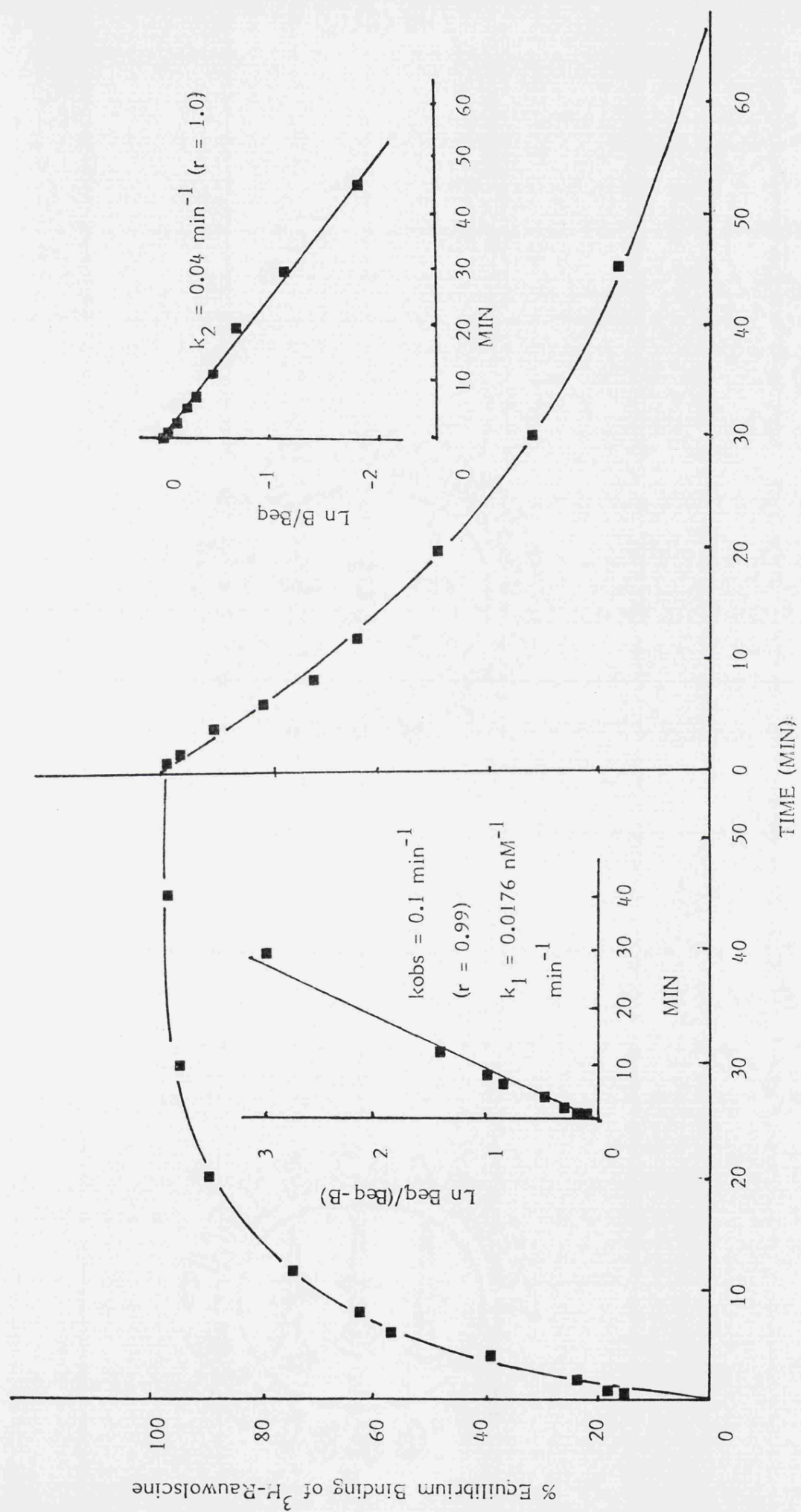
Rat kidney membranes were prepared and extracted with digitonin as described in Chapter III and Section VIII.2. of this chapter. Kinetics experiments of ^3H -Rauwolscine binding to soluble preparation were carried out under conditions described in Section VIII.2, with the assay procedures similar to those described in Chapter III for membranes.

Left panel = association curve; the inset shows the pseudo first-order rate plot for the reaction.

Right panel = dissociation of ^3H -Rauwolscine binding; the inset shows the first-order dissociation rate plot.

Data illustrated is from one of two similar experiments performed in duplicate, with results differing by less than 5%.

Figure VIII.8.



VIII.3.2.2. Pharmacological characteristics

Specific ^3H -rauwolscine binding sites of the solubilized rat kidney preparations displayed a pharmacological behaviour similar to that in membranes. Therefore, although the overall pharmacological profile was still one typical of an α_2 -receptor (Table VIII.3., Fig VIII.9.A,B), the rank order of drug potency in inhibiting ^3H -rauwolscine binding was quite different from that in platelet. Yohimbine had a significantly lower affinity than rauwolscine in the soluble rat kidney preparations, compared to the soluble platelet preparations where yohimbine appeared equipotent with rauwolscine. In addition, although the α_1 -antagonists were significantly weaker than yohimbine or rauwolscine, they were relatively more potent inhibitors of ^3H -rauwolscine binding in the soluble rat kidney receptor preparations (Table VIII.3., Fig. VIII.10. A,B,C and D) than in the soluble platelet receptor preparations. It is important to note that almost all the antagonists displaced ^3H -rauwolscine binding with curves of slope close to unity, suggesting that interaction occurred at a homogenous population of sites or affinity states. However, the curves for clonidine, adrenaline and noradrenaline were rather shallow, even though no modulation in affinity by guanine nucleotides was apparent (Fig. VIII.14.A.B.).

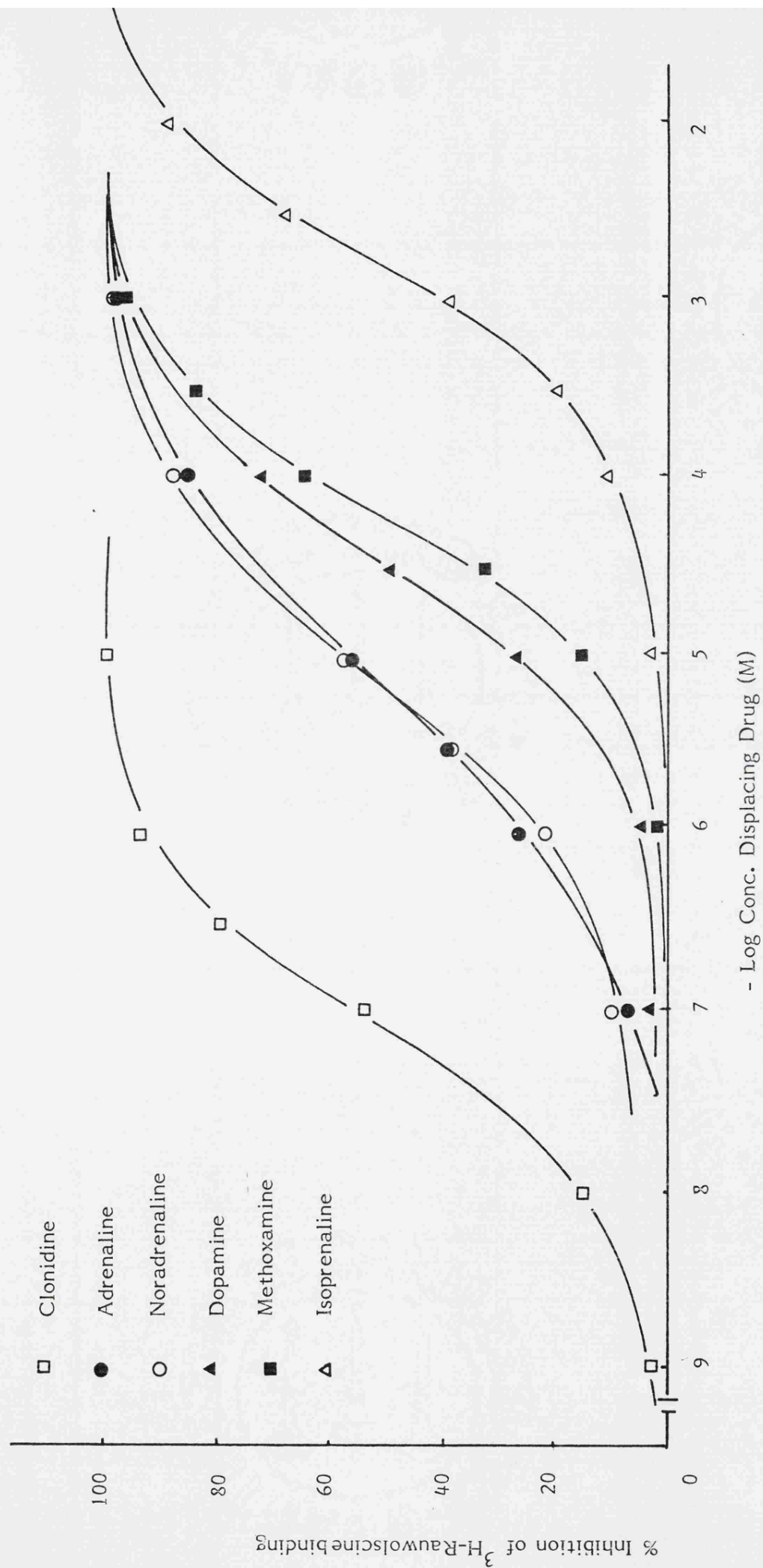
Legend to figures VIII.9.a. and b.

Displacement of specific ^3H -Rauwolscine binding to soluble rat renal cortical preparation by drug agents.

- a) Antagonist displacement
- b) Agonist displacement

Soluble rat kidney preparations were prepared as described in Section VIII.2. Displacement experiments were carried out at 4 - 6 nM ^3H -rauwolscine. The data points are means of 3 - 6 experiments performed in duplicate with S.E.M. around $\pm 5 - 7\%$.

Fig. VIII.9.b.



Legend to Figures VIII.10.a, b, c and d

Comparison of K_i values of selective antagonists at ^3H -Rauwolscine binding sites in particulate and soluble preparations of human platelet, rat renal cortex and rabbit renal cortex.

- a) Rauwolscine
- b) Yohimbine
- c) Prazosin
- d) Indoramin

K_i values were determined in drug displacement experiments of ^3H -Rauwolscine binding to membranes (Chapters IV & V) and soluble preparation (table VIII.3, this chapter) of the above three tissues.

Fig. VIII.10.a.

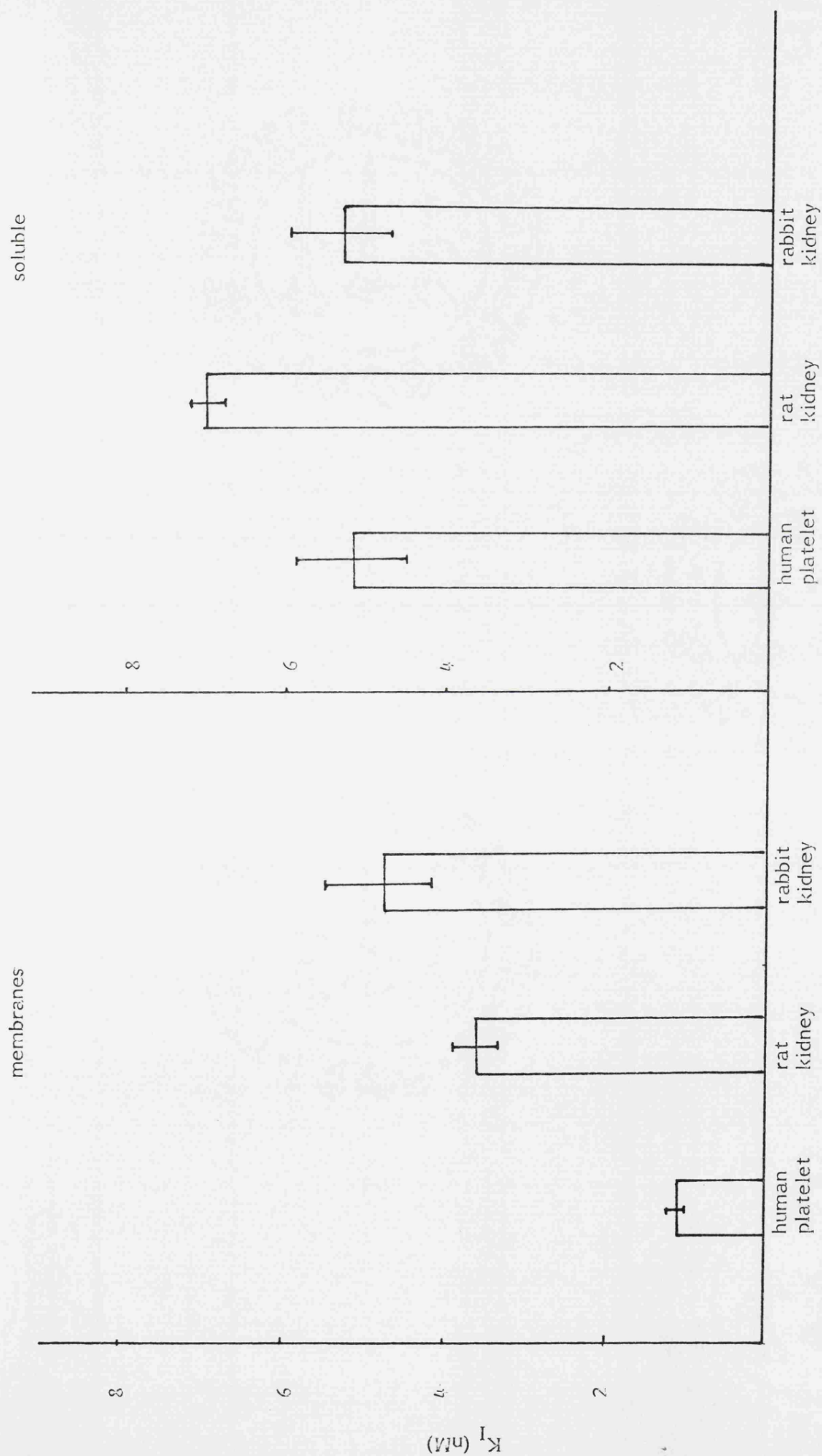


Fig. VIII.10.b.

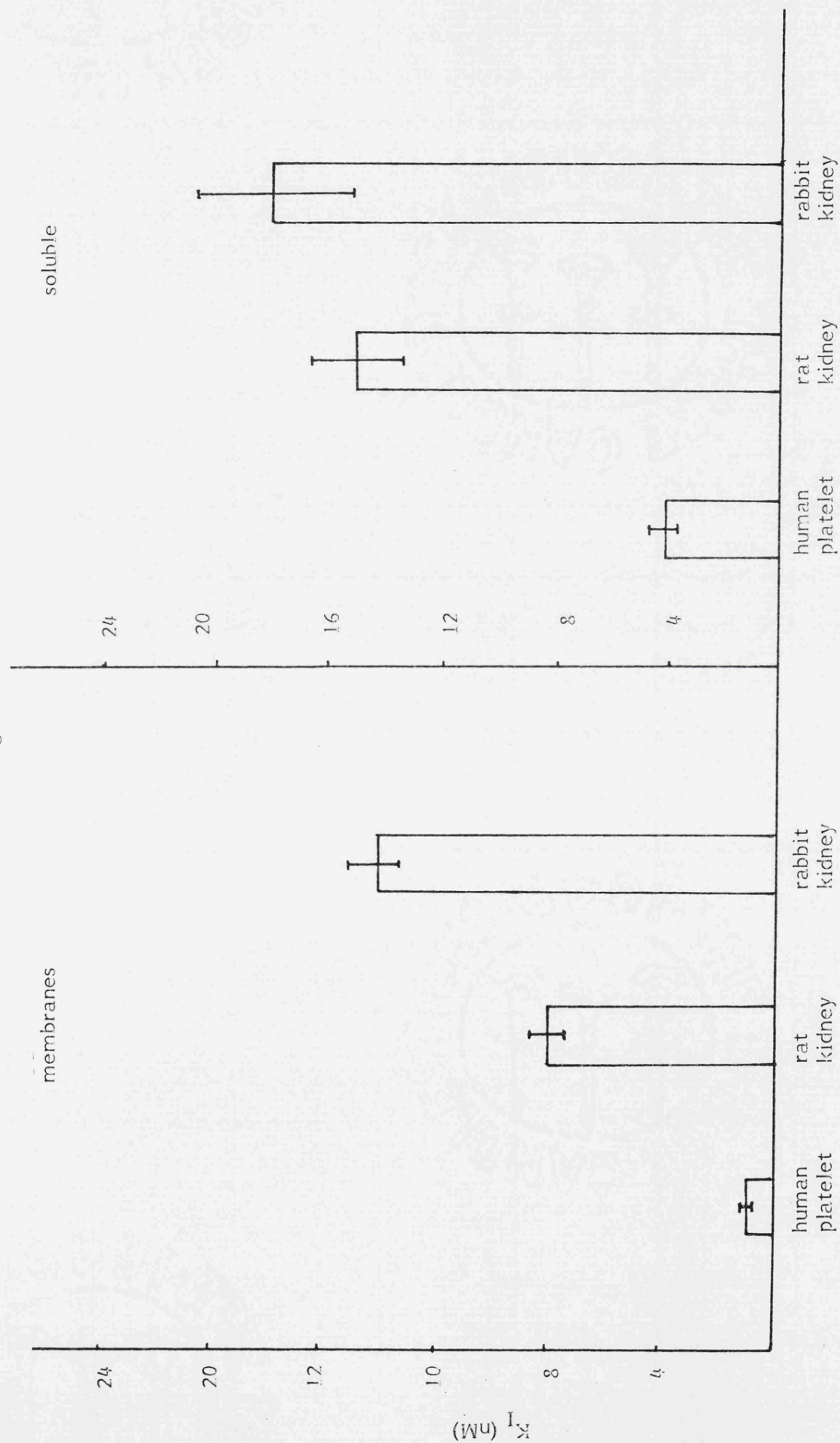


Fig. VIII.10.c.

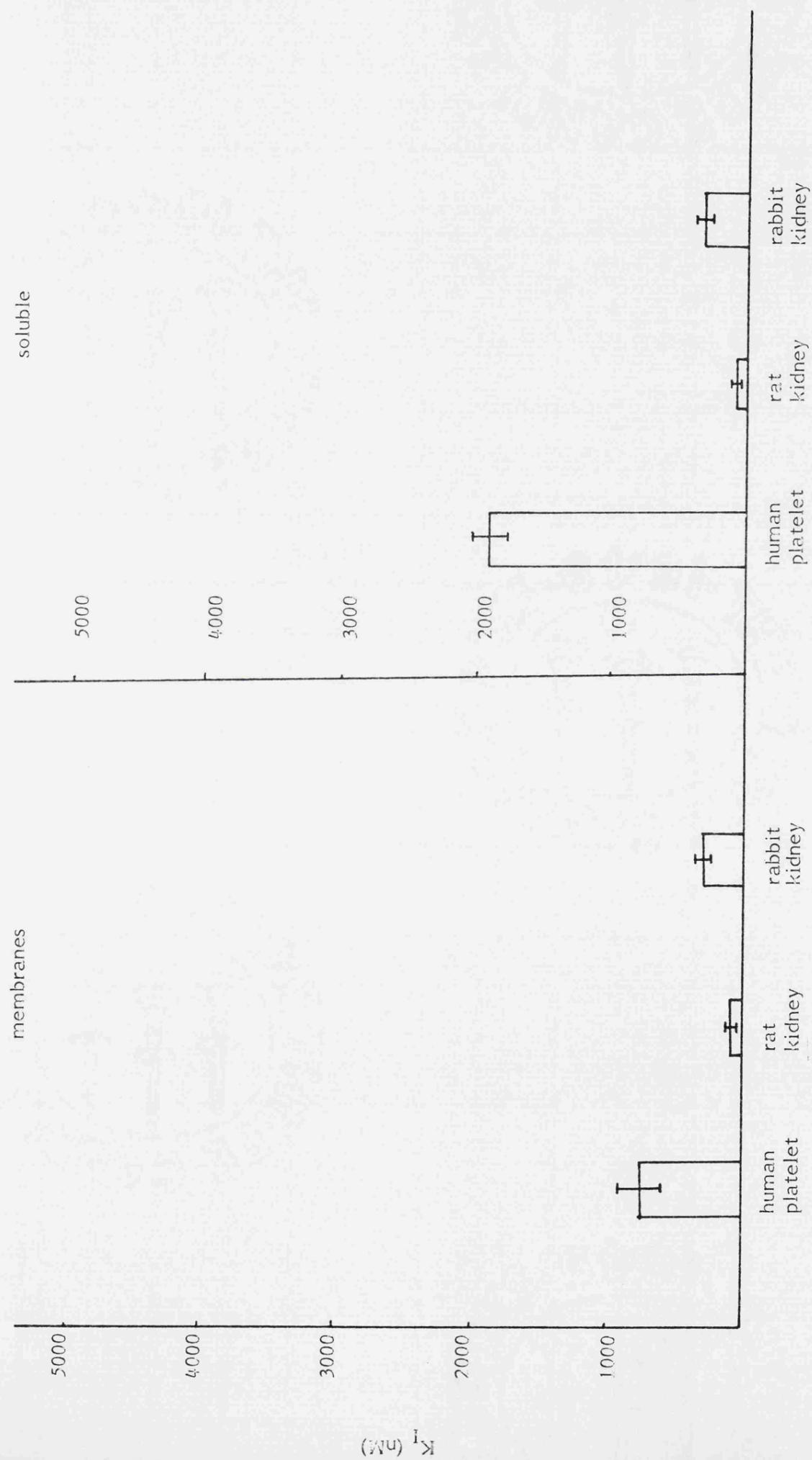
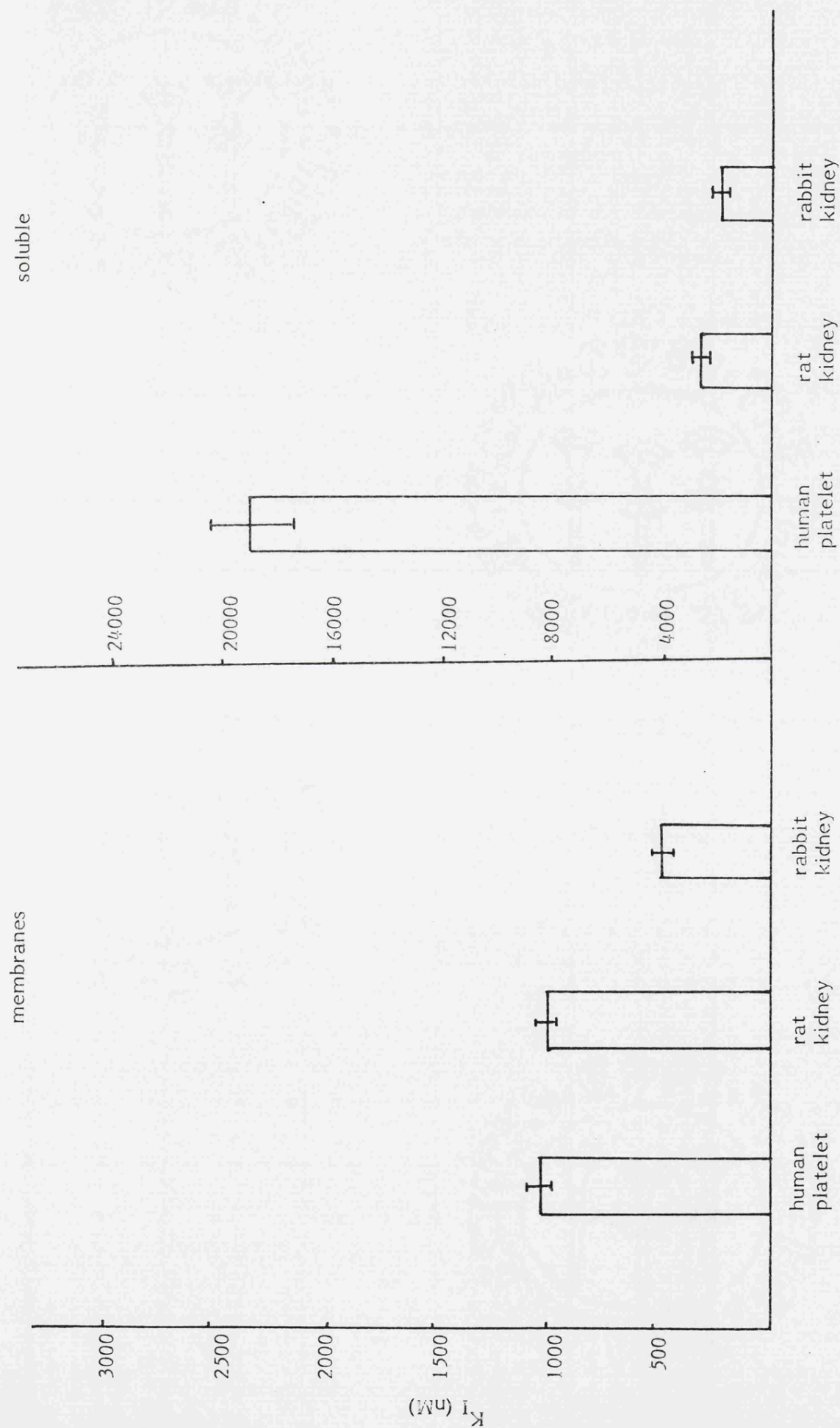


Fig. VIII.10.d.



VIII.3.3. Characterisation of soluble α_2 -adrenoceptors of the rabbit renal cortex

VIII.3.3.1. Equilibrium binding and kinetics studies with ^3H -rauwolscine

^3H -rauwolscine bound to solubilized rabbit renal cortical preparations at least as rapidly as to solubilized platelet or rat kidney preparations (Fig. VIII.12), reaching equilibrium in 30 - 40 minutes at R.T. The Scatchard analysis of specific binding yielded an apparent binding capacity (B_{max}) of 33.8 ± 5.32 f.mole mg protein ($n = 3$), and an apparent dissociation constant (K_d) of 7.02 ± 1.43 (Table VIII.2, Fig. VIII.11). The Hill coefficient was 1.07 ± 0.01 , suggesting that binding occurred at a homogeneous population of binding sites. The apparently lower receptor density in the solubilized receptor preparations, compared to the particulate preparations, may be due to a low α_2 -receptor yield relative to total protein yield.

The kinetics of ^3H -rauwolscine binding to solubilized rabbit kidney preparations resembled that of either platelet or rat kidney preparations. For instance, the apparent K_1 and K_2 values were found to be $0.0154 \text{ nM}^{-1} \text{ min}^{-1}$ and 0.047 min^{-1} respectively, yielding a K_d (K_1/K_2) of 3.05 nM . These values were in fact also similar to the corresponding ones in the particulate preparations.

VIII.3.3.2. Pharmacological characteristics

Results from displacement of ^3H -rauwolscine binding to solubilized rabbit kidney preparations by various drugs are shown in Table VIII.3. and Figures VIII.13A and B. It is clear that the overall pharmacological profile resembled one expected of an α_2 -adrenoceptor, with the α_2 -drugs such as yohimbine and rauwolscine being more potent than the α_1 -drugs such as prazosin, corynanthine and indoramin. In addition, the rank order of drug potency in inhibiting ^3H -rauwolscine binding to the soluble receptor preparations was identical to that in the membranous preparations (see Chapter V). Thus, yohimbine displayed a significantly lower affinity

Legend to Figure VIII.11.

³H-rauwolscine specific binding to soluble rabbit renal cortical preparation.

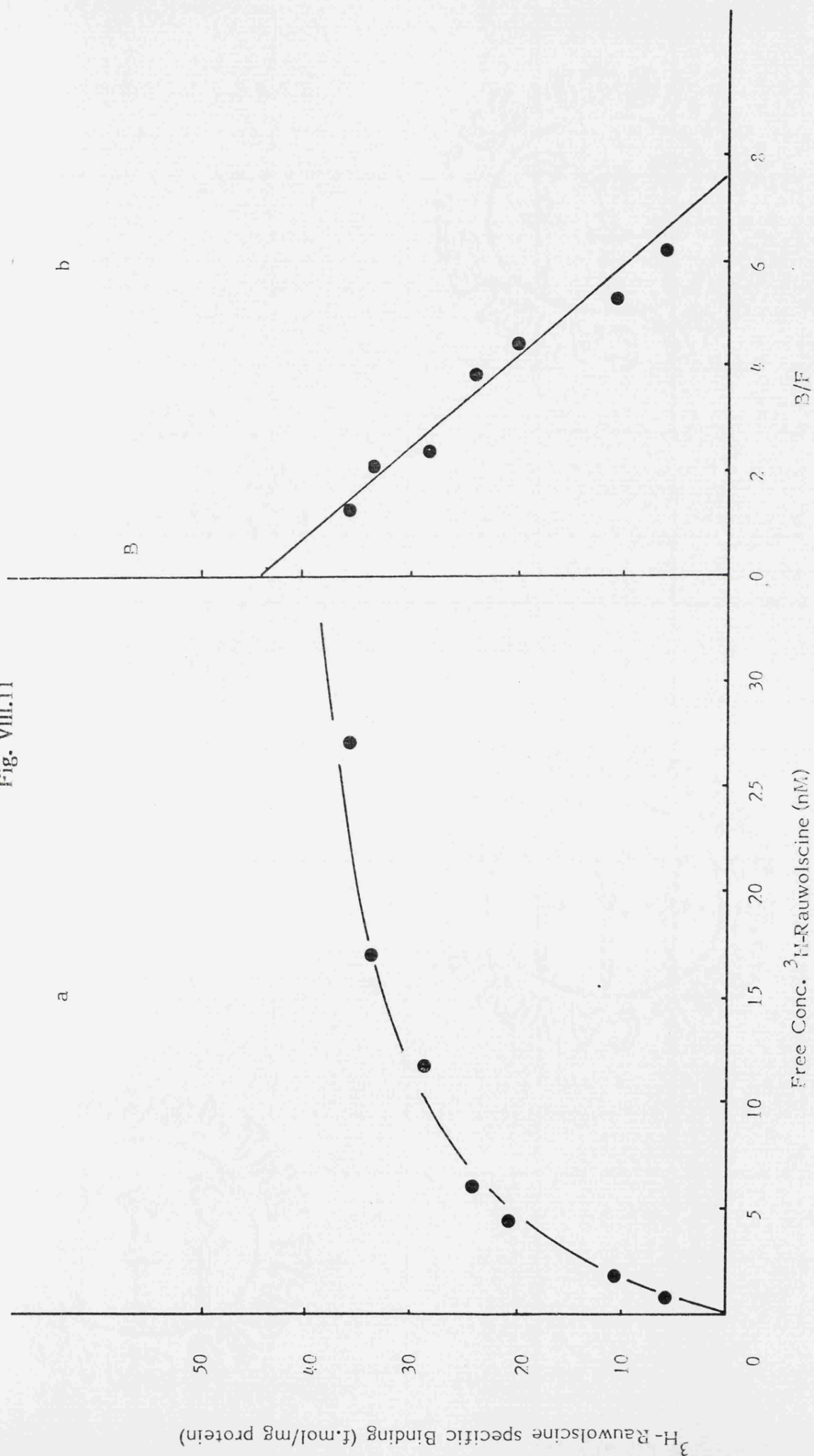
Soluble rabbit renal cortical preparations were prepared with digitonin-NaCl as described in Methods section (VIII.2.). Saturation binding assays of ³H-rauwolscine were carried out at approximately - 35 nM, with a protein concentration of 6 - 7 mg/ml.

a) Saturation

b) Scatchard plot of binding data

Results are from one representative experiment typical of three similar experiments performed in duplicate. Bmax = 44.4 f.mol/mg protein; Kd = 6.03 nM.

Fig. VIII.11



Legend to Figure VIII.12

Kinetics of ^3H -Rauwolscine binding to soluble rabbit renal cortical preparation at R.T.

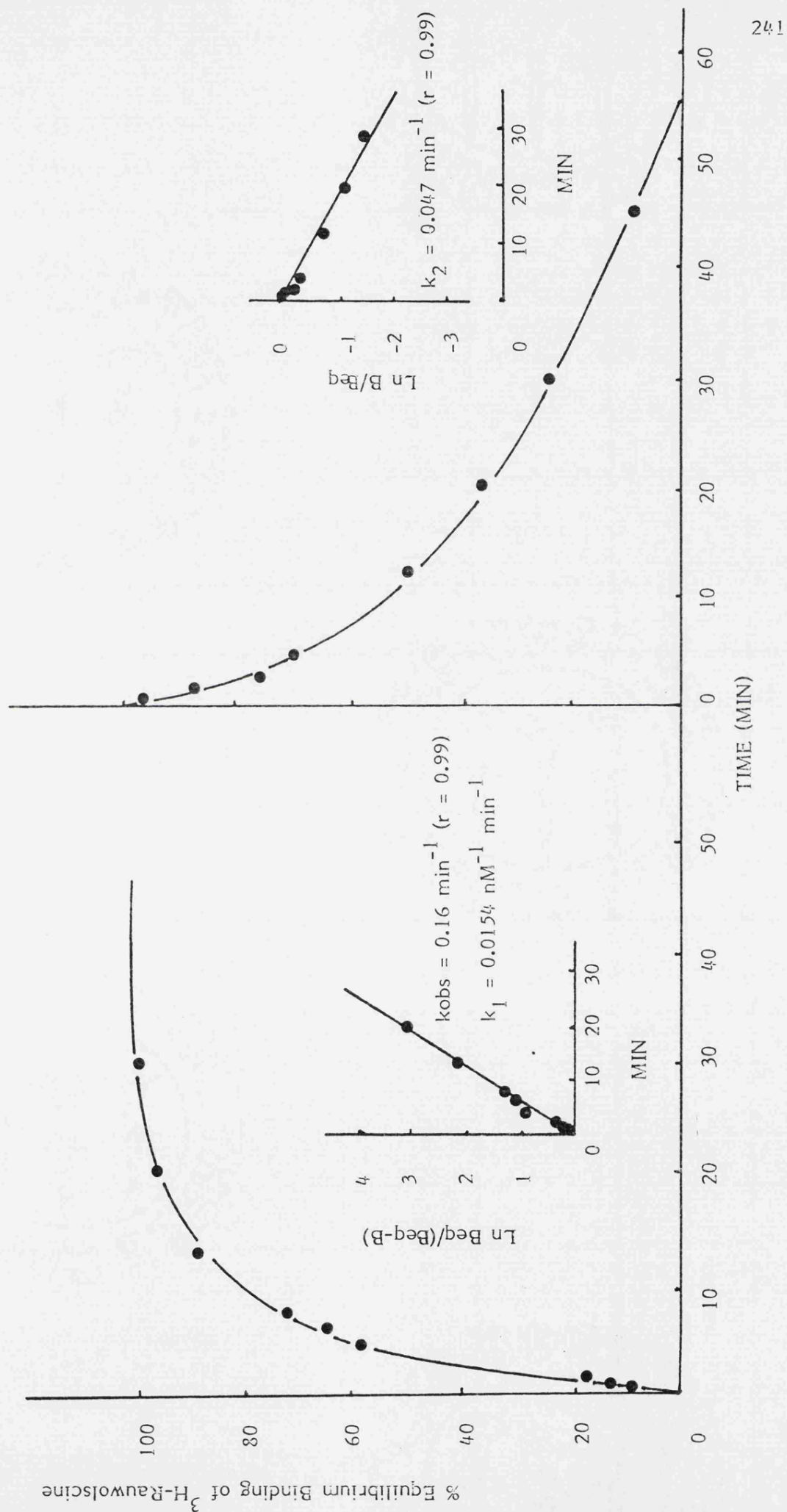
Soluble rabbit kidney membrane extracts were prepared as described in Section VIII.2. of this chapter. Kinetics experiments of ^3H -Rauwolscine binding were conducted in a manner similar to that described for particulate receptors (Chapter III).

Left panel = association curve; inset shows the pseudo first-order rate plot for the association reaction.

Right panel = dissociation of ^3H -Rauwolscine binding; the inset shows the first-order rate plot.

Data presented is from one of two similar experiments performed in duplicate, both yielding almost identical results.

Fig. VIII.12



Legend to figures VIII.13.a., and b.

Displacement of specific ^3H -Rauwolscine binding to soluble rabbit cerebral cortical preparation by antagonists and agonists.

- a) Antagonists
- b) Agonists

Soluble rabbit cerebral cortical preparations were prepared as described in Section VIII.2. Drug displacement experiments were carried out at 4 - 6 nM ^3H -Rauwolscine with increasing concentrations of displacing drugs. The data points are means of 3 - 6 separate experiments performed in duplicate. The S.E.M. of each data point is usually less than $\pm 5\%$.

Fig. VIII.13.a.

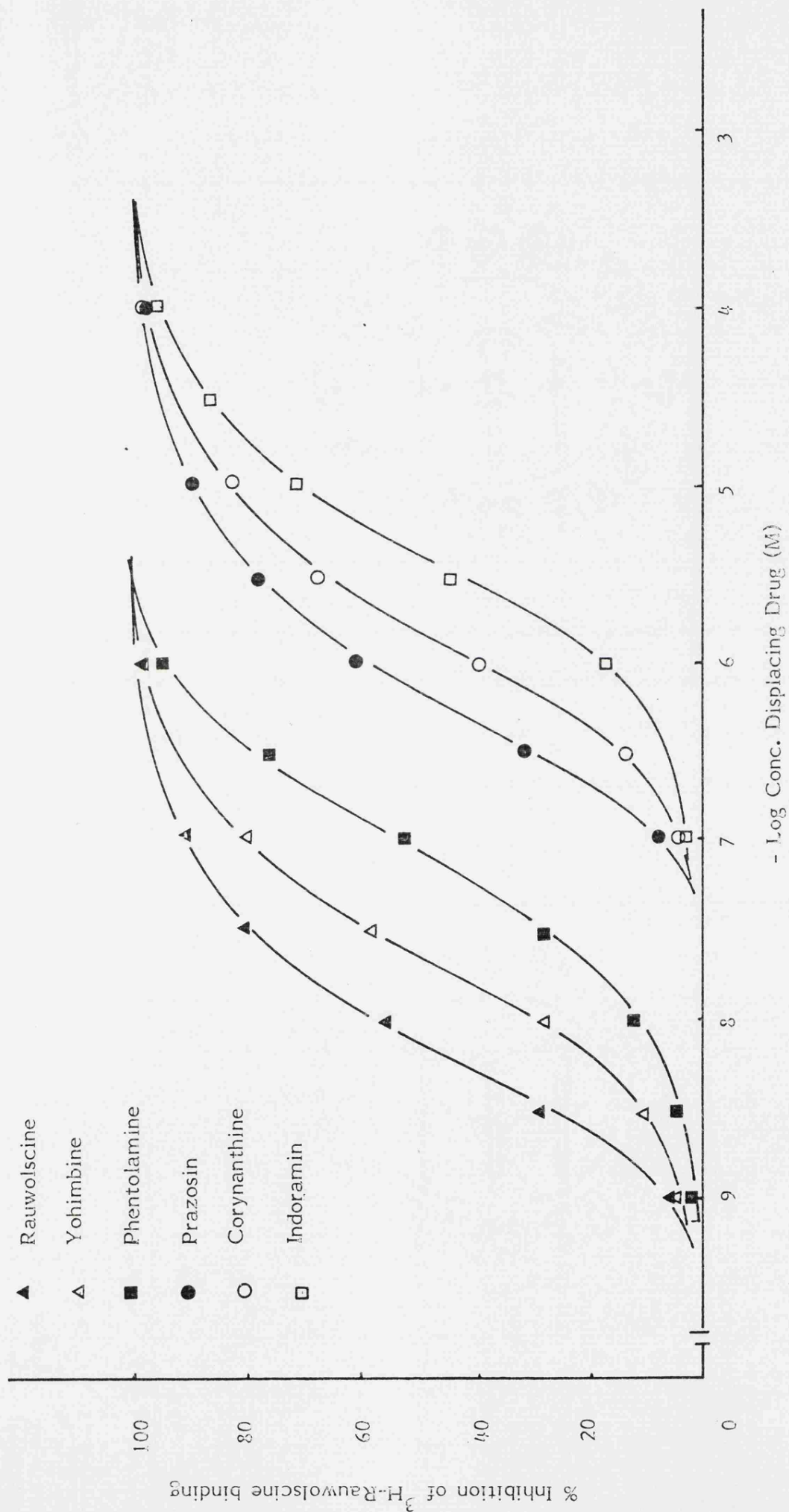
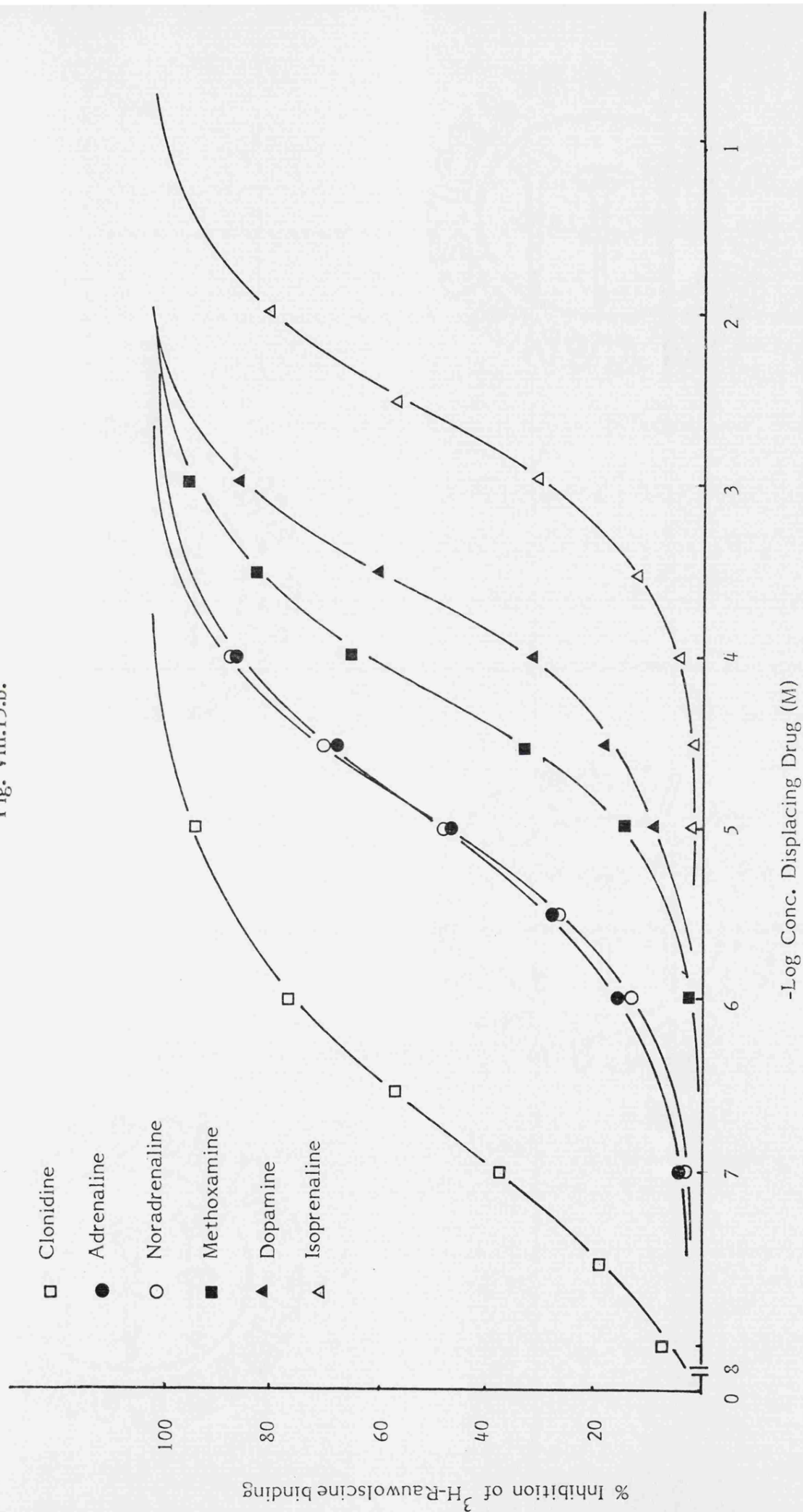


Fig. VIII.13.b.



than rauwolscine but prazosin appeared relatively potent.

In general, the pharmacological behaviour of soluble α_2 -receptors of the rabbit kidney resembled that of the rat kidney. Therefore, in comparison with the soluble platelet α_2 -receptors, those in the rabbit kidney had distinctly weakened affinity for the α_2 -antagonist yohimbine, but exhibited increased affinity for the α_1 -antagonists, such as prazosin and indoramin (Fig. VIII.10.B,C and D). All antagonists generated curves with slopes close to unity suggesting an interaction with ^3H -rauwolscine at a single class of affinity states or sites

The rank order of potency for the agonists was also indicative of interaction with α_2 -adrenoceptors (Table VIII.3 Fig VIII.13.b). Thus clonidine was most potent, followed by adrenaline and noradrenaline in that order. The α_1 -agonist methoxamine and dopamine were relatively weak competitors of ^3H -rauwolscine binding as was the beta-agonist isoprenaline. However, as in the soluble rat kidney receptor preparations, the agonists (clonidine, adrenaline and noradrenaline) all displaced ^3H -rauwolscine binding to soluble rabbit kidney preparations with slopes significantly less than one.

VIII.3.4. Ionic and guanine nucleotide influences on agonist/antagonist interaction with soluble α_2 -adrenoceptors

As demonstrated in Chapter VI, divalent cations, such as Mg^{++} , can modulate the affinity of antagonists at particulate α_2 -adrenoceptors. It was therefore of interest to examine if such effect may still be present when α_2 -receptors are in the solubilized form.

Saturation experiments were performed by incubating 3H -rauwolscine with solubilized platelet receptor preparations in the absence and presence of 10 mM $MgCl_2$. Mg^{++} did not produce any change in the apparent B_{max} (203 ± 34 f.mole/mg protein in the presence of Mg^{++} , compared to 187 ± 20 f.mole/mg protein in the control, $n = 3$). The apparent K_d , however, was increased by about 2-fold (8.3 ± 0.16 nM, compared to 4.3 ± 0.47 nM, $p < 0.05$). Therefore, this effect of Mg^{++} appeared qualitatively and quantitatively similar to that observed in platelet membranes. However, Mg^{++} was not found to enhance the affinity of agonist, at least under the present solubilization and assay conditions. The presence of a final assay concentration of 200 mM NaCl did reduce the affinity of adrenaline by about 2 - 3 fold in either rat kidney or platelet soluble receptor preparation, compared to that in the presence of 80 mM Na^+ or in the absence of this ion. In contrast, there was no apparent difference in the affinity of yohimbine in the absence or presence of Na^+ (data not shown).

In both platelet and rat kidney membranes, guanine nucleotides reduced the affinity of agonist at α_2 -receptors, manifested by a characteristic shift of the displacement curve to the right (see Chapter VI). Such an effect appeared qualitatively similar in the absence or presence of 200 mM NaCl, though in the rat kidney membranes, the presence of high concentrations of Na^+ actually enhanced the modulating effect of guanine nucleotides. However, under similar assay conditions no modulatory effect of agonist affinity by guanine nucleotides was observed in the soluble platelet and rat kidney preparations (Fig. VIII.14.A and B). Therefore,

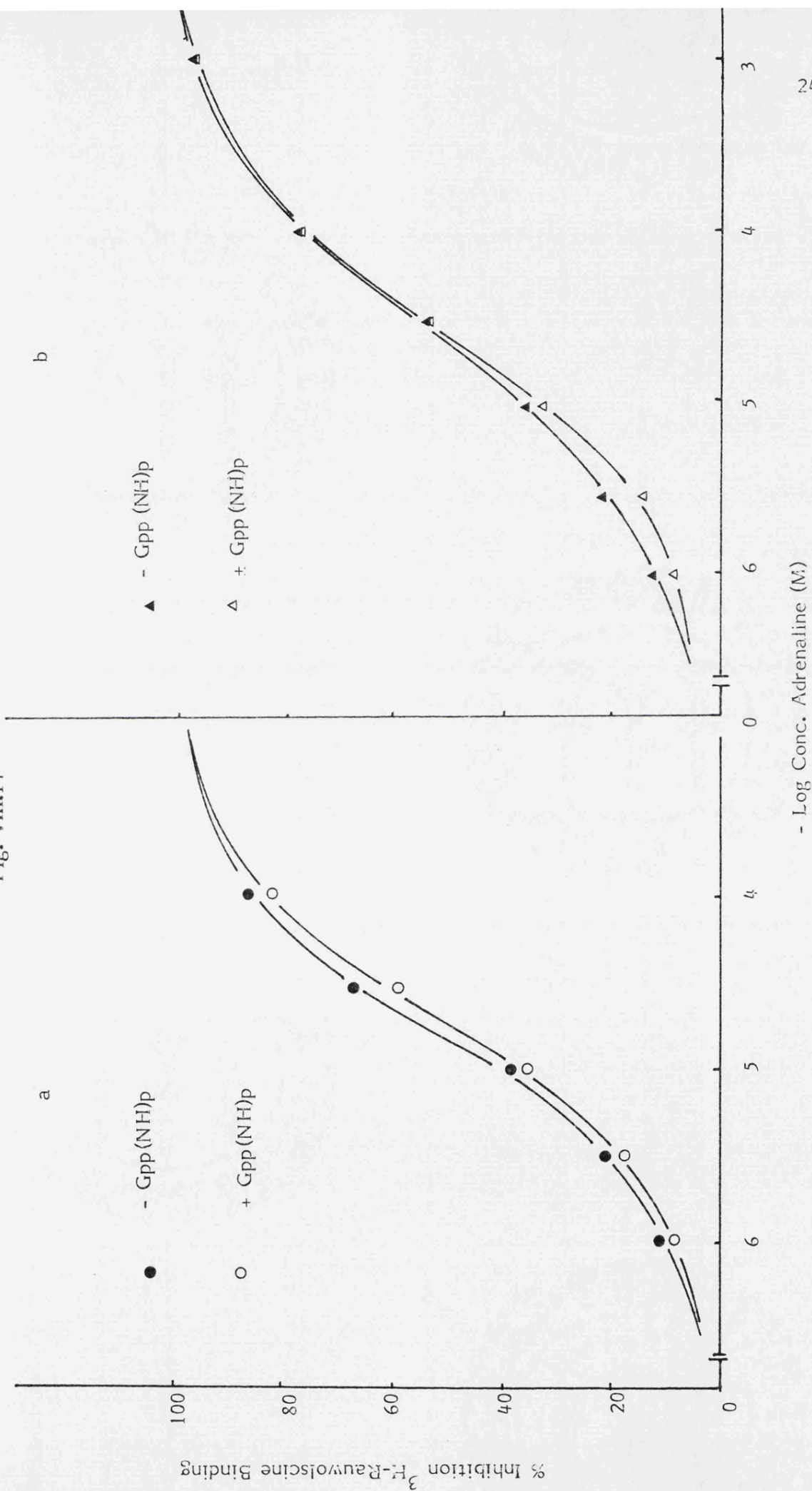
Legend to Figures VIII.14.a and b

Effect of guanine nucleotide on adrenaline displacement of ^3H -Rauwolscine binding to soluble human platelet and rat renal cortical preparations.

- a) Human platelet
- b) Rat renal cortex

Human platelet and rat renal cortical membranes were treated with digitonin (final concentration = 5 mM and 10 mM respectively) in the absence of NaCl. Method of solubilization was identical to that described in Section VIII.2. The resultant soluble preparations were used for studying the effect of 0.1 mM Gpp(NH)p on adrenaline displacement of ^3H -Rauwolscine binding at a ligand concentration of 6 - 7 nM in the presence of 10 mM MgCl_2 and 200 mM NaCl. Data illustrated represents the mean of 3 separate experiments performed in duplicate, with S.E.M. $< \pm 5\%$.

Fig. VIII.14



these findings seem to suggest that, at least in these tissues, guanine-nucleotide sensitive high-affinity agonist states have been 'lost' during the solubilization process.

VIII.3.5. Effect of agonist pre-occupancy of membrane α_2 -receptor on guanine nucleotide modulation of agonist interaction with soluble α_2 -receptors

As mentioned in the last section, agonist interaction with soluble α_2 -receptors no longer appeared to be influenced by guanine nucleotides. This phenomenon suggests an uncoupling of α_2 -receptors from the N-protein during the solubilization process. Smith & Limbird (1981) have demonstrated that pre-incubation of membranes with agonists, though not antagonists prior to solubilization, appeared to be able to stabilize the agonist high-affinity complex, as manifested by an apparently larger molecular size of the agonist-labelled solubilized receptor. Therefore, it was considered worthwhile to investigate if guanine nucleotides can also modulate the interaction of agonist with ^3H -rauwolscine at soluble α_2 -receptors, if membranes have been pre-incubated with agonists prior to solubilization.

Rat kidney membranes were pre-incubated with 100 nM noradrenaline for 30 minutes and then solubilized with digitonin after washing. Fig. VIII.15 shows the results of adrenaline displacement of ^3H -rauwolscine binding to such soluble receptor preparations in the absence or presence of 5'-guanylyl imidodiphosphate (10^{-4}M). The data apparently indicates that the adrenaline displacement curve was shifted to a larger extent by guanine nucleotides in soluble extracts obtained from agonist-treated membranes, compared to preparations from the control membranes. These preliminary results therefore appeared to provide support for the views of Michel et. al. (1981) and Smith & Limbird (1981), that agonist occupancy of α_2 -receptors stabilizes the high-affinity ternary complex, thus maintaining its partial sensitivity toward guanine nucleotide modulation.

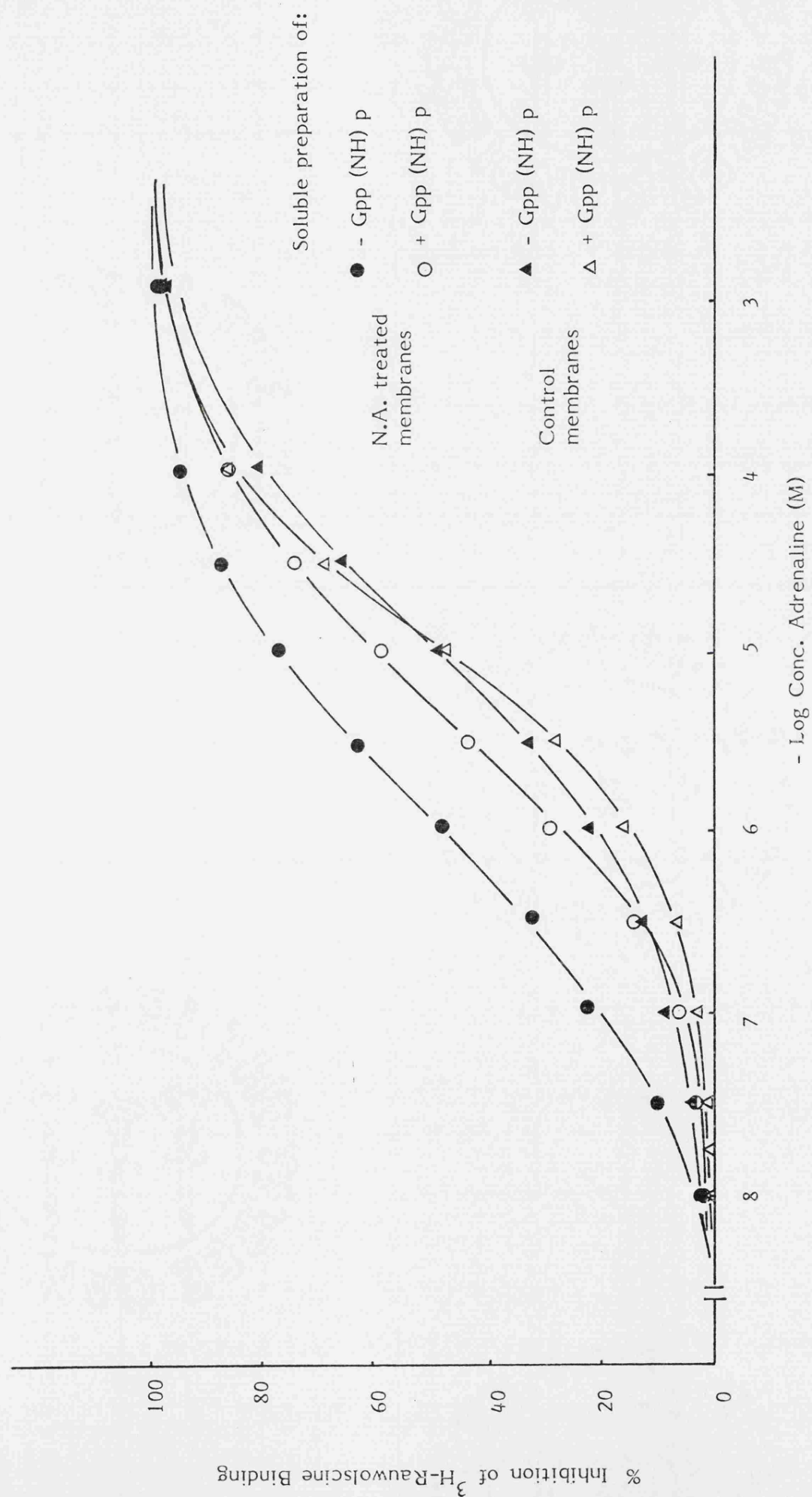
Legend to Figure VIII.15**Effect of agonist pre-occupancy of membrane α_2 -receptors on guanine nucleotide modulation of agonist interaction with soluble α_2 -receptors**

Rat renal cortical membrane preparation was divided into two equal portions. One portion was incubated at R.T. with a final concentration of 100nM norepinephrine (noradrenaline) for 30 minutes (10 mM MgCl_2 was also present). Subsequently, the membrane suspension was centrifuged at 50,000 xg for 10 minutes. The pellet was washed two times with ice-cold assay buffer by gentle resuspension, followed by centrifugation. The resultant membrane suspension was solubilized with digitonin-NaCl by Ultra-Turrax homogenization as described in the Methods section of this chapter. The non-noradrenaline treated membrane suspension was also solubilized in the identical manner. The supernatants obtained after one hour centrifugation at 50,000 xg were used to examine the effect of 0.1 mM Gpp (NH)p, on adrenaline displacement of ^3H -Rauwolscine binding.

Displacements were carried out in standard conditions (Section VIII.2) at 4 - 6 nM ^3H -Rauwolscine and increasing concentrations of adrenaline in the absence and presence of 0.1 mM Gpp (NH)p. 10 mM MgCl_2 was present in all assays.

Data illustrated is the mean of three separate experiments performed in duplicate. S.E.M. is \pm 5 - 10%

Fig. VIII.15



VIII.4. Discussion

The characterisation of solubilized α_2 -receptors have previously been reported in two tissues: an adrenocortical carcinoma (Nambi, et. a. 1981), and human platelet (Smith & Limbird 1981; Michel et. al. 1981). In the present studies, attempts have been made to solubilize α_2 -receptors from human platelet, rat renal cortex, and rabbit renal cortex, using the glycoside detergent digitonin. The solubilized receptors have also been characterized using the α_2 -antagonist ligand ^3H -rauwolscine.

Digitonin generally proved to be quite effective in extracting α_2 -receptors, producing protein and specific receptor yields in the region of 50-60% and 30-40% respectively in human platelet, rat kidney and rabbit kidney. However, there was little success in solubilizing detectable α_2 -receptors from the rat and rabbit cerebral cortex using this detergent. Other detergents, such as CHAPS and cholate, were also ineffective in solubilizing assayable α_2 -receptors from these tissues. In this connection it is of interest to note that Sladeczek et. al. (1984) have recently reported successful solubilization of calf brain α_2 -receptors using CHAPS. However, differences in the solubilization methods might be important here.

The digitonin solubilized α_2 -receptors are truly soluble, as they satisfy most of the criteria for soluble receptors. They were not sedimentable at 100,000 xg for 60 minutes, and they also completely passed through 0.2 μm . pore size membranes filters.

The presence of NaCl can markedly increase the α_2 -receptor yield by digitonin. This enhancement of receptor solubilization turns out to be a rather common phenomenon, as it occurs in different receptor systems and with different detergents (Aronstram et al. 1978; Dickinson 1982; Howells et. al. 1982; Hall et. al. 1983; Kuno et. al 1983). The exact mechanism of NaCl in promoting receptor solubilization is not clear.

However, one of the main factors may be that it protects the receptors during the solubilization process (Kuno et. al., 1983). It is clear that although high salt concentrations alone could apparently extract quite a good amount of membrane protein, there was a negligible yield of specific receptor sites. It is also interesting to note that an optimal yield of receptors appeared to occur over a rather narrow range of NaCl concentrations, particularly with rat and rabbit kidney α_2 -receptors. This may suggest that NaCl can also have influence on the physico-chemical properties of the detergent micelles (eg size, bulk concentration, etc) and that a suitable balance between detergent and salt contents provides the optimal access of drugs to the solubilized receptor sites.

The results of the present studies indicate that the overall pharmacological characteristics of particulate α_2 -receptors are well maintained when the receptors are in the soluble form. However, most drugs did exhibit weaker affinities in the soluble preparation than in the particulate preparation. The discrepancies in drug affinity appeared larger with the agonists than with the antagonists. Moreover, compared to the soluble rat or rabbit kidney preparation, the soluble platelet preparation exhibited relatively large decreases in drug affinities. The presence of NaCl in the soluble preparations does not fully account for the marked decreases in drug affinities, since equivalent concentrations of NaCl produced relatively less influence on drug affinities in membranes.

The observation that decreases in affinities occurred to both agonists and antagonists in soluble platelet preparations is apparently contradictory to the earlier reports (Michel et. al., 1981; Smith & Limbird, 1981; Limbird et. al., 1982) which suggested that only the affinities of agonists, but not antagonists, were significantly reduced in these preparations. However, unlike the present studies, these previous studies only examined the affinities of two or three antagonists. In fact, the affinity of yohimbine at the

soluble ^3H -yohimbine binding sites, as reported by Michel et. al. (1981) was at least 3 - 5 fold weaker than that at the particulate ^3H -yohimbine binding sites (Hoffman et. al., 1981), although comparatively, decreases in agonist affinities were more pronounced. The yohimbine K_d values reported by Smith and Limbird (1981) were indeed quite similar between the membranous and soluble platelet preparations. However, it is interesting to note that the K_d value for yohimbine in their platelet membranes was already quite considerably higher than those from similar studies (Motulsky et. al., 1981; Daiguji et. al., 1981; Hoffman et. al., 1982). In any case, the results from a recent study on soluble α_2 -receptors in the cerebral cortex also indicated a significant reduction in affinity for both agonists and antagonists in the soluble preparation, compared to the membranes (Sladeczek et. al., 1984). Therefore, an overall reduction in affinity for drugs appears to be a common phenomenon occurring to α_2 -receptors upon solubilization.

Changes in receptor affinity for drugs following solubilization may be expected in view of the potential conformational changes resulting from perturbation of the membrane environment by detergent. In fact, overall decreases in drug affinities have also been observed for the digitonin-solubilized muscarinic receptor (Beld & Arien, 1974) and the cholate-solubilized dopamine D_2 receptor (Hall et. al., 1983). As for the soluble platelet α_2 -receptors, the relatively large overall decreases in drug affinities may suggest that α_2 -receptors are normally subjected to a tighter constraint within the platelet membrane lipid environment.

In agreement with the findings of earlier studies (Smith & Limbird 1981; Michel et. al., 1981), the agonists do exhibit markedly reduced affinities at soluble platelet α_2 -receptors compared to the membranes. In fact, all of the agonists generated displacement curves with slopes close to unity, suggesting an interaction with a homogeneous class of low affinity sites. A reasonable interpretation of these findings may be that

platelet α_2 -receptors have been 'uncoupled' from the guanine nucleotide regulating protein during the solubilization process. This is supported by the finding that the adrenaline interaction with the solubilized platelet receptor sites labelled by ^3H -rauwolscine was not modulated by guanine nucleotides. Indeed, it was suggested that agonist pre-occupation of platelet α_2 -receptors prior to solubilization was required to stabilize the ternary high affinity receptor complex, which subsequently exhibited sensitivity to guanine nucleotides (Limbird et. al., 1982). In this connection, it was surprising to notice that the α_2 -agonists still generated rather shallow displacement curves in the rat and rabbit kidney soluble preparations, although no guanine-nucleotide 'shift' of the curves was observed. Interpretation for this phenomenon is not available at the moment. However, it is possible that, compared to the platelet, α_2 -receptors in the rat and rabbit kidneys may be 'coupled' to membrane regulatory components other than the Ni-protein, all exhibiting different sensitivity to the guanine nucleotides. Some receptors may still be linked to regulatory components weakly sensitive to guanine-nucleotides after solubilization. Therefore, there could be apparent co-operative interaction of agonist with these solubilized receptor sites, but such interaction could not be modulated by guanine nucleotides. The preliminary results from these studies have however suggested that pre-incubation of rat kidney membranes with agonist prior to solubilization did appear to yield soluble preparations in which adrenaline displacement curves were more significantly shifted by guanine nucleotide, although there was not much steepening of the 'shifted' curves. Nevertheless, this finding favors the view that pre-occupation of α_2 -receptors by agonist prior to solubilization can indeed stabilize the agonist-receptor-N protein complexes exhibiting guanine nucleotide sensitivity in the soluble preparations.

The recent findings of Sladeczek et. al. (1984) indicating that the partial agonist ligand ^3H -PAC (p-amino clonidine) could label CHAPS-solubilized

alpha₂-receptors from the calf cerebral cortex is indeed very interesting as it suggests that such receptor sites may be of the high-affinity agonist state of the alpha₂-receptors. Indeed, these workers found that the soluble ³H-PAC binding sites were sensitive to modulation by guanine nucleotides. It is not clear why the present work failed to solubilize assayable ³H-rauwolscine binding sites from rat and rabbit brains using CHAPS. Moreover, it was also unsuccessful to observe guanine nucleotide modulation of agonist affinity in the digitonin-solubilized receptor preparations without agonist pre-occupancy of membrane receptors. The nature of those CHAPS-solubilized high-affinity agonist sites would certainly require further evaluation. However, apart from tissue differences, the solubilization method adopted by these workers, compared to the present studies, might be a significant factor for successful solubilization of high-affinity agonist states of the alpha₂-receptors.

The main aim of the present studies was to compare the general pharmacological properties of the soluble alpha₂-receptors in light of the differences between the particulate alpha₂-receptors. The results have indeed identified differences in pharmacological behaviour between the soluble alpha₂-receptors. Thus, the soluble platelet alpha₂-receptor processes an equal affinity for rauwolscine and yohimbine, but exhibits a relatively weak affinity for the alpha₁ antagonists such as prazosin and indoramin. In contrast, yohimbine displays a significantly weaker affinity than rauwolscine in the soluble rat or rabbit kidney alpha₂-receptor, at which prazosin, indoramin, corynanthine and labetalol all appear to be of relatively higher potency than at the soluble platelet alpha₂-receptor. It should be noted that the displacement curves of these antagonists had slopes close to unity, suggesting that interaction with ³H-rauwolscine occurred at a single class of sites in all cases. The possibility that ³H-rauwolscine bound in part to alpha₁-receptors in the soluble rat or rabbit kidney preparation can be totally excluded, as it was further found that no ³H-prazosin binding could be detected at concentrations

as high as 15 nM, which is generally far beyond that required to saturate the α_1 -receptor population.

Therefore, in conclusion, differences in pharmacological behaviour of soluble α_2 -receptors parallel differences observed between particulate α_2 -receptors (See Chapter V). This provides further evidence that α_2 -receptors are not a homogenous population and that their differences in behaviour may truly reflect molecular differences in the receptor proteins.

Chapter IX
CONCLUDING DISCUSSION

The characterisation of α_2 -adrenoceptors can now be effectively achieved by means of functional methods and radioligand binding assays. The present work has primarily involved the development and application of radioligand binding techniques to examine the pharmacological characteristics of α_2 -adrenoceptors.

Prior to this study, pharmacological characterisation of α_2 -receptors relied exclusively on the use of ^3H -agonists and the non-selective antagonist ligand, ^3H -DHE (see Chapter II). This work was undertaken using for the first time the α_2 -selective antagonist ligands, ^3H -yohimbine and ^3H -rauwolscine.

In agreement with earlier studies using ^3H -DHE (Tsai & Lefkowitz, 1978, 1979; Michel et. al., 1980), agonists but not antagonists, interact with ^3H -yohimbine or ^3H -rauwolscine binding sites on human platelet membranes in two distinct affinity states, which can be demonstrated by curve-fitting analysis of displacement data. Mg^{++} and other divalent cations can enhance agonist binding by promoting the formation of the high-affinity states. In contrast, monovalent cations, such as Na^+ markedly reduce the overall affinities of agonists at α_2 -receptors, possibly in part involving a destabilization of the high-affinity states. High concentrations of guanine nucleotides, such as GTP and Gpp(NH)p appear to convert the high-affinity states to the low-affinity states. At the human platelet α_2 -receptor, the modulatory effects of Na^+ and GTP are additive. However, at other α_2 -receptors, including those of the rat and rabbit cerebral cortex and rat and rabbit renal cortex Na^+ synergistically enhances the modulatory effect of GTP. The resultant effect of GTP or Gpp(NH)p on agonist interactions appears qualitatively similar between the α_2 -receptors. The apparent requirement for Na^+ for the full expression of the guanine nucleotide modulatory effect at some but not all α_2 -receptors may reflect some differences in the receptor-effector coupling mechanisms in different systems.

In any case, the overall characteristics of agonist/antagonist interactions with most α_2 -receptors appear to fit in well with the 'two-state' or 'dynamic receptor affinity' model (see Chapters II & VI) that has now become generally accepted to describe the agonist/antagonist interactions with the beta-receptors. Therefore, although α_2 -receptors are coupled to adenylate cyclase in a negative manner, the molecular receptor-effector coupling mechanisms appear similar to those involved in cyclase stimulation. There is, however, increasing evidence suggesting that two distinct guanine nucleotide binding proteins (N_s and N_i) are respectively involved in cyclase stimulation and inhibition (Steer & Wood, 1979; Smith & Limbird, 1981; Hoffman et. al., 1981; Jakobs et. al., 1983_a). Recent progress in the isolation and purification of the N-proteins has provided evidence suggesting that the N_s and N_i proteins are composed of different subunits. The N_s protein appears to be composed of a 35000-Da and a 45000-Da subunit, whereas the N_i protein seems to have an identical 35000-Da subunit, but instead of the 45000-Da subunit, it has a 41000-Da subunit (Northup et. al., 1983; Manning & Gilman, 1983; Katada et. al., 1984_a). Both the 45000-Da and the 41000-Da subunits are responsible for GTP binding and appear to be able to modulate cyclase activity independently of the 35000-Da subunit which possesses inhibitory effects (Katada et. al., 1984_{a,b,c}). It is now postulated that α_2 -receptor activation leads to a dissociation of the 41000- and 35000-Da subunits of N_i . Inhibition of cyclase activity may either result from a direct or indirect effect of the 41000-Da subunit on the catalytic subunit of the enzyme, or from a reduced concentration of the stimulatory 45000-Da subunit of N_s following its increased interaction with the 35000-Da subunit from N_i (Katada et. al., 1984_c).

The major aim of the present work, however, has been to examine the overall pharmacological behaviour of the α_2 -receptors, including those in the human platelet, rat and rabbit cerebral cortex and rat and rabbit renal cortex.

The results have indicated that ^3H -yohimbine and ^3H -rauwolscine both specifically label equal quantities of pharmacological sites of the α_2 -type in each of the tissues examined. However, significant differences in behaviour were observed between the α_2 -receptors. These receptors differ not only with respect to drug affinities, but also to the relative drug potencies. Basically, it is possible to classify them into two main types. One group is represented by those in the human platelet, human cerebral cortex and rabbit cerebral cortex, and is chiefly characterized by equipotency of the yohimbine and rauwolscine diastereoisomers, and also low affinities for the α_1 -antagonists, such as prazosin, indoramin and corynanthine. The other group is represented by α_2 -receptors of the rat brain, rat kidney and rabbit kidney, and is characterized by a significantly reduced affinity of yohimbine, compared to rauwolscine, and also a markedly increased potency of the α_1 -antagonists, particularly prazosin. These features provide the basic evidence for a heterogeneity of α_2 -receptors. Those α_2 -receptors exhibiting reduced affinities for the α_2 -antagonists and increased affinities for the α_1 -antagonists can be viewed as an 'atypical' type, and can be described as having a 'mixed α_1/α_2 ' behaviour. It must be emphasized however, that the results from the present study indicated that it is highly unlikely that ^3H -yohimbine and ^3H -rauwolscine might have labelled α_1 -receptors (see Chapter V). As shown in table IX, most tissues in the rat, including the neuroblastoma X glioma cells appear to possess α_2 -receptors of this type. In no less than five different tissues of this species, results from separate laboratories have indicated (generally without comment) a relatively weak affinity for yohimbine and a high affinity for prazosin, such that the relative K_i ratios of prazosin to yohimbine are of a low value. The high K_i value of prazosin reported by Woodcock & Murley appears to be the only exception. However, this may perhaps only relate to the method of membrane preparation adopted by these workers (see Chapter VII). Indeed, a reduced affinity for yohimbine at ^3H -PAC sites

Table IX Relative affinities of prazosin and yohimbine at ³H-yohimbine labelled sites

Tissue	Ki or Kd (nM)		Reference
	Prazosin	Yohimbine	
Rat Cerebral Cortex	25000	14	Woodcock & Murley, 1982
Rat Cerebral Cortex	138	9.12	Rouot et. al., 1982
Rat Cerebral Cortex	199	11.25	Chapter V (table V.2)
Rat Kidney	142	19	Schmitz et. al., 1981
Rat Renal Cortex	80	11	Snively & Insel, 1982
Rat Renal Cortex	170	12.1	Woodcock & Johnston, 1982
Rat Renal Cortex	53	7.98	Chapter V (table V.2)
Rat Liver	100	11	Hoffman et. al., 1981 _b
Rat (Neonatal) Lung	5.4	0.99	Latifpour et. al., 1982
Rat Submandibular Gland	169	13	Feller & Bylund, 1984
Rabbit Uterus	1300	24	Lavin et. al., 1981
Rabbit Cerebral Cortex	4555	6.9	Chapter V (table V.3)
Rabbit Renal Cortex	303	14.5	Chapter V (table V.3)
Dog Artery	490	1.5	Bobik, 1982
Canine Tracheal Smooth Muscle	2700	5.9	Barnes et. al., 1983
Pig Lung	175	0.22	Feller & Bylund, 1984
Pig Submandibular Gland	223	0.45	
Human Adipocyte	1625	3.9	Tharp et. al., 1981
Human Platelet	2000	2	Motulsky et. al., 1980
Human Platelet	784	0.99	Chapter V (table V.4)
Human Cerebral Cortex	887	3.3	Chapter V (table V.4); Summers et. al., 1982 (at ³ H-Rauwolscine sites)
Neuroblastoma X Glioma	48	9.1	Kahn et. al., 1982
		5.3	

was also observed in the rat tissues, compared to the pig tissues (Feller & Bylund, 1984). In complete contrast, the α_2 -receptors in the human tissues appear to exhibit high affinities for yohimbine and low affinities for prazosin, thus exhibiting large relative K_i ratios of prazosin to yohimbine. α_2 -receptors in tissues of the dog, pig and rabbit appear generally like those of the human. However, the similar behaviour of the α_2 -receptors in the rabbit kidney and rat tissues indicates that pharmacological differences in α_2 -receptors occur both between species and between tissues of a given species.

Apparent differences in drug affinities between α_2 -receptors have been further evaluated with respect to the possible influences of cations and allosteric interaction. Although cations generally have some variable effect on the affinities of antagonists, as well as agonists, such influence is considered to be insufficient to account for the marked differences in affinities for drugs like prazosin and yohimbine. For instance, the presence of up to 200 mM Na^+ or 20 mM Mg^{++} only reduced the K_i or K_d of yohimbine from about 0.6 - 1.0 nM in the human platelet to about 2 - 2.5 nM. This value is still distant from the K_i or K_d value of yohimbine in tissues such as rat brain and rat kidney, which is already around 5 - 10 nM in the absence of those cations. As for the affinities of prazosin, the slight increases by Na^+ and the slight decreases by Mg^{++} , which occurred in all the tissue examined, also cannot account for its marked affinity differences between the α_2 -receptors. The competitive nature of the interaction between prazosin and ^3H -yohimbine or ^3H -rauwolscine binding sites has also been confirmed from saturation experiments and kinetics studies. For instance, the presence of increasing concentrations of prazosin increased ^3H -yohimbine or ^3H -rauwolscine K_d in the human platelet, rat brain and rat kidney without changing the B_{max} . The Schild plots were all linear with slopes not distant from one (Chapter V). Furthermore, the presence of prazosin did not alter the dissociation kinetics of ^3H -yohimbine

or ^3H -rauwolscine binding to the human platelet and rat kidney, where prazosin exhibits marked affinity difference at the labelled sites.

In an attempt to evaluate the differences in membrane constraint or lipid environment as a possible cause for pharmacological differences between tissues, digitonin-solubilized α_2 -receptors from the human platelet, rat kidney and rabbit kidney have been characterized. The results clearly indicated that although the soluble α_2 -receptors generally exhibit somewhat reduced drug affinities, the overall pharmacological profiles remain identical to those in membranes. Therefore, the differences in pharmacological properties between the particulate α_2 -receptors can also be revealed in the soluble receptors. These results therefore provide further evidence for the heterogeneity of α_2 -receptors, and strongly suggest that structurally different α_2 -receptor proteins may exist in mammalian tissues.

Recently, U'Prichard and co-workers have raised the question of whether the 'two-state' model is totally applicable to describe the agonist/antagonist interactions with α_2 -receptors. Based on their data mainly obtained from ^3H -agonist binding to the human platelet membranes, these workers suggest that there may be an additional Mg^{++} -promoted, guanine nucleotide-sensitive 'super-high' agonist affinity state (SH), in addition to the high- and low-affinity states already recognized to exist at the α_2 -receptors (U'Prichard et. al., 1983). Moreover, a model has been developed which suggests that some, but not all antagonists may also exhibit selectivity for the different agonist states of the α_2 -receptors. Accordingly, antagonist binding to α_2 -receptors would be expected to be modulated under the influence of guanine nucleotides and cations in a manner opposite to that with the agonists (Bylund & U'Prichard 1983; U'Prichard, 1983; see fig.IX). These particular concepts may indeed be relevant to the apparent observed drug affinity differences between tissues. Different tissues may contain the same α_2 -receptor existing in different proportions of

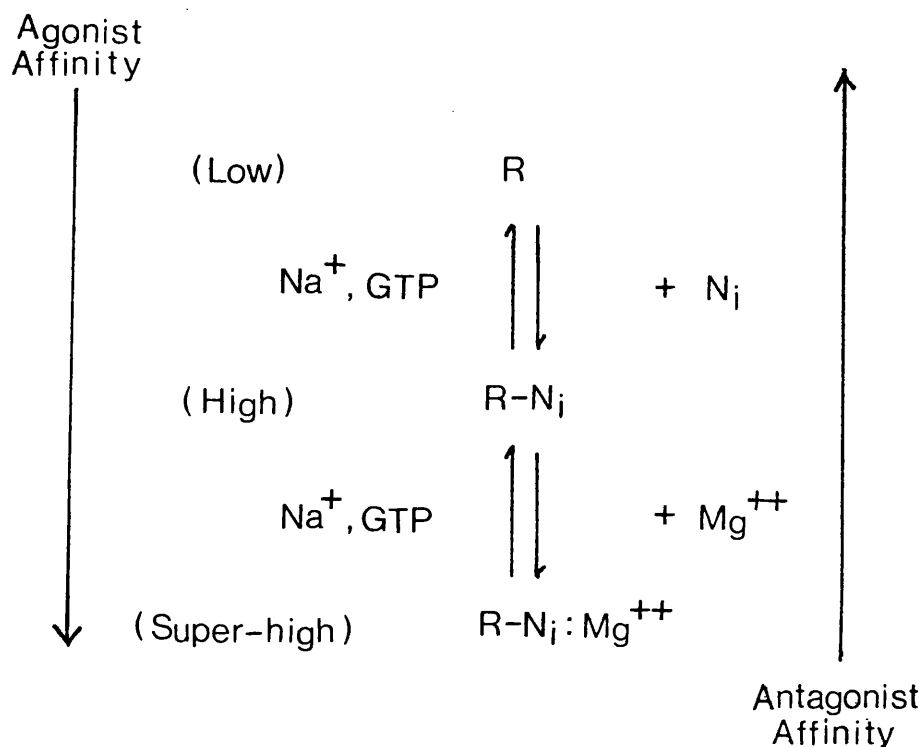


Figure IX Diagrammatic interpretation of the U'Prichard model of agonist/antagonist interactions with α_2 -receptor

Free α_2 -receptor (R) exhibits 'low' affinity for agonists. However, the complexation of Ni protein with the receptor to form (R-Ni) results in a 'high' affinity state for the agonists. The presence of Mg^{++} promotes the formation of (R-Ni : Mg^{++}) complex which represents the 'super-high' affinity state for the agonists. The antagonists would exhibit lower affinities for the 'super-high' and 'high' affinity states compared to the 'low' affinity state. The presence of Na^+ and GTP would shift the equilibrium between the agonist states in favour of the 'low' affinity state. Hence, the effect of Na^+ and GTP on antagonist affinities would be just opposite to that on agonist affinities.

the agonist high and low- affinity states. Therefore, affinity differences between tissues may only reflect the relative degree of drug interaction with different proportions of the agonist high and low-affinity states of the same α_2 -receptor. However, before considering this possibility further, it may be important to evaluate the validity of this U'Prichard model first.

The apparent evidence in support of this model is:

- 1) Divalent cations, such as Mg^{++} , promote high-affinity agonist binding but conversely increase the K_d of 3H -yohimbine or 3H -rauwolscine without altering the B_{max} (Daiguji et. al., 1981; U'Prichard et. al., 1983; also see Chapter VI).
- 2) The inhibitory affinities of some, but not all antagonists at 3H -clonidine sites in the rat cerebral cortex appear to be reduced in the presence of Mg^{++} (Salama et. al., 1982).
- 3) Although the rank orders of drug potencies at 3H -agonist and 3H -antagonist sites are similar within a given tissue, the affinities of agonists are usually more potent than antagonists at 3H -agonist binding sites; and conversely, many antagonists exhibit relatively high affinities at 3H -antagonist labelled sites, compared to the agonists (see review by Bylund & U'Prichard, 1983).
- 4) Whereas Na^+ and GTP reduce agonist affinities and destabilize high-affinity states at α_2 -receptors; these modulators also appear to produce a 'reciprocal' effect on antagonist binding, as reflected in apparent increases in antagonist affinities and/or binding capacities of 3H -antagonists (Woodcock & Murley, 1982; Limbird et. al., 1982; U'Prichard et. al., 1983, Bylund & U'Prichard, 1983).

The above findings, however, have not been totally supported by results of the present work. For instance, the presence of 100-200 mM NaCl and/or 0.1 mM GTP or Gpp(NH)p, was not found to significantly alter the B_{max} of 3H -yohimbine or 3H -rauwolscine in all the tissues examined at least under the tissue preparation and assay conditions adopted in this

study in which there is little retained endogenous agonist in the membranes. The effects of Na^+ on antagonist binding appeared variable in different tissues. The presence of 100 - 200 mM Na^+ reduced the affinities of ^3H -yohimbine and ^3H -rauwolscine in the platelet membranes without altering the B_{max} . Similar concentrations of Na^+ did not produce any significant change in either the B_{max} or K_d of these two antagonist labels in rat and rabbit kidneys, as well as rat brain (see Chapter VI). Interestingly though, while the affinities of prazosin at ^3H -yohimbine or ^3H -rauwolscine sites appeared slightly increased in all the tissues examined, the affinities of indoramin were reduced and those of corynanthine were not affected. Furthermore, the presence of 0.1 mM GTP or Gpp(NH)p did not change the position and slope of the displacement curve for either yohimbine or prazosin at ^3H -rauwolscine labelled sites (data not shown). In fact, the absence of a change in ^3H -yohimbine K_d by Na^+ and/or GTP has also been indicated in the studies of other laboratories involving the rat cerebral cortex (Woodcock & Murley, 1982), rat renal cortex (Woodcock & Johnston, 1982), rat and pig submandibular glands (Feller & Bylund, 1984), and neonatal rat lung (Latifpour et. al., 1982), although increases in the binding capacities of ^3H -yohimbine were observed in some of these tissues. However, the present work (Chapter VII) has further provided strong evidence suggesting that apparent decreases in the binding capacities of ^3H -antagonists can be induced by endogenous or retained agonists and that this interference can be reversed by Na^+ and guanine nucleotides. It appears that even a small quantity of residual agonist can produce a marked influence on antagonist binding in what can be described as a 'pseudo non-competitive' fashion. This effect may even be potentiated in the presence of retained endogenous cations. The presence of an agonist appeared to be able to 'reduce' the apparent affinity of an antagonist for only a proportion of the receptor sites, because of the preferential high-affinity agonist-receptor interaction. It is not clear if the apparently weaker affinities of the antagonists

in competing for ^3H -agonist binding may relate to this type of complicated interactions. Similarly the observed apparent increases of ^3H -antagonist binding sites in the presence of Na^+ and guanine nucleotides may also relate in part to the reversal of the influence of endogenous or retained agonists by these modulators.

Indeed, there are other unexplained phenomena which are not easily accommodated within the U'Prichard 'three-state' model of 'reciprocal' agonist and antagonist interactions with α_2 -receptors. The following arguments can illustrate that:

- 1) From the results of U'Prichard et. al., (1983), there appears to be some discrepancy between the ^3H -agonist binding data to human platelet and the agonist displacement data of ^3H -yohimbine binding in the same tissue, with respect to the influence of GTP (U'Prichard et. al., 1983). For instance, it was found that $10\ \mu\text{M}$ GTP already produced a maximal 'shift' of the adrenaline displacement curve to the right, apparently rendering the receptor population into a predominantly low-affinity state. Surprisingly, this same concentration of GTP was only found to slightly alter the ^3H -adrenaline K_d , but not B_{max} , which still represents about 70-80% of the ^3H -yohimbine B_{max} . In fact, one might reasonably expect that a significant quantity of the 'super-high' and 'high' affinity states should have been converted to the low-affinity states. The presence of 'super-high' and 'high' affinity ^3H -adrenaline binding was identified primarily from 'microanalysis' of the kinetics data. Similar agonist 'super-high' and 'high' affinity states have not been readily resolved in other tissues, for example, the neuroblastoma X glioma cells (U'Prichard et al., 1983). Feller & Bylund (1984) also found that ^3H -PAC labelled almost the same homogeneous quantity of sites as ^3H -yohimbine in the rat and pig lungs and submandibular glands. GTP ($10\ \mu\text{M}$) was found to reduce the ^3H -PAC affinities slightly in the pig lung and gland, but not the B_{max} , and the Scatchard plots were still linear in the presence of this concentration

of GTP. In the rat submandibular gland, GTP even unexpectedly increased the ^3H -PAC sites. These findings clearly suggest that the ^3H -PAC and ^3H -yohimbine binding sites may not necessarily represent the separate states of the same receptor population.

Interestingly, these results appear to contradict the 'two-state' model which would predict curvilinear Scatchard plots for ^3H -PAC binding, if it indeed labelled similarly to ^3H -yohimbine, the entire population of the α_2 -receptors (ie both high and low-affinity states). However, these results neither support the U'Prichard 'three-state' model, which would also predict curvilinear Scatchard plots for the ^3H -PAC and/or ^3H -yohimbine binding data if both ligands labelled all three states of the receptor population.

2) According to the suggestions of U'Prichard & colleagues (Bylund & U'Prichard, 1983) observable enhanced ^3H -rauwolscine binding by Na^+ and GTP appeared to occur at relatively low concentrations of these modulators (eg 0.1 nM to 0.1 μM for the GTP effect; the EC_{50} for Na^+ effect is 5.0mM), and that higher concentrations of Na^+ brought binding back to control levels. However, it must be realised that in fact, much higher concentrations of these modulators are required to maximally reduce agonist affinities. If one assumes that the low-affinity agonist states produced by Na^+ and GTP bind antagonists with higher affinities, one must expect that at concentrations of Na^+ and GTP which maximally reduce agonist affinities, a maximal enhancement of antagonist binding should also be observed. However, in this study it was found that at 100 - 200 mM Na^+ and 0.1 mM GTP which produced maximal reduction of agonist affinities at α_2 -receptors, no change in the B_{max} and K_d of ^3H -rauwolscine or ^3H -yohimbine binding was observed (see Chapter VI).

3) It is proposed in the U'Prichard model that the Mg^{++} -liganded high affinity states (or 'super high' affinity states) may be expected to

exhibit lower affinity for the antagonists. However, in the present work, all the Scatchard plots for ^3H -yohimbine or ^3H -rauwolscine binding to different tissues were linear in the absence or presence of Mg^{++} . According to U'Prichard and co-workers, one might expect that the ^3H -antagonists would, within a limited concentration range, 'selectively' label only the agonist low-affinity states of the α_2 -receptors. If one accepts this interpretation, one must be doubtful how the ^3H -antagonist labelled sites in the presence of Mg^{++} should be classified.

4) The U'Prichard model predicts that at low concentrations of the ^3H -antagonists (^3H -yohimbine or ^3H -rauwolscine), only low-affinity agonist states of the receptors would be labelled. One might expect this to happen particularly in competition experiments in which relatively low concentrations of radiolabels are used. However, if this was true, agonists would not be expected to exhibit heterotropic interactions with the ^3H -antagonist sites, and no guanine nucleotide modulation of agonist affinity should be observed. In fact, one might expect that all agonists generate steep displacement curves with low overall affinities even in the absence of GTP. This prediction obviously contradicts the results of the present work (Chapter VI). The results of ^3H -agonist and ^3H -antagonist labelling of α_2 -receptors in the rat neonatal lung membranes have further refuted this model. In this tissue, no ^3H -agonist binding, but only ^3H -yohimbine binding was detected (Latifpour et. al., 1983). Yet, contrary to the prediction that the ^3H -yohimbine sites must represent exclusively low-affinity agonist states, guanine nucleotide modulation of adrenaline displacement curve was demonstrable.

In view of the above reasoning, it is considered appropriate that much more conclusive evidence is required before the view that antagonists exhibit selectivity for different agonist states of α_2 -receptors can be accepted. In any case, it is clear that inter-tissue drug affinity differences

observed at ^3H -yohimbine and ^3H -rauwolscine sites cannot be explained by the different degree of drug interactions with agonist high and low-affinity states of the same α_2 -receptor. There are two obvious reasons:

1) As pointed out before, at low concentrations of ^3H -yohimbine or ^3H -rauwolscine, such as those used in the present competition studies, the U'Prichard model would predict that only the agonist low-affinity states in each tissue are selectively labelled. Indeed, in all tissues, antagonists generated displacement curves with slope of unity, suggesting interaction with a single class of sites or states. However, if these sites/states were identical, there is no reason whatsoever to expect any drug affinity differences between tissues. Moreover, while the affinities for α_2 drugs like yohimbine and rauwolscine appeared reduced in the rat kidney, compared to human platelet, the affinities of α_1 drugs such as prazosin, were markedly higher in the rat kidney than in the platelet. These findings are clearly inconsistent with the predictions from the U'Prichard model.

2) In the soluble receptor preparations, the α_2 -receptors appeared to be predominantly in the agonist low-affinity states, since no guanine nucleotide modulation of agonist affinity was observed (Chapter VIII). Again, according to the U'Prichard model, it would be expected that antagonists exhibited relatively higher affinities at these sites. Moreover, one should also expect the relative drug potencies to be the same between the soluble receptor preparations. However, the present study indicated that the pharmacological behaviour of the soluble receptors was identical to that in membranes. Therefore, the relative affinities for drugs like yohimbine and prazosin were still markedly different between the soluble preparations of the platelet and rat kidney or rabbit kidney, although drug affinities were generally somewhat reduced, rather than increased.

In conclusion, there is now enough evidence to indicate that the molecular mechanisms of agonist and antagonist interaction with α_2 -receptors are basically different. The differential influences of guanine

nucleotides and cations on the binding characteristics of agonists and antagonists have provided substantial evidence in this respect. In the present work, similar evidence has also been obtained on the basis of thermodynamic differences. However, the hypothesis that antagonists may also interact with α_2 -receptor in a 'reciprocal' manner to the agonists, and may therefore allosterically modulate the functional activities, requires further verification. In future studies, a better understanding of the molecular mechanisms of drug-receptor interactions, coupling and regulation should benefit from the development and application of more purified receptor preparations from homogeneous cell population. The application of genetic manipulation to develop homogeneous cell line and variants, the isolation and purification of the receptor components, and their application to reconstitution studies should eventually allow a full dissection of the α_2 -receptor system(s) and provide conclusive answers to the receptor-effector coupling mechanisms. Indeed, some progress toward these aims has already been made recently (Katada et. al., 1984 a, b, c).

The results of the present work have clearly provided strong evidence for the possible heterogeneity of α_2 -receptors in mammalian tissues. It appears likely that the observed differences in pharmacological behaviour between α_2 -receptors may reflect structural rather than merely conformational differences of the receptor proteins. Naturally, identification of molecular differences between the α_2 -receptor proteins will require future isolation and purification of the soluble receptors. The development of an affinity chromatography technique appears essential to achieve such a goal. Techniques such as photo-affinity labelling or 'target size analysis' can be useful to assess the differences in certain molecular properties of the receptor proteins.

If α_2 -receptors subtypes do independently exist, it is very important that any possible differences in functional and/or coupling mechanisms must be identified and evaluated. It is perhaps possible that some α_2 -

receptors may mediate functional responses primarily through the inhibition of adenylate cyclase activity, whereas others do so at least partly through some Ca^{++} gating mechanism. At this moment, the functional evidence for α_2 -receptor heterogeneity is still somewhat limited. Indeed, more carefully designed studies will be essential to identify α_2 -receptor subtypes of true physiological significance. For instance, it may be interesting to see if α_1 -antagonists such as prazosin might indeed exhibit significant potency differences in antagonizing α_2 -mediated inhibition of C'AMP accumulation in the human platelets and rat brain slices or rat kidney preparations, in view of their marked affinity differences at ^3H -yohimbine or ^3H -rauwolscine sites. Likewise, the classical physiological approach will be a useful alternative. It may be interesting to note that Ruffolo et. al. (1980, 1981, 1982) have indeed identified three different types of postsynaptic α_2 receptors both between the rat tissues and between same tissues across different species based on the affinities of yohimbine and clonidine determined in functional (contractile) responses. Postsynaptic α receptor (eg in rat aorta) exhibiting high affinities for both α_2 -drugs and α_1 -drugs (eg prazosin), and hence possessing 'mixed α_1/α_2 ' properties, was described. Such findings may in fact be of relevance to results of the present work.

APPENDICES

Appendix I

A Drugs

(-) Adrenaline	(Sigma)
Clonidine	(Boehringer)
Corynanthine	(Carl Roth)
Dopamine	(Sigma)
5-HT	(Sigma)
(-) Isoprenaline	(Sigma)
Indoramin	(Wyeth)
Methoxamine	(Burroughs Wellcome)
(-) Noradrenaline	(Sigma)
Phentolamine	(Ciba-Geigy)
Prazosin	(Pfizer)
Rauwolscine	(Carl Roth)
WB4101	(Ward, Blenkinshop)
Wy 26703	
Wy25309	(Wyeth)
Wy 26392	
Yohimbine	(Medochem)

Others

5'-G.T.P.	
5'-guanylyl imidodiphosphate [Gpp(NH)p]	(Boehringer Mannheim)

B Radiochemicals

³ H-Yohimbine	(New England Nuclear; specific activity = 80 - 90 Ci/m.mole)
³ H-Rauwolscine	
³ H-Prazosin	(Pfizer, specific activity = 33 Ci/m.mole)

C Chemicals

All are of analytical reagent grade.

Digitonin	(Sigma)
Cholate	(Sigma)
Deoxycholate	(Sigma)
CHAPS	(Sigma)
Bovine γ -globulin (Fraction II)	(Sigma)
Polyethylene Glycol 6000 (M.W.6-8000)	(Sigma, BDH Chemicals)
Bovine Serum Albumin	(Sigma)

D Instruments**Centrifuges**

Beckman T J-6

Sorvall R C-3

Sorvall R C-5 (super speed)) (Du Pont)

Homogenizer

Ultra-Turrax (Jankel & Kunkel)

Spectrophotometer

SP6-500 (Pye Unicam)

Liquid Scintillation Counter

LKB-Wallac 1216 Rackbeta

Appendix II**Reagents for Lowry protein analysis**

A Na_2CO_3 in 0.1 N NaOH 2%

B $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 1%

C Sodium potassium tartrate 2%

D Freshly made from -
200 ml. A
+ 2 ml. B
+ 2 ml. C

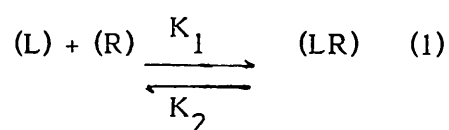
E Folin-Ciocalteu Reagent diluted 1 : 3 with distilled water.

F Protein standard = Bovine Serum Albumin 10%

Appendix III

Derivation of equations for Scatchard and Hill plots

The binding of a ligand L to a receptor R can be described by:



$$K_d = K_1 = \frac{(L)(R)}{\overline{K_2} (LR)} \quad (2)$$

$$(LR) + (R) = B_{\max} \quad (3) \quad (\text{where } B_{\max} = \text{total receptor density})$$

$$(L) (LR) + (L)(R) = B_{\max} (L) \quad (4)$$

$$\text{and } (L)(LR) + (LR) \frac{(L)(R)}{(LR)} = B_{\max} (L) \quad (5)$$

from (2), (5) becomes

$$(L)(LR) + (LR) K_d = B_{\max} (L)$$

$$(LR)((L) + K_d) = B_{\max} (L) \quad (6)$$

$$\text{if } (LR) = B \quad (\text{receptor or ligand bound})$$

$$\text{and } (L) = F \quad (\text{free ligand concentration})$$

then (6) becomes

$$B (F + K_d) = B_{\max} F$$

$$BF + BK_d = B_{\max} F$$

By rearrangement,

$$(B_{\max} - B) F = BK_d$$

$$\frac{(B_{\max} - B)}{K_d} = \frac{B}{F} \quad (7) \quad (\text{Scatchard})$$

A plot of $\frac{B}{F}$ against B yields a slope the reciprocal of which is the K_d .

The X-intercept yields the B_{\max} . Alternatively (6) can be rearranged to

$$B = B_{\max} - \frac{B}{F} K_d \quad (8)$$

A plot of B against $\frac{B}{F}$ yields a negative slope which is equal to K_d .

The Y-intercept is the B_{\max} .

The binding isotherm can be described by

$$B = \frac{B_{\max} (L)^n}{K_d + (L)^n}$$

$$B_{\max} (L)^n = B K_d + B (L)^n$$

$$(L)^n \frac{(B_{\max} - B)}{B} = K_d$$

$$n \log (L) + \log \frac{(B_{\max} - B)}{B} = \log K_d \quad (\text{Hill})$$

$$\log \frac{(B_{\max} - B)}{B} = \log K_d - n \log (L)$$

A plot of $\log \frac{B}{(B_{\max} - B)}$ against $\log (L)$

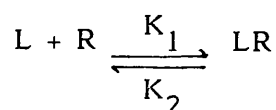
yields the slope n , which is known as the Hill coefficient (n_H).

Appendix IV

Derivation of association and dissociation kinetics equations

Association reaction

The binding of the ligand L to the receptor R can be described by the following equation:



where k_1 = second-order association rate constant

k_2 = first-order dissociation rate constant.

Let (a), (b) and (c) be the free concentrations of L, R and LR respectively at a particular time.

Therefore, $-\frac{d(b)}{dt} = k_1'(b) - k_2(c)$

(where $k_1' = k_1(a)$, if $(a) \gg (b)$)

since $(c) = (b)_0 - (b)$ (where $(b)_0$ = initial concentration of R at time zero)

therefore, $-\frac{d(b)}{dt} = k_1'(b) - k_2[(b)_0 - (b)]$ (1)

$$\frac{-d(b)}{(k_1' + k_2)(b) - k_2(b)_0} = dt$$

Let $(k_1' + k_2)(b) - k_2(b)_0 = v$

then, $\frac{dv}{d(b)} = (k_1' + k_2)$

Equation (1) becomes

$$\frac{-dv}{(k_1' + k_2)v} = dt, \text{ and } \frac{-dv}{v} = (k_1' + k_2) dt$$

By integration between time zero $\rightarrow t$, and by rearrangement, equation

(1) becomes

$$\ln \left[\frac{k_1'(b)_0}{(k_1' + k_2)(b) - k_2(b)_0} \right] = (k_1' + k_2) t \quad (2)$$

At equilibrium,

$$k' (b)_{eq} = k_2 (c)_{eq}$$

$$\text{but } (c)_{eq} = (b)_0 - (b)_{eq}$$

$$\text{therefore, } k' (b)_{eq} = k_2 (b)_0 - k_2 (b)_{eq} \quad (3)$$

It follows that

$$(b)_0 = \frac{(b)_{eq} (k' + k_2)}{k_2} \quad (4)$$

Substituting (4) into (2), one gets

$$\ln \left[\frac{\frac{k' (b)_{eq}}{k_2}}{(b) - (b)_{eq}} \right] = (k' + k_2) t \quad (5)$$

Substituting (3) into (5),

$$\ln \left[\frac{(b)_0 - (b)_{eq}}{(b) - (b)_{eq}} \right] = (k' + k_2) t \quad (6)$$

Let $(b)_0 - (b)_{eq} = B_{eq}$ (ie total amount of ligand bound at equilibrium)

then, $(b) - (b)_{eq} = B_{eq} - B$ (where B is the amount bound at time t)

Therefore, equation (6) becomes

$$\ln \frac{B_{eq}}{B_{eq} - B} = (k' + k_2) t \quad (7)$$

A plot of $\ln \frac{B_{eq}}{(B_{eq} - B)}$ against t yields a slope $(k' + k_2)$ or K_{obs} , which

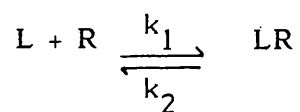
is the pseudo first-order association rate constant.

Since $k' = k_1 (a)$

Therefore, k_1 , the second-order association rate constant

$$= \frac{K_{obs} - k_2}{(a)}$$

Since $(a) \gg (b)$ and can be regarded as relatively unchanged throughout the reaction, $(a) \approx (a)_0$, which is the initial ligand concentration used.

Dissociation reaction

If association can be experimentally stopped at equilibrium, ligand dissociation would be expected to follow a first-order kinetics.

$$\text{Therefore, } -\frac{d(c)}{dt} = k_2(c)$$

$$\text{or } \frac{-d(c)}{(c)} = k_2 dt \quad (1)$$

After integration between time zero $\rightarrow t$ (where time zero refers to the equilibrium state before dissociation is initiated), equation (1) becomes

$$\text{Ln} \left[\frac{(c)_t}{(c)_{eq}} \right] = -k_2 t \quad (2)$$

$$(c)_{eq} = B_{eq} \quad \text{and} \quad (c)_t = B$$

$$\text{Therefore, } \text{Ln} \left(\frac{B}{B_{eq}} \right) = -k_2 t$$

A plot of $\text{Ln} \left(\frac{B}{B_{eq}} \right)$ against t yields a negative slope k_2 , which is the first-order rate constant for the dissociation reaction.

Appendix V

I Calculation of K_I from IC_{50} (from Cheng & Prusoff, 1973)

For radioligand binding to receptor,

$$B_o = \frac{B_{max} S}{K_d + S}$$

where B_o = Bound radioligand in the absence of competing drug, B_{max} = maximal binding capacity, K_d = dissociation constant of the radioligand and S = concentration of free radioligand.

When competitive inhibitor is present,

$$B_I = \frac{B_{max} S}{K_d \left(1 + \frac{I}{K_I}\right) + S} \quad \text{WHERE}$$

B_I = Bound radioligand in the presence of competing drug, I = competing drug concentration, and K_I = dissociation constant of the competing drug.

When $I = IC_{50}$, $2B_I = B_o$,

$$\text{therefore, } \frac{2B_{max} S}{K_d \left(1 + \frac{IC_{50}}{K_I}\right) + S} = \frac{B_{max} S}{K_d + S}$$

By rearrangement,

$$IC_{50} = K_I \left(1 + \frac{S}{K_d}\right)$$

$$\text{or } K_I = \frac{IC_{50}}{\left(1 + \frac{S}{K_d}\right)}$$

II Determination of slope factor (nH) for displacement curve

$$\% I = \frac{100\% (L)^n}{IC_{50} + (L)^n}$$

where $\% I$ = % radioligand displaced.

(L) = concentration of inhibitor

IC_{50} = concentration of inhibitor causing 50% Inhibition/displacement.

$$100\% (L)^n = \% I (IC_{50}) + (L)^n \% I$$

$$(L)^n \frac{(100\% - \%I)}{\%I} = IC_{50}$$

$$n \text{ Log } (L) + \text{Log} \left(\frac{100\% - \%I}{\%I} \right) = \text{Log } IC_{50}$$

$$\text{Log} \left(\frac{\%I}{100\% - \%I} \right) = n \text{ Log } (L) - \text{Log } IC_{50}$$

A plot of $\text{Log} \left(\frac{\%I}{100\% - \%I} \right)$ against $\text{Log } (L)$ yields n , which is the 'Hill' slope

factor. The X-intercept yields the IC_{50} .

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