BIOCHEMICAL STUDIES OF BETA-ADRENOCEPTOR HETEROGENEITY

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

by

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SUMMARY

The properties of the specific $^3$H-labelled (-)-dihydroalprenolol ($^3$H-DHA) binding sites on membrane preparations from a number of mammalian tissues have been examined. Specific $^3$H-DHA binding to all membrane sites was rapid, reversible, saturable, stereoselective and displaceable by beta-adrenoceptor agents with affinities which were similar to those found using intact tissue preparations. These findings indicated that $^3$H-DHA binding was to sites that had all the characteristics of beta-adrenoceptors. The pharmacological characteristics of these sites have been examined using highly selective beta-adrenoceptor agents. The results indicated that membranes of rat and rabbit lung, rat cerebral cortex, and rat myocardium possessed heterogeneous sites which were pharmacologically equivalent to $\beta_1$ and $\beta_2$-adrenoceptors. The single cell types rat erythrocytes and reticulocytes possessed a homogeneous population of $\beta_2$-adrenoceptors. The pharmacological properties of the beta-adrenoceptor subtypes were conserved between tissue and species and they were independent of the proportion of the subtypes in the preparation. This finding suggested that there were only two mammalian beta-adrenoceptors.

The rat erythrocyte and reticulocyte beta-adrenoceptor systems have been compared. The results indicated that these cell types possessed identical beta-adrenoceptor binding sites but markedly different receptor-adenylate cyclase coupling relationships. Rat reticulocytes contained approximately four-fold greater density of beta-adrenoceptors, and approximately twenty-fold greater maximum isoprenaline stimulated adenylate cyclase activity. The intrinsic activities of the partial agonists salbutamol and procaterol were
higher for rat reticulocytes than erythrocyte membranes. Guanine nucleotides modulated agonist binding to beta-adrenoceptors of rat reticulocytes but not to rat erythrocytes. These results suggested that maturation of the rat reticulocyte was associated with functional uncoupling of the receptor-effector system.

The beta-adrenoceptors of non-mammalian tissues have also been characterised. Chick and frog erythrocytes were shown to possess beta-adrenoceptor binding sites which did not correspond exactly to mammalian beta_1 and beta_2-adrenoceptors. The atypical nature of these non-mammalian beta-adrenoceptors is discussed in relation to their previous extensive use as model beta-adrenoceptor systems.

Beta_1 and beta_2-adrenoceptors have been solubilised from rabbit and rat lung using the detergent digitonin. A charcoal separation technique has been developed for the assay of soluble beta-adrenoceptors and its application indicated that the major pharmacological characteristics of the beta-adrenoceptors were conserved upon solubilisation. The potency of several catecholamine agonists and the steepness of the displacement curves were greater in the solubilised preparations. The co-presence of beta_1 and beta_2-adrenoceptors has been demonstrated in solubilised preparations of rabbit lung. Soluble beta_1-adrenoceptors appeared to be more labile than soluble beta_2-adrenoceptors, which provided support for the concept that the beta-adrenoceptor subtypes were separate entities. The hydrodynamic properties of soluble rat and rabbit lung beta-adrenoceptors have been examined using gel exclusion chromatography.

Agonist interaction with membrane preparations of beta_1 and beta_2-adrenoceptors have been examined, and the results are discussed in relation to current models of beta-adrenoceptor effector coupling. Beta_1 and beta_2-adrenoceptor preparations exhibited differential ion and temperature requirements in order for guanine nucleotide modulation of
agonist binding to be expressed. Agonist binding to beta₁-adrenoceptors was characterised by thermodynamic parameters which differed from those of beta₂-adrenoceptors. The partial agonist salbutamol activated the adenylate cyclase coupled to beta₂-adrenoceptors but not that coupled to beta₁-adrenoceptors. These findings suggested that there may be different receptor-effector coupling relationships between the two beta-adrenoceptor subtypes. Evidence is also presented which suggested that the in vivo selectivity of salbutamol was related not to selective affinity, but to selective efficacy at beta₂-adrenoceptors.
The following publications resulted from work described in this thesis:


CHAPTER 1

Introduction
General Introduction

A large number of tissues undergo alterations in their functional state upon exposure to drug or hormone molecule. The molecular components of the effector cells which interact with these agents, or 'receptive substances' were studied as early as 1905 by Langley. His studies of the site of action of nicotine and curare at the neuromuscular junction have been the keystone of modern theories of drug action. For many years the 'receptor' has been a useful operational concept, with no direct proof of its existence, although the consequences of drug-receptor interaction have been investigated in great detail (Gaddum, 1936; Clark, 1937; Stephenson, 1956; Ariens et al, 1960; Paton, 1961). These classical receptor theories have been formulated from tissue responses which represented amplified signals of the initial drug-receptor interaction (see Moran, 1966; Ariens and Simonis, 1976), and conclusions about the initial step were, therefore, indirect inferences. The biochemical approach to this problem has been to study in vitro preparations of receptors and to attempt their isolation. Such work had as its aims: the provision of conditions for the direct study of drug-receptor interactions; the proof of receptors as discrete molecular entities; the development of methods for receptor localisation in tissues; and, ultimately, the reconstitution of functional receptor-effector systems.

In its simplest form the receptor system can be considered to be composed of two components, a receptor or recognition site which binds specific ligands and an effector system which responds to the binding of agonist drugs. Agonists have affinity for the receptor and intrinsic activity, i.e. a capacity to activate the effector system, whereas antagonists have affinity but lack intrinsic activity. Agonist occupation of receptors is thought to result in specific conformational changes in the receptor which are propagated to adjacent effector molecules. This implies a close association of receptor and effector units for functional
activity, and it makes the task of receptor isolation and reconstitution difficult. In the last decade, however, substantial advances have been made in the characterisation and purification of receptors. In particular, the nicotinic acetylcholine receptor has been shown to be a discrete molecular entity which was located at cell surface sites of the neuromuscular junction (Raftery et al., 1976).

The work to be described will apply biochemical techniques to the in vitro study of isolated preparations of beta-adrenoceptors. A major portion of the work will be directed at investigations of the pharmacological characteristics of the receptor binding sites. Attempts to isolate and characterise these receptors will also be described.

In recent years, considerable advances have been made in the delineation of the effector system linked to beta-adrenoceptors. This system, which appears to be less direct than the ion channel-linked nicotinic acetylcholine receptor (Fatt and Katz, 1951), is thought to consist of a receptor, a guanine nucleotide regulatory protein and adenylate cyclase (Rodbell, 1980). In the light of these recent findings, the classical pharmacological observations of partial agonist activity (Stephenson, 1956) and tachyphylaxis may be explained in molecular terms. The work to be described will also investigate the biochemical consequences of receptor occupation by agonist drugs.

1:2 The general classification of adrenoceptors

The concept of adrenoceptors arose from attempts to subdivide the autonomic nervous system into functional components. The classification of this system into sympathetic and parasympathetic was based on the anatomy of the efferent nerves from the central nervous system. Thus, sympathetic nerves had their cell bodies in the thoracolumbar segments of the spinal cord, whereas parasympathetic pathways originated from neurons that had their cell bodies in the medulla, midbrain and sacral
regions of the spinal cord. The end organ receptors for the sympathetic system have been termed adrenergic receptors or adrenoceptors, and they responded to nerve stimulation, circulating catecholamines, and exogenously applied catecholamines and related agents. Some non-innervated tissues also contained adrenoceptors, which responded with an identical specificity to the adrenoceptors of innervated tissues.

The first experimental evidence for two distinct classes of adrenoceptors was provided by the classical studies of Dale (1906). His results demonstrated that the excitatory motor responses of various organs to adrenaline, and sympathetic nerve stimulation were inhibited by ergot alkaloids, whereas the inhibitory responses were unaffected. The full significance of this result was not appreciated until much later, largely because of the uncertainty of the transmitter released from sympathetic nerves. However, the early work of Dale introduced two methods for the differentiation of adrenoceptors - the selective blockade of responses by antagonists, and the relative potencies of a series of adrenoceptor sympathomimetic agents. The latter methodology was used by Ahlquist (1948) to differentiate the tissue responses from a number of species on the basis of the order of potencies of five catecholamines. His classical studies demonstrated that the order of potencies of these drugs for eliciting vasoconstriction, and excitation of the smooth muscle of nictitating membrane, uterus, dilator pupillae, fundus and ureter differed from that producing inhibition of vascular, bronchial and uterine smooth muscle and excitation of the heart. These results were interpreted as indicating that there were two types of adrenoceptor which mediated these effects, the former adrenoceptors were termed alpha and the latter beta. The responses elicited by alpha-receptor stimulation were inhibited by the known 'adrenergic blocking' agents, whereas, no beta-adrenoceptor stimulated responses could be antagonised.
Ahlquist's concept was substantiated by the subsequent discovery of dichloroisoprenaline (DCI), an agent which showed selective antagonism for beta-adrenoceptors (Powell and Slater, 1958). DCI was shown to antagonise the 'adrenergic inhibitory receptor sites' in smooth muscle, and adrenergic stimulatory responses in the heart (Moran and Perkins, 1958), whereas adrenergic stimulatory responses in smooth muscles were unaffected. The availability of selective antagonists for alpha- and beta-adrenoceptors enabled Ahlquist and Levy (1959) to demonstrate that intestinal relaxation was mediated through alpha and beta-adrenoceptors.

1:3 The dual beta-adrenoceptor hypothesis
1:3:1 Pharmacological studies

(A) Classification

The first indication that beta-adrenoceptors in different tissues might not be homogeneous came from work with newly synthesised beta-adrenoceptor antagonists (Levy, 1966; Moran, 1967). Thus, the beta-adrenoceptor antagonists butoxamine and alpha-methyl-DCI blocked isoprenaline induced vasodilatation but not cardiac stimulation and intestinal inhibition in cats and dogs. Moreover, butoxamine blocked isoprenaline-induced relaxation of isolated rat uterus, but not that of the isolated rabbit jejunum (Levy, 1967). Furthermore, isopropylmethoxamine and butoxamine blocked catecholamine-stimulated increases in plasma glucose, lactic acid, and free fatty acid in anaesthetised dogs, without antagonising the cardiac stimulating effects (Salvador et al, 1964; Burns et al, 1967).

The work of Lands and co-workers provided additional evidence for the heterogeneity of beta-adrenoceptors in tissues and a nomenclature for these receptor subtypes. These investigators studied the relative potencies of a series of sympathomimetic agents in producing beta-adrenoceptors mediated responses in both isolated tissues and intact animals (Lands et al, 1967a,b). They concluded that the receptors could be divided into two
distinct groups: those concerned with cardiac stimulation, lipolysis, and intestinal relaxation were termed beta$_1$, whilst those mediating smooth muscle relaxation in the trachea and blood vessels were termed beta$_2$.

Support for the dual beta-adrenoceptor concept has come from the introduction of beta-adrenoceptor agonist and antagonist drugs which showed selectivity for beta$_1$ or beta$_2$-adrenoceptors. Thus, practolol, a beta$_1$-selective antagonist was shown to be more potent in blocking the stimulation of cardiac receptors than bronchial and vascular smooth muscle receptors (Dunlop and Shanks, 1968). Salbutamol, which was reported to be more potent in activating vascular and bronchial smooth muscle beta-adrenoceptors than cardiac beta-adrenoceptors (Brittain et al., 1968) has been termed a beta$_2$-selective partial agonist. These agents were the archetypes of the many selective drugs which presently abound (for review see Clark, 1976). Perhaps the most notable of the 'cardioselective' beta-adrenoceptor antagonist were atenolol and betaxolol which showed considerable degrees of selectivity for beta$_1$-adrenoceptors (Barrett et al., 1973; Boudot et al., 1979). Few highly beta$_2$-adrenoceptor-selective agents were reported until the recent introduction of the partial agonist procaterol (Yabuuchi et al., 1977) and the antagonist ICI 118 551 (Bilski et al., 1980).

Although most workers agreed that the original classification of Ahlquist for a single class of beta-adrenoceptor represented an over-simplification, the concept of only two beta-adrenoceptors has also received some criticism. Thus, Furchgott, (1967) investigated the relative potencies of adrenoceptor agonists and the affinities of pronethalol and phentolamine in isolated tissues of the guinea pig and rabbit. He concluded that in the tissues studied, there was one type of alpha-adrenoceptor but at least three types of beta-adrenoceptor. Bristow et al. (1970) supported this contention by showing that the affinities of three beta-adrenoceptor antagonists determined
in rabbit atria, trachea, aorta and stomach varied from tissue to tissue, which suggested that many different subtypes existed. Others have also proposed that there may be a large spectrum of beta-adrenoceptors (Brittain et al, 1970; Boissier et al, 1971; Ahlquist, 1976).

These variations in the pharmacological specificities of receptor-mediated tissue responses may have resulted from factors other than receptor differences. The importance of adequately controlled experimental conditions has been stressed by Furchgott (1972), and in particular the need to eliminate the distorting influences of alpha-adrenoceptors, and neuronal and extraneuronal uptake of catecholamines. Indeed, the apparent selectivity of certain drugs for tracheal compared to vascular beta-adrenoceptors has been shown to result from differences in the extraneuronal uptake of these compounds (O'Donnell and Wanstell, 1976).

(B) The co-existence of beta\textsubscript{1} and beta\textsubscript{2}-adrenoceptors in tissues

The concept of dual beta-adrenoceptors which can mediate the same response in a tissue was first suggested by Carlsson and co-workers (Carlsson et al, 1972). They showed that equal submaximal responses of the cat heart to certain beta-adrenoceptor agonists were differentially antagonised by selective beta-adrenoceptor blocking drugs. Thus, practolol blocked responses to the agonists with an order: noradrenaline > isoprenaline > adrenaline > salbutamol, whereas the beta\textsubscript{2}-selective antagonist H35/25 blocked responses with an order which was the exact reverse. The non-selective antagonist propranolol blocked all responses equally. These results were interpreted as indicating that both beta\textsubscript{1} and beta\textsubscript{2}-adrenoceptors mediated chronotropic responses in the cat heart, but that beta\textsubscript{1}-adrenoceptors comprised the majority of the beta-adrenoceptor population.

The co-presence of beta-adrenoceptor subtypes in a tissue has also been delineated by comparing the relative blockade of non-selective and selective
agonist dose-response curves by selective antagonists. Guinea pig trachea has been reported to contain functionally coupled beta_1 and beta_2-adrenoceptors, with the latter subtype predominating. Some variation in the proportion of the subtypes between animals was also apparent, (Furchgott and Wakade, 1975; Zaagsma et al, 1979; O'Donnell and Wanstall, 1979a).

The proportions of subtypes has also been shown to vary between regions of the same organ. Thus, although the predominant beta-adrenoceptor subtype in regions of the cat heart was beta_1, the sinus node contained a higher proportion of beta_2-adrenoceptors (Carlsson et al, 1977). Cat lung also exhibited a differential location of functional beta-adrenoceptor subtypes, with beta_1-adrenoceptors predominant in the trachea and beta_2-adrenoceptors predominant on the small airways smooth muscle (Lulich et al, 1976). Both beta_1 and beta_2-adrenoceptors were present in guinea pig trachea, whereas the lung strip possessed exclusively beta_2-adrenoceptors (Zaagsma et al, 1979). A number of other systems have also been described, which added to the growing list of tissue responses which were mediated by both beta_1 and beta_2-adrenoceptors. These included: vasodilatation of canine renal vasculature (Taira et al, 1977); and rat jugular vein (Cohen et al, 1978) and inhibition of gastric acid secretion in the dog (Daly et al, 1978).

1:3:2 In vitro biochemical studies

(A) Adenylate cyclase measurements

As a result of the pioneering work of Sutherland and co-workers the stimulation of adenylate cyclase by catecholamines was shown to be mediated through beta-adrenoceptors (Sutherland and Robison, 1966). This result meant that the pharmacological effects of catecholamines could be determined proximal to the drug-receptor interaction. Measurements of adenylate cyclase therefore, represented an excellent means of testing the extent to which cell-free preparations reflected the drug specificity of the intact tissue.

Burges and Blackburn (1972) examined the effects of some beta-adrenoceptor
agonists and antagonists on adenylate cyclase activity in homogenates of rat heart and lung. The order of potencies of the catecholamines in rat heart homogenates was isoprenaline > noradrenaline > adrenaline, whereas in rat lung preparations the order was isoprenaline > adrenaline > noradrenaline. These adenylate cyclase responses were consistent with beta₁ and beta₂-adrenoceptor classifications as proposed by Lands et al (1967a). In support of this conclusion, the selective beta-adrenoceptor antagonists practolol and butoxamine exhibited greater potencies for heart and lung adenylate cyclase respectively.

Lefkowitz (1975a) determined the potencies of catecholamines and selective antagonists on adenylate cyclase activity in homogenates of canine myocardium, liver, lung and skeletal muscle, rat paraovarian fat and frog erythrocytes. The order of potencies of the catecholamines for the stimulation of adenylate cyclase in cardiac and adipose tissue was isoprenaline > noradrenaline > adrenaline and in all other tissues the order was isoprenaline > adrenaline > noradrenaline. Practolol had greater potency for cardiac and fat tissue stimulated adenylate cyclases, whereas the reverse was the case for butoxamine. These results suggested that the adenylate cyclase coupled beta-adrenoceptors reflected the specificities of the intact tissue, and that receptor heterogeneity was apparent at the level of adenylate cyclase. Other studies have shown that the beta-adrenoceptor mediated adenylate cyclase responses of cardiac tissue differed from those of liver, trachea and lung (Mayer, 1977; Murad, 1973; Minneman et al, 1979a).

Recently the presence of beta₁ and beta₂-adrenoceptors in homogenates of brain tissue have been postulated, following the analysis of adenylate cyclase data by computer modelling (Ebersolt et al, 1981). Thus, the dose-response curve obtained for practolol inhibition of isoprenaline stimulated adenylate cyclase was biphasic, and it modelled to a two site system. Data obtained for the stimulation of adenylate cyclase by selective agonists also modelled to a two-site system whereas the non-selective agonist
isoprenaline generated a dose-response curve which was best fitted to a single site model.

(B) Radiolabelled ligand binding studies

The most direct way of characterising a receptor was to measure the interaction of a specific ligand with the receptor. This method has the advantage that the event being monitored was a simple bimolecular reaction, which was not influenced by drug effects distal to the receptor. Paton and Rang (1965) pioneered this approach for neurotransmitter receptors by labelling the muscarinic receptors in slices of guinea pig ileum with $^3$H-atropine. These studies prompted the development of further muscarinic radiolabelled ligands, which allowed these receptors to be characterised and their tissue location delineated (Miledi et al, 1971; Burgen et al, 1974; Yamamura and Snyder, 1974).

The characterisation of beta-adrenoceptors were not, initially, as successful as that of cholinergic receptors. Marinetti and co-workers showed that $^3$H-adrenaline labelled putative beta-adrenoceptors of rat liver membranes, and the binding was inhibited by catecholamines (Lesko and Marinetti, 1975). However, the binding was also inhibited by a variety of catechol-containing compounds that were inactive at beta-adrenoceptors. Moreover, stereoselectivity was not apparent, and the bound radioactivity could be dissociated with strong acid. These observations prompted Bilezikian and Aurbach (1973) to suggest the existence of a beta-adrenoceptor possessing dual binding sites for the catechol and ethanolamine moieties, where occupation of both sites would lead to adenylate cyclase activation. This concept could not be supported, however, since many catechol derivatives bound to the putative receptor, but they were not effective antagonists at beta-adrenoceptors.
Most of the studies of $^{3}\text{H}$-catecholamine binding to putative beta-adrenoceptors showed that binding was unaffected by potent beta-adrenoceptor antagonists and, with few exceptions was irreversible (Haber and Wrenn, 1976). Cuatracasas et al (1974) proposed that the catecholamine binding site was related to the enzyme catechol-O-methyltransferase (COMT), although it was later shown that potent inhibitors of COMT activity had no effect on the binding (Koretz and Marinetti, 1974). Maguire et al (1974) concluded that the binding of catecholamines involved a non-specific, covalent, interaction of oxidised degradation products of catechols with tissue components.

The problems associated with catecholamine binding were circumvented by the introduction of radiolabelled antagonists. These labels were more suitable because of their greater affinities for beta-adrenoceptors, enhanced specificity, resistance to oxidation and greater specific activities. The most widely used radioligands are $^{3}\text{H}$-dihydroalprenolol ($^{3}\text{H}$-DHA) and $^{125}\text{I}$-hydroxybenzylpindolol ($^{125}\text{I}$-HYP), which were introduced in 1974. Since that time the binding of these ligands to membrane fractions from a wide range of tissues has been shown to satisfy the criteria expected for binding to a beta-adrenoceptor (for review see Maguire et al, 1977; Wolfe et al, 1977). In addition, the abilities of drugs to compete with radioligand binding to beta-adrenoceptors has been shown to correlate directly with their abilities to interact with adenylate cyclase (Mukherjee et al, 1976).

The availability of these ligands provided the perfect tools with which to study beta-adrenoceptors in tissues. Snyder and co-workers used $^{3}\text{H}$-DHA to investigate the beta-adrenoceptors of the mammalian central nervous system, and demonstrated beta$_1$-adrenoceptors in cerebral cortex and beta$_2$-adrenoceptors in cerebellum (Bylund and Snyder, 1976; U'Pritchard et al, 1978). This latter finding confirmed the earlier reports of Chasin et al (1974), who showed that the adenylate cyclase responses of guinea pig
cerebellar homogenates were characteristic of beta\textsubscript{2}-adrenoceptors.

The beta-adrenoceptors of rat heart and lung were examined by U'Pritchard \textit{et al} (1978), and later by Minneman \textit{et al} (1979a). These investigators showed that the pharmacological specificities of the tissue preparations were consistent with overall beta\textsubscript{1} and beta\textsubscript{2}-adrenoceptors respectively. Thus, the catecholamines inhibited radioligand binding to heart membranes with an order of potency isoprenaline > adrenaline = noradrenaline and to rat lung membranes with an order isoprenaline > adrenaline > noradrenaline. Moreover, a number of selective beta-adrenoceptor agonists and antagonists exerted greater potencies for certain of the preparations which correlated with their known selectivity for the intact tissues.

In complementary studies, Nahorski and co-workers examined the beta\textsubscript{1} and beta\textsubscript{2}-adrenoceptor specificities of brain and lung homogenates (Nahorski, 1978; Barnett \textit{et al}, 1978; Rugg \textit{et al}, 1978). They showed that selective beta-adrenoceptor antagonists displaced \textsuperscript{3}H-DHA binding in a manner which suggested the existence of heterogeneous sites for the antagonists. Moreover, the inhibition constants of drugs for the sites was similar in lung and cerebral cortex, although the proportions of the sites differed. These results were interpreted as indicating the co-existence of beta\textsubscript{1} and beta\textsubscript{2}-adrenoceptors in these tissue preparations.

1:4 \textbf{The aims of this study}

The aims of the studies to be described are threefold.

i) Radiolabelled ligand binding techniques will be used to characterise the pharmacological specificities of tissue preparations. The purpose of this work is to examine the dual beta-adrenoceptor hypothesis in the light of recent work which suggested that the proportions of beta\textsubscript{1} and beta\textsubscript{2}-adrenoceptors in tissues could vary. The quantification of beta-adrenoceptor heterogeneity in membrane preparations will use the technique of monitoring the competition between non-selective radioligand and selective displacing
drug. This method was first applied to studies of beta-adrenoceptor heterogeneity by Nahorski and co-workers (Barnett et al., 1978) and later by Minneman and co-workers (Minneman et al., 1979b). The criterion used for the following classification studies was that identical receptors had identical pharmacological specificities for antagonists.

ii) Beta₁ and beta₂-adrenoceptors will be solubilised from appropriate mammalian tissue homogenates, in order to determine whether their pharmacological properties are conserved in solution. Attempts will be made to isolate soluble beta₁ and beta₂-adrenoceptors and to determine their physical and molecular properties. These studies have as their ultimate aim the provision of evidence that beta₁ and beta₂-adrenoceptors represent independent molecular entities.

iii) The interaction of agonists with beta₁ and beta₂-adrenoceptor preparations will be determined and the influences of various interventions (guanine nucleotides, ions, temperature) will be compared. The major challenge in adrenergic pharmacology today is to resolve the molecular events which occur between agonist occupation of receptors and activation of adenylate cyclase. In particular, a molecular explanation needs to be found for the differences in the intrinsic activities of certain beta-adrenoceptor agonists. The studies to be described will attempt to provide some information on the relative abilities of selected agonists to couple receptor occupation to adenylate cyclase stimulation in beta₁ and beta₂-adrenoceptor preparations.
CHAPTER 2

Methods
Introduction

The basic requirements for the biochemical identification of specific beta-adrenoceptor binding sites in tissues involved (i) a suitable radioactively labelled, biologically active, ligand; (ii) an effective method for separating bound and free radiolabelled ligand; and (iii) a tissue preparation containing beta-adrenoceptors. Taking appropriate criteria into consideration (see Lefkowitz, 1975b; Wolfe et al, 1977; Maguire et al, 1977; Burt, 1978) these 'tools' could then be used to establish whether interaction of ligand occurs to a physiologically relevant receptor.

The introduction of high affinity, high specific activity, radio-labelled beta-adrenoceptor antagonists has allowed major advances in the identification of specific beta-adrenoceptor binding sites. At the onset of these studies three radiolabelled ligands were commercially available: (+)-[^3]H-propranolol; (-)-[^3]H-dihydroalprenolol ([^3]H-DHA); and (+)-[^125]I-iodohydroxybenzylpindolol ([^125]I-HYP). (+)-[^3]H-Propranolol had been used to label beta-adrenoceptor binding sites in turkey erythrocytes (Levitzki et al, 1974) and chick cerebral cortex (Nahorski, 1976), but this ligand was associated with considerable degrees of non-specific binding. (-)[^3]H-Dihydroalprenolol, a ligand introduced by Lefkowitz et al in 1974, had the advantage of higher specific activity (\( \approx 50 \) Ci/m mole); bound to tissue preparations with good specific/non-specific ratios; and was chemically resolved into a single (biologically active) stereoisomer.

[^125]I-HYP, a high affinity beta-adrenoceptor antagonist which was introduced by Aurbach et al in 1974, had the advantage of possessing an atom of[^125]I which produced a theoretical specific activity of 2200 Ci/m mole. The disadvantages of[^125]I-HYP were the short half life of the isotope; the possibility of radiolysis of the ligand; the uncertainty of the iodination reaction which could result in multiple iodination products and therefore
the need for considerable quality control (see Bearer et al, 1980); and the availability of the ligand as the racemate, the problems associated with which have been analysed by Bürgisser et al (1981).

A further consideration for the choice of radiolabelled ligand were the specificity with which the ligand bound to the receptor under study. The literature was replete with examples of ligands which bound to more than one receptor type - the dopamine receptor ligand/neuroleptic $^3$H-spiperone bound additionally to serotonin sites (Leysen et al, 1978); $^3$H-dihydroergocryptine bound to alpha-adrenoceptors and dopamine receptors (Caron et al, 1978); and $^3$H-catecholamines bound to catechol recognition sites which are unassociated with beta-adrenoceptors (Haber & Wrenn, 1976). Furthermore, recent work has demonstrated that the beta-adrenoceptor antagonist ligand $^{125}$I-HYP bound to indole recognition sites in brain tissue (Dickinson et al, 1981).

In view of the former observations, the present studies of beta-adrenoceptor binding sites in membrane and soluble preparations have utilised, as the radiolabelled ligand, (-)-$[^3$H]$\cdot$dihydroalprenolol. Separation of $^3$H-DHA bound to membrane preparations from free ligand was accomplished by filtration under reduced pressure through glass-fibre filters and subsequent washing. This technique was suitable because dissociation of ligand from receptor during the time taken to separate bound/free ligand (<10 sec for most membrane preparations) was negligible. This method had the further advantage of being simpler and more rapid than the alternative techniques of equilibrium dialysis or centrifugation.

Studies of ligand binding to soluble beta-adrenoceptors has required, in some cases, modified methods to separate bound from free ligand. Equilibrium dialysis, sephadex G50 chromatography (Caron & Lefkowitz, 1976), precipitation techniques (Haga et al, 1977) and adsorption of free ligand (Clement-Cormier et al, 1980) have all been used effectively. The latter two techniques have been adopted for the present studies of
soluble beta-adrenoceptors and their relative merits compared.

One of the fundamental problems associated with binding studies has been the presence, in tissue preparations, of non-receptor sites to which the radiolabelled ligand bound. Consequently, binding studies generally accounted for this problem by including, in parallel incubations, an excess (usually 100 x K_d concentration) of unlabelled ligand or competing drug to define this non-specific binding (NSB). The choice of this drug, and its concentration, were critical for a proper estimation of specific binding, since a great excess of competing drug would also compete for non-specific sites. Where possible it was always advisable to use a drug which was chemically dissimilar to the ligand. The pitfalls associated with calculating NSB have been defined recently (Nahorski & Richardson, 1979).

The nature of non-specific binding has been suggested to relate to hydrophobic interactions of radiolabelled ligand with membrane constituents (Mendel et al, 1979). It was usually non-saturable and occurred instantaneously. Some reduction of tissue NSB has been accomplished by purification of plasma membrane enriched fractions from homogenates, since beta-adrenoceptors with few exceptions (Liao et al, 1971; Entman et al, 1976) were thought to be located on plasma membranes (Williams et al, 1976). Utilising a different approach, Baker et al (1980) have reduced the non-specific binding sites in cardiac tissue homogenates by dissolution of contractile proteins using high salt concentrations. Sporn et al (1976) utilised the alpha-adrenoceptor antagonist phentolamine to decrease NSB of 125I-HYP to rat brain membranes. These techniques will be applied to certain tissue preparations in order to produce as 'clean' a membrane preparation as possible.
2:2 Animals

Male Wistar rats were used for experiments in the weight range 150-200g, and were supplied from Bantin and Kingman Limited, or were bred from stock animals. Rabbits were obtained from a local supplier and were generally of the New Zealand White strain, weight 1-2 Kg. Frogs, obtained from Xenopus Limited, either Rana esculenta or Rana pipiens, were maintained at 4°C overnight before use. Male Ranger chicks were obtained from Ross Poultry Limited, and were 1-2 days old. Animals were fed ad lib before treatments or sacrifice.

2:3 Animal Treatments

Induction of reticulocytosis in rats.

Wistar rats (150-200g) were injected intra-peritoneally with a sodium bicarbonate-neutralised solution of phenylhydrazine HCl according to the following dosing regime: - Day 1, 40mg/Kg; Day 3, 60mg/Kg; Day 5 80mg/Kg. Control rats received saline injections. Blood was obtained by cardiac puncture on Day 8.

Aliquots of blood were diluted in isotonic NaCl/5mMTrisHCl pH7.4 and reticulocyte counts were determined in blood smears after staining with brilliant cresyl blue. Results were expressed as a percentage of the total red blood cells. This dosing regime produced a red cell population which was >85% reticulocytes. Control, erythrocyte, preparations had <5% reticulocytes.

2:4 Binding Studies

1 Membrane Preparation

(A) Rat and Rabbit Lung Membranes

Rats were killed by cervical dislocation and decapitation, rabbits by cervical dislocation and exsanguinisation. Lungs were removed from the thorax, and cleaned in cold 150 mM NaCl/5mMTrisHCl pH 7.5. Major bronchi and connective tissue were removed and the lungs weighed, and
-roughly minced with scissors. The tissue was homogenised in 10 volumes of 50 mM Tris-HCl, pH 7.8, at 4° using a Polytron homogeniser (setting 5, 3 x 5 sec bursts) or an Ultra-Turrax homogeniser (3 x 10 sec bursts). Homogenisation with the Ultra-Turrax was followed by 3 x 4 strokes of a motor-driven glass-teflon (Potter-Elvehjem) homogeniser. The resultant homogenate was centrifuged at 500 g in a Sorvall RC3 centrifuge for 10 min at 4°, in order to remove fibrous material. The supernatant from this centrifugation was passed through a double layer of cheese cloth and the filtrate was then centrifuged at 50,000 g in a Sorvall RC5 for 20 min at 4°. The pellet was resuspended in 50 mM Tris-HCl, pH 7.8, and washed three times before final suspension in the same buffer to a protein concentration of 3-5 mg/ml. Using this procedure routine yields of 6-8mg of protein were obtained/gm original wet weight of lung tissue.

Membranes were either snap-frozen in liquid nitrogen or placed at -50° until frozen. Membranes were stored, until required for assay, at -50°. Prepared in this manner, binding sites were stable for prolonged periods (up to one year).

Following thawing of stored lung membranes, preparations were observed to show signs of particulate aggregation. Dispersal of these clumped membranes by a 2 sec burst of the Polytron homogeniser resulted in a preparation which could be easily pipetted. This procedure did not appear to affect the binding parameters of the membranes, as evidenced by similar results obtained with these and freshly prepared membranes.

(B) Preparation of membranes from non-nucleated red cells: rat erythrocytes and reticulocytes

Rats (pretreated with phenylhydrazine or saline, (see 2:3) were anaesthetised with an intraperitoneal injection of sodium pentobarbital, 50mg/kg, and blood (8ml) was obtained by cardiac puncture using 0.6ml of 0.2 M EDTA, pH 7.5, as the anticoagulant. The blood was centrifuged at
500g for 10 min in a Sorvall RC3 at 4°, after which the plasma was removed by aspiration. The cells were washed in cold isotonic 150 mM NaCl/5 mM Tris-HCl, pH 7.6, and recentrifuged at 500g. The supernatant was removed and the cells were washed three more times in 150 mM NaCl/5 mM Tris-HCl, pH 7.6, by centrifugation at 1500g. During the course of these washings, the buffy coat layer was removed leaving a relatively pure population of washed red cells. Aliquots (1ml) of packed cells were removed from the bottom of the centrifuge tube and lysed in 40ml of 5 mM Tris-HCl, pH 7.8. After 10 min at 4°, 1ml of 4 M KCl/40 mM MgCl₂ was added and the lysate was centrifuged at 50,000g for 30 min at 4°. The pellet was resuspended in 50 mM Tris-HCl, pH 7.8, and resuspended. Following centrifugation at 50,000g for 20 min, the upper layer was removed (measurements of binding site maxima suggested that this fraction was enriched in plasma membranes: upper layer: 185 (+ 20) fmoles/mg protein; lower layer: 15 (+ 2) fmoles/mg protein; n=3). These membranes were briefly homogenised (Ultra-Turrax 1-2 sec), centrifuged at 50,000g for 20 min, and the pellet washed a further two more times in 50 mM Tris-HCl, pH 7.8, by centrifugation at 50,000g. The final pellet was taken up in 50 mM Tris-HCl, pH 7.8, at a protein concentration of 3-5mg/ml, rapidly frozen in liquid nitrogen and stored at -50° until use.

(C) (i) Preparation of membranes from nucleated red cells: chick and frog erythrocytes

Chicks and frogs were decapitated and blood collected into cooled centrifuged tubes containing a neutralised solution of 0.2 M EDTA (0.6ml/8ml blood) to prevent coagulation. The blood was filtered through a single layer of cheese cloth and centrifuged at 500g in a Sorvall RC3 for 10min at 4°, after which the plasma was removed by aspiration. Avian erythrocytes were washed twice in cold isotonic 150 mM NaCl/5 mM Tris-HCl, pH 7.6, by centrifugation at 500g for 10 min at 4° and three times in the same buffer by centrifugation at 1,500g for 10 min. Frog erythrocytes were washed in amphibian saline, 110 mM NaCl/10 mM Tris-HCl, pH 7.6.
using an identical experimental protocol. The white cells were easily removed during these washings by suction, leaving a red cell population which was essentially buffy coat layer-free.

The nucleated cells of frog and chick erythrocytes were lysed in 40ml of 5 mM Tris-HCl, pH 7.8 (20-40 vols). After 10 min at 4°, 1ml of 4 M KCl/40 mM MgCl₂ was added and the lysate was centrifuged at 50,000g for 30 min at 4°. The pellet was resuspended in 5 mM Tris-HCl, pH 7.8, and relysed. The lysate was centrifuged at 50,000g for 20 min at 4° and the pellet homogenised briefly using a Polytron homogeniser (setting 5, 2 sec). The homogenate was washed three times in 50 mM Tris-HCl, pH 7.8, and the final pellet taken up in this buffer at a protein concentration of 2-5 mg/ml.

(ii) Preparation of purified membrane fractions of chick and frog erythrocytes

Purified preparations of avian and amphibian erythrocytes were obtained by separation of the layers of the pellet which resulted from centrifugation of the homogenised lysate. This pellet contained a lower 'nuclear' layer and an upper, pink, plasma membrane layer which sedimented at higher centrifugation forces. Application of suction to the surface of the pellet resulted in removal of this top layer, which was subsequently assayed for specific ³H-DHA binding capacity, and compared with that of the nuclear fraction. Table 2:1 shows data obtained from frog erythrocyte preparations, where 'purified' preparations contained a ten-fold enrichment of specific binding sites compared to whole homogenates.
Table 2:1

$^3$H-DHA binding capacity of fractions of centrifuged frog erythrocyte lysate

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Max binding capacity of $^3$H-DHA fmoles/mg prot.</th>
<th>Number of determinations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower 'nuclear' fraction</td>
<td>37 (+ 2)</td>
<td>3</td>
</tr>
<tr>
<td>Upper 'plasma membrane' enriched layer</td>
<td>922 (+ 40)</td>
<td>6</td>
</tr>
<tr>
<td>Whole homogenate</td>
<td>86 (+ 6)</td>
<td>3</td>
</tr>
</tbody>
</table>

Caron et al (1976) have reported the successful use of centrifugation through buffered sucrose to purify frog erythrocyte homogenates. The specific activity of $^3$H-DHA binding and adenylate cyclase activity of their purified preparations were enhanced 5-10 fold. The major advantage of these preparations were their lower non-specific binding and faster filtration rates.

(D) (i) Preparation of membranes from rat heart

Rats were killed by cervical dislocation and decapitation, their hearts removed and placed in cold 150 mM NaCl/5 mM Tris-HCl, pH 7.6. The atria were dissected away and the ventricles washed free of blood. The ventricles were weighed, roughly minced with scissors and homogenised in 10 vols of ice-cold 10 mM Tris-HCl, pH 7.8, using a Polytron homogeniser (setting 5, 3 x 5 sec bursts with cooling). The homogenate was filtered through two layers of cheese cloth and added to an equal volume of 1 M KCl/10 mM Tris-HCl, pH 7.8, at $4^\circ$. Extraction and solubilisation of contractile proteins proceeded for 10 min at the temperature of melting ice, after which the homogenate was centrifuged at 50,000g for 15 min at $4^\circ$. The supernatant was discarded and the pellet washed three times in 50 mM Tris-HCl, pH 7.8, by centrifugation. The final pellet was taken up in 50 mM Tris-HCl, pH 7.8, at a protein concentration of 5-8mg/ml, and assayed immediately or snap frozen in liquid nitrogen and stored at -50$^\circ$ until use.
(D) (ii) Problems associated with membrane preparations of rat myocardial tissue

Cardiac tissue has been reported to be difficult to use for binding studies in view of the low number of specific beta-adrenoceptor binding sites present, and the high amounts of non-specific binding associated with homogenates (Winek & Bhalla, 1979). In addition, freezing of cardiac membrane preparations often resulted in loss of specific binding and increased non-specific binding (Nahorski, personal communication).

A recent report suggested, however, that NSB associated with canine ventricular preparations could be attenuated by KCl extraction (Baker et al, 1980). Experiments were therefore conducted to test the effect of such treatment on the binding parameters of freshly prepared and frozen preparations of rat myocardial homogenates.

Results suggested that KCl extraction of cardiac homogenates had no influence on non-specific binding of the resultant preparations, but increased by $\pm 40\%$ the specific activity of the preparation for $^3$H-DHA binding to beta-adrenoceptors. Since protein yields of treated preparations were 73 ($\pm 3\%$ of controls ($n=3$), this improvement in specific activity probably resulted from removal of non-receptor (contractile) protein by KCl.

The effect of freezing KCl-treated and control preparations was also investigated. Unlike the membrane preparations described previously (2:4:1, A-C) where freezing had no influence on the binding parameters, freezing of cardiac membranes resulted in a 10-15% loss of specific binding sites and an increase in non-specific binding. Preparations which had been KCl-extracted were slightly more resistant to this freezing-induced increase in NSB.
**Preparation of membranes from rat cerebral cortex**

Wistar rats (150-200g) were killed and the brains placed on a cooled glass plate. The brain was sectioned according to the method of Glowinski and Iversen (1966), the parietal cortex dissected out, and placed into 30-40 vols of ice cold 50 mM-Tris HCl pH 7.8. The tissue was homogenised using a Polytron homogeniser (setting 6, 10 secs) and the homogenate centrifuged at 50,000g for 15 min at 4°. The pellet was resuspended in the same volume of buffer and washed by centrifugation a further three times. The final pellet was taken up in Tris buffer to a protein concentration of ±10mg/ml and stored at -50° until use.

**Assay of radiolabelled ligand binding**

(A) **To particulate membrane preparations**

100 μl of membrane suspension (100-500 μg protein, depending upon the density of binding sites) was added to 50 mM Tris-HCl, pH 7.8. 1 mM Ascorbic acid, pH 7.6, radiolabelled ligand, (-)-^3^H dihydroalprenolol (50-100 Ci/m mole) and displacing drug or buffer in a total volume of 250μl. All constituents of the assay were dissolved in Tris buffer. Incubations were carried out, under standard conditions, at room temperature (22°) for 30 min, after which the reaction was terminated by the addition of 1ml ice-cold 50 mM Tris-HCl, pH 7.8, followed by rapid filtration through glass-fibre filters (Whatman GF/B) under reduced pressure. The filters were washed with 3 x 5ml of 50 mM Tris-HCl, pH 7.8 (at room temperature), placed in insert vials, into which 3.5ml of a toluene/triton X 100 based scintillation cocktail (Appendix 2 ) or Fisofluor scintillation cocktail (Fisons Limited) was added. Following extraction of the mixture for at least 6 hours the radioactivity associated with the filters was counted in a Packard Tricarb liquid scintillation spectrometer, at 40% counting efficiency.
Non-specific binding of radiolabelled ligand was assessed by parallel incubations of radiolabelled ligand with a suitable concentration of non-radioactive displacing drug (routinely 200µM (-)-isoprenaline, see below) which removed all radiolabelled ligand bound to receptor. Specific binding to beta-adrenoceptors was defined as the difference between total and NSB.

(A) (ii) Non-specific binding (NSB) of $^3$H-DHA: validation of the use of (-)-isoprenaline to define NSB

Non-specific binding of radiolabelled ligands consisted of a number of components - true non-specific binding to tissue, free radiolabelled ligand not effectively removed, and binding to separation materials e.g. filters. For filtration assays the latter two cases were accounted for by optimising the washing procedure which gave the maximum reduction of NSB without reducing the level of specific binding. Three washes of Tris buffer (5ml each) proved to be an effective protocol for the membrane preparations described previously.

Non-specific binding of labelled ligand to tissue preparation has been measured in a number of ways. Appropriate (and inappropriate) concentrations of (+)-propranolol (Vallieres et al, 1979); (-)-alprenolol (Bylund et al, 1976), (Reddy et al, 1979) and (-)-isoprenaline (Nahorski, 1978) have generally been used. The use of high concentrations of antagonists generally resulted in values of $K_D$ for $^3$H-ligand which were high and which suggested displacement of $^3$H-ligand from non-specific binding sites by the competing antagonist.

The abilities of (-)-alprenolol and (-)-isoprenaline to displace $^3$H-DHA binding to non-specific sites were compared in a rat erythrocyte preparation, where NSB had been increased by (i) boiling the membranes and (ii) the use of high ligand concentration. Figure 2:1 demonstrated the absence of competition between isoprenaline and $^3$H-DHA for the non-specific
Displacement of $^3$H-DHA binding to non-specific sites of boiled rat erythrocyte membranes by (-)-isoprenaline ($\triangle$), and (-)-alprenolol ($\blacksquare$). Specific binding sites for $^3$H-DHA were destroyed, and NSB sites increased (approx. 120%) by boiling rat erythrocyte membranes for 5 min. $^3$H-DHA binding to these membranes, in the presence of increasing concentrations of competing drug, was then measured. 5-6 nM $^3$H-DHA was used and incubations were performed for 30 min at 22°C. Filter blanks, which contained all incubants except membranes, were run in parallel. Neither drug affected this blank value. $^3$H-DHA binding to membranes was calculated as per cent of the binding in the absence of displacing drugs. Results shown were the mean of two experiments performed in duplicate.
sites over the entire concentration range tested. (-)-Alprenolol, on the other hand, caused a dose-related displacement of $^3$H-DHA binding to these sites, with an $IC_{50}$ of $\approx 0.1$ mM. The Hill slope factor of (-)-alprenolol for the non-specific sites in boiled erythrocyte membranes ($n_H = 0.255; r = 0.964$) agreed well with the Hill slope of this drug determined for non-specific sites in bovine lung membranes ($n_H = 0.205; IC_{50} = 7 \times 10^{-4}$) (Richardson, 1979). In the latter system, NSB was thought to consist of multiple low affinity sites together with physico-chemical partitioning. It was perhaps not surprising, therefore, that the chemically related drug alprenolol was found to compete with $^3$H-DHA binding to non-specific sites. These results, together with those of Richardson, clearly demonstrated the superiority of using isoprenaline displaceable binding to define specific binding to beta-adrenoceptors.

(A) (iii) **Specific $^3$H-DHA binding to particulate preparations: tissue linearity and pH dependence**

Specific $^3$H-DHA binding increased linearly with tissue concentration over the range 0-300$\mu$g protein for all preparations assayed (using 1.5 nM $^3$H-DHA). Receptor concentrations used for binding studies were well within the linear range and generally bound $<10\%$ of the added ligand.

The effect of pH on $^3$H-DHA binding to rat and rabbit lung preparations was investigated using buffered solutions of Tris-HCl (pH 7-9). Non-specific binding was unaffected by changes in the pH range tested (Figure 2:2). The pH curves of total and specific $^3$H-DHA binding demonstrated a small but consistent increase as the pH was raised towards 9. The maximal specific binding to both preparations occurred at pH 9. However, in view of the susceptibility of catecholamines to oxidation above pH 8, binding assays were conducted at pH 7.8.
Figure 2:2

$^3$H-DHA binding to particulate preparations of rat lung (A) and rabbit lung (B) as a function of pH. Membrane preparations were diluted in Tris-buffer of varying pH values and added to incubations made up in similar buffers. The final pH of the mixture was monitored using a Pye pH meter fitted with a microelectrode. $^3$H-DHA ($\approx 1.5$ nM) binding to the preparations in the absence (total) and presence (NSB) of 200 $\mu$M (-)-isoprenaline was determined and specific binding calculated by subtraction. Incubations were performed under standard conditions at 22°C. Values represented the mean of two experiments performed for each preparation. Experimental determinations were performed in duplicate.
A rather broad pH optimum for specific $^3$H-DHA binding to rat cerebral cortex membranes has also been reported by Bylund et al. (1976). Therefore, binding of this radio-labelled antagonist to beta-adrenoceptors may not exclusively depend on the drug possessing a protonated amine. In contrast, activation of beta-adrenoceptors by adrenaline seemed to be proportional to the protonated, cationic, form of the agonist (Hardman & Reynolds, 1965; Reynolds & Hardman, 1972).

(B) (i) Assay of radiolabelled ligand binding to soluble preparations of beta-adrenoceptors: polyethylene glycol (PEG) precipitation technique

Soluble receptor (100-300μg protein) was added to tubes containing 50 mM Tris-HCl, pH 7.8, 1 mM ascorbic acid; (-)-$^3$H-DHA and displacing drug in a total volume of 500μl. Incubations were carried out at 22°C for 30 minutes, after which the tubes were placed in an ice bath and 0.5ml ice-cold 16% polyethylene glycol 6000, 50 mM Tris-HCl, pH 7.8, 100 mM NaCl was added to each tube. The tubes were vortexed and allowed to stand on ice for 15 min in order to precipitate the solubilised receptor protein. One ml of 8% PEG 6000, 50 mM Tris-HCl, pH 7.8, 100 mM NaCl was added followed by rapid filtration, under reduced pressure, through Whatman GF/B filters. The filters were washed twice with cold 8% PEG 6000, NaCl, Tris buffer and placed in insert vials. Scintillation fluid (3.5ml) was added, and after extraction for 6 hours at 4°C, the radioactivity associated with the filters was counted in a Packard Tricarb liquid scintillation counter at 40% efficiency.

Specific and non-specific binding of $^3$H-DHA was defined as described in section 2:4:2 (A) (i).
(B) (ii) **Charcoal separation technique**

Solubilised membranes (100-300μg protein) were added to tubes containing \(^{3}\text{H}-\text{DHA}\), 50 mM Tris-\(\text{HCl}\), pH 7.8, 1 mM ascorbic acid and competing drugs in a total volume of 500μl. Incubations were performed at 22° for 30 min, and bound and free radiolabelled ligand were separated by the addition of 0.25ml ice-cold mixture of 1.8% Norit GSX charcoal, 0.4% bovine serum albumin in 50 mM Tris-\(\text{HCl}\), pH 7.8. After 21 min at 4° the tubes were centrifuged at 2200g in a Sorvall RC3 for 10 min at 4°. Aliquots (500μl) of supernatant were removed in the cold, and placed in insert vials. Scintillation fluid (4.5ml) was added, the vials shaken and counted in a Packard liquid scintillation counter at an efficiency of 30%.

Specific binding of \(^{3}\text{H}-\text{DHA}\) was defined as previously described (2:4:2 (A) (i)).

(B) (iii) **Comparison of the methods used to determine \(^{3}\text{H}-\text{DHA}\) binding to soluble beta-adrenoceptors**

Accurate determination of radiolabelled ligand binding to soluble beta-adrenoceptors depended on effective separation of bound from free ligand. Two separation methods have been investigated (i) PEG precipitation of \(^{3}\text{H}\)-ligand-receptor complex and (ii) charcoal adsorption of free \(^{3}\text{H}\)-ligand. The former method has found extensive use for measurements of \(^{3}\text{H}\)-ligand binding to solubilised insulin receptors (Cuatrecasas, 1972); beta-adrenoceptors (Haga et al, 1977); Vauquelin et al, 1977; Strauss et al, 1979; Kleinstein et al, 1978); alpha-adrenoceptors (Guellaen et al, 1979); and benzodiazepine receptors (Gavish, 1980). Charcoal separation techniques have been used to assay soluble melatonin receptors (Cohen et al, 1978); \(^{3}\text{H}\)-spiroperidol binding sites (Clement-Cormier et al, 1980); and muscarinic acetylcholine receptors (Gorissen et al, 1981); but have not been applied for the assay of soluble beta-adrenoceptors.
The above methods were compared for their ability to monitor $^3$H-DHA binding sites in digitonin solubilised preparations of rat lung. Protein dilution curves were obtained by measuring the specific binding of $^3$H-DHA to increasing amounts of soluble receptor protein (obtained initially by increasing the volume of soluble preparations used). The curves obtained for soluble rat lung using the charcoal adsorption technique were linear up to 300μg protein (Figure 2:3). In marked contrast, the curves obtained for the same soluble preparation using PEG precipitation deviated markedly from linearity, and appeared to plateau at the higher protein concentrations.

Supplementing all receptor concentrations to include 0.3% digitonin linearised the protein dilution curve, but gave values of $^3$H-DHA binding which were 75% lower than those determined using charcoal separation (Figure 2:3). These results suggested that digitonin concentration greater than approximately 0.06% diminished the capacity of PEG to precipitate the receptor-ligand complex. Furthermore, the inclusion of carrier protein, bovine gamma globulin (200μg) failed to improve the precipitation.

These findings clearly indicated the charcoal adsorption technique as the method of choice, and the need for caution when using the PEG method for soluble preparations containing detergent.

(B) (iv) Optimisation of the charcoal separation technique for the measurement of soluble beta-adrenoceptors

Selective adsorption of free ligand by charcoal has formed the basis for separation techniques which were widely used in radioimmunoassay procedures. The addition of protein or carbohydrate polymer has provided additional improvements, provided the adsorbent/protein ratio was optimum (O'Dell, 1980). Effective separation of free from bound ligand was usually obtained upon subsequent centrifugation, provided the dissociation rate of receptor-ligand complex was slow enough
Figure 2:3
Comparison of PEG and charcoal separation techniques to measure $^{3}$H-DHA binding to digitonin solubilised preparation of rat lung. Specific binding of $^{3}$H-DHA (≈ 1 nM) to increasing amounts of soluble protein was measured using charcoal separation (A) or PEG precipitation (B). Open symbols: increases in soluble protein were obtained by increasing the volume of soluble preparation assayed (0-300 µl), tubes therefore contained 0-0.3% digitonin. Closed symbols: incubation tubes were supplemented with buffered digitonin to maintain a constant proportion of digitonin (0.3%) in each tube. Results were the mean of duplicate determinations of a representative experiment (n = 2).
relative to the total separation time.

Experiments were conducted to optimise the charcoal/protein ratio and to investigate the effect of separation time upon the observed ligand binding to soluble receptors. Figure 2:4 shows the effect of increasing the charcoal/protein ratio, upon the $^3$H-DHA binding (total and non-specific) to a soluble preparation of rat lung. The values of non-specific binding in these systems were equivalent to blanks determined in the absence of soluble receptors. As the charcoal concentration was increased, so the adsorption capacity increased as shown by the declining values of NSB. 'Specific binding', on the other hand, showed a bell-shaped response to increasing charcoal/protein ratios. The optimum ratio from these data appeared to be 4.5:1.

Figure 2:5 demonstrates the effect of increasing the separation time, between charcoal addition and centrifugation, upon the observed $^3$H-DHA binding to soluble preparations of rat lung. Values of non-specific $^3$H-DHA binding to soluble receptors were unaffected by increases in the separation time (up to 15 min). However, the total and specific $^3$H-DHA binding observed after 15 min was approximately 80% of their initial values. This probably resulted from dissociation of $^3$H-DHA from the receptor protein, thereby producing an underestimate of the radiolabelled ligand bound to receptor. Bennett (1978) has calculated that the total separation time of free from bound ligand should be at least 1/7th of the half life of the ligand-receptor complex to avoid significant (> 10%) loss of bound ligand. These studies suggested that, for this system, periods of up to five minutes could elapse before significant (>5%) loss of $^3$H-ligand from receptor occurred.
Figure 2:4

Histogram of $^3$H-DHA binding (total and non-specific) to soluble rat lung using different proportions of charcoal/bovine serum albumin (BSA) to separate free ligand. Soluble rat lung was incubated to equilibrium with $^3$H-DHA ($\pm 30,000$ cpm), and the reaction terminated by the addition of 0.25 ml of cold buffered charcoal/BSA mixtures. The BSA concentration was maintained constant at 0.4%. After 3 min at $4^\circ$ the mixture was centrifuged at 2,200 g for 10 min at $4^\circ$ and 0.5 ml aliquots of supernatant were assayed for radioactivity. Inset - specific binding of $^3$H-DHA to soluble rat lung has been plotted against the charcoal/BSA ratio used. Results shown were the mean $\pm$ SEM of triplicate determinations of a representative experiment ($n = 2$).
Figure 2:5
The effect of increasing the separation time upon the observed $^3$H-DHA binding to a soluble rat lung preparation.

Soluble rat lung preparations were incubated to equilibrium with $^3$H-DHA, charcoal/BSA was added, and the mixture allowed to stand at $4^\circ$ for the indicated times. Following centrifugation, total ($\bullet$), and non-specific binding ($\triangle$) were measured, and specific binding ($\square$) was calculated by subtraction. Blanks ($\triangle$) contained, in the absence of soluble receptor, equivalent amounts of digitonin. The results show the mean ($\pm$ SEM) of triplicate determinations of a representative experiment ($n=2$).
2:5:1 Measurement of adenylate cyclase

Assays were conducted in a total volume of 250μl in tubes containing 80 mM Tris-maleate, pH 7.4; 4 mM MgSO₄; 0.2 mM EGTA; 1 mM 3-isobutyl-1-methylxanthine; 50 μl homogenate (50-400μg protein); 10 μM GTP; and agonist drugs. The reaction was started by the addition of ATP (1 mM final concentration) and the tubes were incubated for 4-6 min at 37°C in a water bath. The reaction was terminated by plunging the tubes into a boiling water bath for 3-4 min, followed by centrifugation at 2200g for 10 min in a Sorvall RC3. The supernatants were removed and aliquots assayed for adenosine 3', 5'-cyclic monophosphate (c'AMP) directly or frozen and stored at -20°C until assays could be performed.

Basal levels of c'AMP were determined by assaying homogenates in the absence of activating drugs. Blank values were determined by adding ATP and immediately placing the tubes in the boiling water bath.

Production of c'AMP was linearly related to the time of incubation for each adenylate cyclase preparations.

2:5:2 (A) (i) Measurement of c'AMP

Cyclic AMP was measured using the protein binding method of Brown et al, (1971). This procedure relied on competition between ³H-c'AMP and c'AMP present in samples for binding to a c'AMP dependent protein kinase derived from bovine adrenals. The binding protein was prepared in bulk by Mrs N. Cook using the method described by Richardson (1979). Dilutions of binding protein were used which gave 30-40% binding of the total added ³H-c'AMP (≈0.5pmoles). The lower limit of detection using these conditions was ≈0.25pmoles c'AMP.

Each assay tube contained 100μl blank, sample, c'AMP standard or 1 mM c'AMP (for NSB); 10μl ³H-c'AMP (≈0.5pmoles, 10-15000cpm) and 'c'AMP assay' buffer (50 mM Tris-HCl, pH 7.4, 4 mM EDTA). The reaction was started by the addition of 200μl of adrenal binding protein in assay
buffer and allowed to proceed to equilibrium at 4° for 3-24hr. The incubation was terminated by the addition of 0.5ml of 2% Norit GSX charcoal/0.2% BSA in assay buffer, followed by centrifugation at 2200g for 10 min at 4° in a Sorvall RC3. The supernatant was decanted, 4.5ml of scintillation fluid (Fisofluor) added and the radioactivity counted using scintillation spectrometry.

2:5:2 (A) (ii) Calculation of results

Cyclic AMP values were calculated using a general non-linear, least squares, curve fitting programme (Richardson, 1979), providing the best estimates of sample c'AMP concentrations with 95% confidence limits. This programme was based on the logistic transformation of dose response relationships (De Lean et al., 1978) which has found wide application for radioreceptor assays and radioimmunoassays.

The general form of the function was expressed as:

\[ Y = \frac{a - d}{1 + \left(\frac{x}{c}\right)^b} + d \]

where \( Y \) is response; \( x \) the arithmetic dose; \( a \) the response when \( x = 0 \); \( d \) the response for infinite dose; \( c \) is the \( \text{ED}_{50} \) i.e. the dose resulting in half maximal response (between \( a \) and \( d \)); and \( b \) is the slope of the logit-log plot (slope factor).

2:6 Measurement of protein

Protein was determined by the method of Lowry (1951). The final colour occurred as a result of (i) the biuret reaction of protein with copper ion in alkali and (ii) reduction of the phosphomolybdic-phosphotungstic reagent by the tyrosine and tryptophan present in the treated protein.
Samples and standards were prepared in water. A range of standard concentrations of bovine serum albumin (0-250µg/ml) was prepared, and following treatment with Lowry reagents (Appendix 3), the absorbance was measured at a wavelength of 750nm on a Pye-Unicam SP6-500 spectrophotometer. Sample values of protein concentration were determined from regression lines calculated from the standard curve.

Protein determinations of soluble preparations were obtained by reference to a standard curve of bovine serum albumin which had been supplemented with appropriate amounts of digitonin.

2:7 Statistical treatment of results

In all cases, except where indicated, mean values ± standard error of the mean were presented. Significance between groups was tested using Student's 't' test and differences considered significant when probability was <0.05.
CHAPTER 3

Radiolabelled Ligand Binding to Membrane Preparations of Rat Lung
3:1 Introduction

The identification of beta-adrenoceptors in tissue preparations using direct binding studies required the testing of appropriate criteria. Data obtained from intact tissue preparations of beta-adrenoceptors (Furchgott, 1972) and measurements in vitro of adenylate cyclase (Lefkowitz, 1975b) have provided the following criteria which defined beta-adrenoceptor binding sites.

(i) Binding kinetics and saturability: It should be expected that binding of ligand to beta-adrenoceptors would be at least as fast as the rate of activation of the effector system, adenylate cyclase, and the binding should be rapidly reversible. The binding of radiolabelled ligand to receptor should show saturability in view of the finite (and usually small) number of receptors present.

(ii) Tissue distribution: beta-adrenoceptor binding sites should be located in tissues or upon cells which are pharmacologically or biochemically responsive to catecholamines. In addition, these sites should have a distribution which parallels catecholamine stimulated adenylate cyclase.

(iii) Pharmacological Specificity: Binding to putative beta-adrenoceptors should reflect all the structure-activity relationships which characterised beta-adrenoceptor mediated responses. Furthermore, there should be a reasonable correlation between the affinity constants of drugs for beta-adrenoceptors determined using pharmacological techniques and direct binding measurements. Such a relationship might not be expected for agonists, however, in view of the problems of determining agonist efficacy (see Jenkinson, 1973).

Since agonist and antagonist interactions at beta-adrenoceptors showed stereoselectivity, binding sites should show marked selectivity for the (-) isomers of beta-adrenoceptor agents.
The catecholamines adrenaline and noradrenaline should be equiactive at beta$_1$-adrenoceptors and noradrenaline should be significantly weaker than adrenaline at beta$_2$-adrenoceptors (Lands et al, 1967a). "Cardio-selective" antagonists should demonstrate greater affinities for tissue preparations containing beta$_1$-adrenoceptors.

These criteria will be used to define and characterise the specific $^3$H-DHA binding sites of rat lung homogenates, which have been shown, using biochemical methods, to contain beta$_2$-adrenoceptors (Burges and Blackburn, 1972). The possible co-existence of beta$_1$- and beta$_2$-adrenoceptors in this tissue, with the latter subtype predominating, was suggested following the use of direct radiolabelled ligand binding methods (Barnett et al, 1978). This technique delineated beta-adrenoceptor heterogeneity in tissue homogenates using selective antagonists in competition with non-selective ligand $^3$H-DHA. At the onset of these studies such methods had been used to study the beta-adrenoceptors present in lung and brain (Rugg et al, 1978; Nahorski, 1978). However, in order to quantify the relative proportion of high and low affinity sites observed using this procedure, an effective graphical or mathematical analysis of the data was required. The direct graphical analysis of non-linear Hofstee plots used originally (Barnett et al, 1978) has subsequently been suggested to result in a marked overestimation of the proportion of high affinity sites, and to introduce considerable error in the IC50 values of drugs for these sites (Hancock et al, 1979).

This study was designed to confirm and extend those original findings by using recently synthesised beta-adrenoceptor agents of high selectivity, and by analysis of binding data by computerised curve fitting.
3:2 Results
3:2:1 Binding of $^3$H-DHA to rat lung membranes - Saturability

Specific $^3$H-DHA binding to rat lung membranes (defined as the binding displaceable by 200μM(-) isoprenaline) was saturable (Figure 3:1), whereas non-specific binding increased linearly with $^3$H-DHA concentration. Scatchard analysis of specific binding data revealed a homogeneous population of binding sites with an equilibrium dissociation constant ($K_D$) of 0.35(± 0.02) nM, (n=5); and a binding site maxima (Bmax) of 430 (± 17) fmoles/mg protein. Hill analysis (not shown) revealed an absence of co-operative interactions since the Hill co-efficient ($n_H$) approached unity, $n_H = 1.06$ (± 0.04).

3:2:2 Kinetics of $^3$H-DHA Binding

The specific binding of $^3$H-DHA to rat lung membranes was rapid and reversible (Figure 3:2), with a $t_{1/2}$ (time for half-maximal binding) for association of 1.5 min at 22°. The second order association rate constant, determined using $^3$H-DHA concentrations of 0.9 - 1.3 nM, was 0.262 (± 0.01) nM$^{-1}$ min$^{-1}$ at 22° (n = 5). The mean dissociation rate constant obtained at 22° was 0.069 (± 0.004) min$^{-1}$, (n = 3). The dissociation constant calculated from the fraction $K_1/K_1$ was 0.26nM, in excellent agreement with that obtained from equilibrium experiments.

3:2:3 Pharmacological Specificity

(A) Agonists

Competition experiments, in which adrenergic agonists were used to displace specific $^3$H-DHA binding were performed in order to characterize the high affinity $^3$H-DHA binding sites on rat lung membranes. Figure 3:3 shows displacement curves obtained for a series of synthetic and natural beta-adrenoceptor agonists. The order of potency of these agents was

(-) isoprenaline > (-) adrenaline > (+) salbutamol > isoetharine ≥ (-) noradrenaline, which was suggestive of a beta$_2$-adrenoceptor (Lands et al.,
Figure 3:1
Specific binding of $^3$H-DHA to rat lung membranes. Membranes were incubated with increasing concentrations of $^3$H-DHA for 30 min at 22°C, and binding was determined as described in Methods. Specific binding was defined as that proportion of total binding which was displaceable by 200 μM (−)-isoprenaline, which represented approximately 90% at 1 nM $^3$H-DHA. (A) Binding data plotted directly. (B) Scatchard analysis of data shown in (A). The results shown were representative of five such experiments performed in triplicate.
Figure 3:2
Kinetic analysis of $^3$H-DHA binding to rat lung membranes. (A) Membrane preparations were incubated at $22^\circ$C with 1.3 nM $^3$H-DHA and specific binding was measured as a function of time (■). After 30 min, 200 μM isoprenaline was added and the decrease in specific binding was measured at timed intervals (□). (B) The pseudo first-order plot of specific $^3$H-DHA binding. Where $X_{eq}$ was the amount of specific binding at equilibrium and $X$ was the amount bound at time 't'. The slope of this line was the observed rate constant for the pseudo first-order reaction ($K_{OBS}$). (C) The first-order plot of the dissociation reaction where the slope of the line ($K_{-1}$) equalled the dissociation rate constant. The binding site concentration for these experiments was 0.12 nM. These data were representative of 3-5 experiments performed in duplicate.
Figure 3:3

Inhibition of specific $^3$H-DHA binding to rat lung membranes by (-)-isoprenaline (○), (-)-adrenaline (○), (±)-salbutamol (△), (±)-isoetharine (△) and (-)-noradrenaline (□). $^3$H-DHA, 1.5 nM, and the agonist under investigation (in buffered 1 mM ascorbic acid to prevent oxidation), were incubated with membranes for 30 min at 22°C, and specific binding was assessed as described previously. The data represented mean curves of 3-8 experiments conducted in duplicate. Standard errors of the mean (SEM) were <10% and have been excluded for clarity.
1967a; Daly and Levy, 1979). Values of inhibition constants (Ki) for these agents were calculated from displacement data by the method of Cheng and Prusoff (1973), (Table 3:1).

(B) Antagonists

Specific binding to rat lung membranes was stereoselective since the (-) isomer of propranolol was considerably more potent than the (+) isomer by approximately two orders of magnitude (Table 3:1).

The non-selective beta-adrenoceptor antagonists (-) timolol, (-) alprenolol and (-) propranolol displaced $^3$H-DHA binding with high affinities and in a manner indicating binding to a homogeneous population of sites with binding governed by law of mass action.

The potent alpha-adrenoceptor antagonist phentolamine was a weak inhibitor of $^3$H-DHA binding (Table 3:1).

The availability of beta-adrenoceptor antagonists which showed considerable degrees of selectivity for beta$_1$- or beta$_2$-adrenoceptors allowed further characterisation of the specific binding sites in rat lung. From overall displacement data, inhibition constants (Ki) of these agents were calculated, and pKi's compared to literature pA$_2$ values determined using guinea pig trachea. Table 3:2 showed that binding data for the antagonists correlated well with pharmacologically derived data using intact lung tissue, ($r = 0.968$, slope = 0.85). Since guinea pig trachea contained a predominance of beta$_2$-adrenoceptors (Furchgott and Wakade, 1975) these results strongly suggested a beta$_2$-adrenoceptor classification for the specific $^3$H-DHA binding sites of rat lung membranes.

The competition curves of highly selective beta-adrenoceptor antagonists obtained for rat lung membranes were shallow, with overall pseudo Hill coefficients <1; and inflections especially marked at low (with beta$_1$-selective agents) or high (with beta$_2$-selective agents) drug concentrations (Figure 3:4, Figure 3:5). Agents with less degrees of selectivity
Table 3.1

Inhibition constants of drugs for specific (-)-$^3$H-DHA binding sites on rat lung membranes.

<table>
<thead>
<tr>
<th>Drug</th>
<th>$K_i$ (M)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Beta-adrenoceptor agonists</strong></td>
<td></td>
</tr>
<tr>
<td>(-)-Isoprenaline</td>
<td>$2.7 (\pm 0.3) \times 10^{-8}$</td>
</tr>
<tr>
<td>(-)-Adrenaline</td>
<td>$2.5 (\pm 0.3) \times 10^{-7}$</td>
</tr>
<tr>
<td>(-)-Noradrenaline</td>
<td>$1.4 (\pm 0.2) \times 10^{-6}$</td>
</tr>
<tr>
<td>(+)-Salbutamol</td>
<td>$6.6 (\pm 1.3) \times 10^{-7}$</td>
</tr>
<tr>
<td>(+)-Isoetharine</td>
<td>$9.7 (\pm 0.8) \times 10^{-7}$</td>
</tr>
<tr>
<td><strong>Beta-adrenoceptor antagonists</strong></td>
<td></td>
</tr>
<tr>
<td>(-)-Timolol</td>
<td>$4.6 (\pm 0.4) \times 10^{-10}$</td>
</tr>
<tr>
<td>(-)-Propranolol</td>
<td>$6.9 (\pm 0.5) \times 10^{-10}$</td>
</tr>
<tr>
<td>(+)-Propranolol</td>
<td>$5.5 (\pm 0.2) \times 10^{-8}$</td>
</tr>
<tr>
<td>(-)-Alprenolol</td>
<td>$5.4 (\pm 0.4) \times 10^{-10}$</td>
</tr>
<tr>
<td><strong>Alpha-adrenoceptor antagonists</strong></td>
<td></td>
</tr>
<tr>
<td>Phentolamine</td>
<td>$7.2 (\pm 0.6) \times 10^{-5}$</td>
</tr>
</tbody>
</table>

*IC$_{50}$ values were obtained from Hill plots and $K_i$ values determined using the equation of Cheng & Prusoff. Results were derived from 3-8 experiments performed in duplicate.
Table 3:2
Comparison of $pA_2$ values for beta-adrenoceptor antagonists obtained using agonist-stimulated guinea-pig tracheal chains, with $pK_i$ values determined by competition studies of $^3$H-DHA binding to rat lung membranes.

<table>
<thead>
<tr>
<th>Drug</th>
<th>$pA_2$ values</th>
<th>Reference No.</th>
<th>Mean $pA_2$ values</th>
<th>$pK_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>$(\pm)$-Propranolol</td>
<td>8.25</td>
<td>7.90</td>
<td>8.65</td>
<td>8.27</td>
</tr>
<tr>
<td>$(\pm)$-Practolol</td>
<td>4.87</td>
<td>5.08</td>
<td>-</td>
<td>4.98</td>
</tr>
<tr>
<td>$(\pm)$-Atenolol</td>
<td>5.57</td>
<td>5.80</td>
<td>-</td>
<td>5.69</td>
</tr>
<tr>
<td>$(\pm)$-Metoprolol</td>
<td>6.06</td>
<td>6.14</td>
<td>-</td>
<td>6.10</td>
</tr>
<tr>
<td>$(\pm)$-Betaxolol</td>
<td>-</td>
<td>6.18</td>
<td>-</td>
<td>6.18</td>
</tr>
<tr>
<td>$(\pm)$-ICI 118.551</td>
<td>-</td>
<td>-</td>
<td>8.69</td>
<td>8.69</td>
</tr>
</tbody>
</table>

Inhibition constants ($K_i$) for antagonists were calculated from overall IC$_{50}$ determinations using the equation of Cheng and Prusoff. $pA_2$ values (the dose of drug at which twice the concentration of agonist was required to produce a response equivalent to controls) were obtained for guinea-pig trachea from the following literature sources: Reference No. 1: Harms (1976); 2: Boudot et al. (1979); 3: O'Donnell & Wanstall (1980).
Figure 3:4
Inhibition of specific $^3$H-DHA binding to rat lung membranes by beta$_1$-selective antagonists betaxolol (○), metoprolol (●), atenolol (△) and practolol (■), and the non-selective antagonist timolol (▲). $^3$H-DHA, 1.5 nM, and the antagonist under investigation were incubated for 30 min at 22°C, and specific binding was measured as previously described. The inhibition curves represented the mean (± SEM) of 3-9 experiments performed in duplicate.
Figure 3:5
Inhibition of specific $^3$H-DHA binding to rat lung membranes by beta$_2$-selective agents ICI 118.551 (▲), procaterol (□), and H35/25 (■), and the non-selective antagonist timolol (▲). Experimental conditions were identical to those described in Figure 3:4. The inhibition curves represented the mean (± SEM) of 3-8 experiments performed in duplicate.
(e.g. H35/25, metoprolol) generated curves which deviated less from the theoretical curves expected for binding to homogeneous sites according to mass action law.

Inhibition curves of non-selective and selective beta-adrenoceptor antagonists were analysed using the method of Hofstee and Hill (Figure 3:6). In contrast to the straight line plots obtained for non-selective agents, selective drugs produced curvilinear Hofstee and non-linear Hill plots. This finding suggested heterogeneity of the $^3$H-DHA binding sites on rat lung membranes. From Hofstee plots of highly beta$_1$- or beta$_2$-selective drugs it was apparent that the membranes contained $\approx$ 80% of sites having high affinity for beta$_2$-selectivity agents and $\approx$ 20% of sites having high affinity for beta$_1$-selective agents.

Analysis of inhibition data by computerised iterative curve fitting indicated best fit parameters for a two site, non interacting, model with binding to both sites governed by mass action law (See Appendix 4). The relative proportions of the two sites were 80(±1)% for the site having high affinity for beta$_2$-selective agents (beta$_2$-adrenoceptors) and (20(±1)% for the site having high affinity for beta$_1$-selective agents (beta$_1$-adrenoceptors). Table (3:3).

3:2:4 Selective inhibition of beta-adrenoceptor binding sites on rat lung membranes

In order to examine further these heterogeneous binding sites, lung membranes were incubated with a concentration of the beta$_1$-selective antagonist (+) atenolol which, at 1.5 nM $^3$H-DHA, was predicted to occupy 96% of beta$_1$ sites and 14% of beta$_2$ sites. The displacement of specific $^3$H-DHA binding from the membranes by increasing concentrations of the beta$_2$-selective partial agonist (+) procaterol was then determined. Control membranes produced displacement curves which had a distinct inflection at high drug concentrations, and which analysed as non-linear Hofstee and Hill plots. (Figure 3:7).
Analysis of displacement curves obtained for antagonists in competition with $^3$H-DHA binding to rat lung membranes. (A) Hofstee plots of inhibition data shown in Figure 3:4 for the non-selective beta-adrenoceptor antagonist timolol (▲), and the beta1-selective antagonists atenolol (●) and metoprolol (□). (B) Hill plots of the same data.
Table 3.3

Proportions of beta-adrenoceptor subtypes in rat lung membranes, and affinities of selective agents for these sites.

<table>
<thead>
<tr>
<th>Agent</th>
<th>$K_i$ (nM)</th>
<th>Beta$_2$-component</th>
<th>Beta$_1$-component</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta$_1$-selective</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(±)-Atenolol</td>
<td>5,800 (80%)</td>
<td>40 (20%)</td>
<td></td>
</tr>
<tr>
<td>(±)-Practolol</td>
<td>46,500 (78%)</td>
<td>341 (22%)</td>
<td></td>
</tr>
<tr>
<td>(±)-Betaxolol</td>
<td>201 (80%)</td>
<td>1.9 (20%)</td>
<td></td>
</tr>
<tr>
<td>(±)-Metoprolol</td>
<td>450 (83%)</td>
<td>21 (17%)</td>
<td></td>
</tr>
<tr>
<td>Beta$_2$-selective</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(±)-Procaterol</td>
<td>41 (76%)</td>
<td>4,100 (24%)</td>
<td></td>
</tr>
<tr>
<td>(±)-ICI 118.551</td>
<td>1.7 (83%)</td>
<td>107 (17%)</td>
<td></td>
</tr>
</tbody>
</table>

Mean proportion of sites

| Beta$_2$: 80 (± 1) % | Beta$_1$: 20 (± 1) % |

Inhibition curves of selective agents were analysed by iterative curve fitting using a two-site non-interacting model, and $K_i$ values for the two sites were determined using the Cheng & Prusoff equation. Percentage values in parenthesis represented the proportion of the two sites determined from averaged inhibition curves of 3-9 experiments performed in duplicate. SEM's were <10%.
Figure 3:7
Inhibition of specific $^3$H-DHA binding to rat lung membranes by ($\pm$)-procaterol in the absence (▲) and presence (△) of 5 μM ($\pm$)-atenolol. Rat lung membranes were incubated for 15 min at 22°C with ($\pm$)-atenolol or buffer and added to tubes containing $^3$H-DHA (1.5 nM), and increasing concentrations of ($\pm$)-procaterol, yielding a final concentration of atenolol of 5 μM. After 30 min at 22°C specific $^3$H-DHA binding was measured as previously described.

(A) Inhibition data plotted directly. Hofstee (B) and Hill (C) plots of the data presented in (A). Results represented the mean (± SEM) of 4 (control) and 8 (atenolol treated) experiments performed in duplicate.
In contrast, the inhibition curve for procaterol in the presence of atenolol was shifted to the left and was clearly closer in shape to the theoretical curve expected for a homogeneous receptor population with binding governed by mass action law. Analysis of these data by Hofstee plots (Figure 3:7;B) and iterative curve fitting revealed the presence of approximately 4% of total specific binding which had low affinity for procaterol (beta_1-adrenoceptor), as compared with 23% in control membranes. The Hill plot (Figure 3:7;C) was a sensitive indicator of the small degree of heterogeneity still present in these membranes (n_H overall = 0.91).

3:2:5 Comparison of methods to analyse binding data

Previous workers (Barnett et al., 1978; Nahorski, 1978; Minneman et al., 1979b) have used the Hofstee plot to determine the proportions of beta_1 and beta_2 sites in membrane preparations and the affinities of drugs for each site. Graphical extrapolation of Hofstee plots (Rugg et al., 1978) and computer assisted iterative analysis of these plots (Minneman et al., 1979b) were both subject to certain limitations which were implicit in the data transforms (Hancock et al., 1979).

In order to resolve the possible errors involved in the former analysis, displacement curves of beta-adrenoceptor antagonists with different degrees of selectivity have been analysed by Hofstee plots and iterative curve fitting of the inhibition data. The results shown in Table 3:4 demonstrated that for atenolol and ICI 118551 (60-150 fold selective), the IC50 values and proportions of sites obtained by both methods were in reasonable agreement, although Hofstee analysis of data for atenolol did result in slightly higher proportions of the minor site. These results indicated that Hofstee analysis of data for such drugs gave a reasonable estimate of the two binding sites. However, the serious limitations associated with graphical analysis of Hofstee plots were clearly shown for the data obtained for metoprolol (10-20 fold beta_1-selective). This agent produced less marked curvilinear Hofstee plots which resulted in considerable overestimates of the proportion of beta_1 sites and approximately...
Table 3:4
IC₅₀ values of drugs for beta₁ and beta₂ sites of rat lung membranes, and the proportions of each site.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Hofstee analysis</th>
<th>Iterative curve fitting of inhibition curves</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Beta₂-site</td>
<td>Beta₁-site</td>
</tr>
<tr>
<td></td>
<td>Beta₁-site</td>
<td>Beta₂-site</td>
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<tr>
<td>Beta₁-site</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta₂-site</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(±)-ICI 118.551</td>
<td>7.1 x 10⁻⁹</td>
<td>8.5 x 10⁻⁹</td>
</tr>
<tr>
<td></td>
<td>4.9 x 10⁻⁷</td>
<td>5.3 x 10⁻⁷</td>
</tr>
<tr>
<td></td>
<td>81%</td>
<td>83%</td>
</tr>
<tr>
<td></td>
<td>18%</td>
<td>17%</td>
</tr>
<tr>
<td>(±)-Atenolol</td>
<td>3.1 x 10⁻⁵</td>
<td>3.1 x 10⁻⁵</td>
</tr>
<tr>
<td></td>
<td>2.4 x 10⁻⁷</td>
<td>2.1 x 10⁻⁷</td>
</tr>
<tr>
<td></td>
<td>74%</td>
<td>80%</td>
</tr>
<tr>
<td></td>
<td>24%</td>
<td>20%</td>
</tr>
<tr>
<td>(±)-Metoprolol</td>
<td>6.5 x 10⁻⁶</td>
<td>2.6 x 10⁻⁶</td>
</tr>
<tr>
<td></td>
<td>3.2 x 10⁻⁷</td>
<td>1.2 x 10⁻⁷</td>
</tr>
<tr>
<td></td>
<td>64%</td>
<td>83%</td>
</tr>
<tr>
<td></td>
<td>43%</td>
<td>17%</td>
</tr>
</tbody>
</table>

The mean of 3-9 inhibition curves were analysed by the method of Hofstee (see Rugg et al, 1978) and iterative curve fitting of the inhibition data using a two site non-interacting model. IC₅₀ values (M) and proportions of high and low affinity sites were indicated. The proportion of sites determined by Hofstee analysis represented interceptions on the ordinate (per cent inhibition of specific binding) of the best fit line determined by linear regression analysis.
3-fold errors in IC50 values.

Model inhibition curves have been drawn for competitor drugs which possessed increasing degrees of selectivities for the minor site of a two binding site, non-interacting system, comprising 80% low and 20% high affinity sites (Figure 3:8). The error involved in quantifying these sites by Hofstee analysis was calculated for selective drugs. Both Hofstee and Hill plots of these data (Figure 3:8, B and C) clearly showed the presence of heterogeneous binding sites. However, a good quantification of these sites was only possible using data for the 1000 fold selective drug (20.5% high affinity sites estimated). Hofstee analysis of data for a drug with 100 fold selectivity gave a 15% overestimation of the fraction of high affinity sites (23% sites determined). This error was similar to that determined experimentally for atenolol (See Table 3:4).

For the converse system, with 80% high and 20% low affinity sites, Hofstee analysis of model inhibition data for a 100 fold selective drug resulted in only a 1% overestimation of the high affinity sites (81.5% sites observed).

Hill plots could also be used to estimate the fraction of binding sites by using determinations of the minimum values of the Hill slope (See Birdsall et al, 1978). However, the accuracy of this graphical method was again dependent on the degree of selectivity of the drug (Figure 3:8). Furthermore, drugs with poor degrees of selectivity produced Hill plots which, within experimental error, approximated to a straight line with low overall slope, suggestive of negative co-operative interactions (See also the Hill plot for metoprolol, Figure 3:6).

3:3 Discussion

A large body of pharmacological and biochemical evidence has accumulated which indicated that beta-adrenoceptors of lung and tracheal tissue differ substantially from those present in heart (Lands et al, 1967a; Furchgott, 1972; Lefkowitz, 1975a; U Pritchard et al, 1978).
Figure 3:8
Model binding curves for competitor drugs with increasing degrees of selectivity for the high affinity site of a two-site system. (A) Occupancy data calculated for drugs binding to a two-site system consisting of 20% high affinity, 80% low affinity sites. The IC$_{50}$ of drugs for the low affinity site was maintained constant at $2 \times 10^{-6}$ M, and for the high affinity site IC$_{50}$'s were: $2 \times 10^{-9}$ M (a), $2 \times 10^{-8}$ M (b), $2 \times 10^{-7}$ M (c), $2 \times 10^{-6}$ M (d). (B) Hofstee plots of the same data, where I was the % occupancy and C was the concentration of competitor drug. Abscissae scales differed for curves a-d and were indicated. (C) Hill plots of the data presented in (A).
The beta-adrenoceptors regulating cardiac function have been termed beta₁ whilst those mediating bronchodilation beta₂. There is, however, some controversy regarding the absolute number of beta-adrenoceptor subtypes and whether they can be differentiated on a strict organ-specific basis (Boissier et al., 1971; Barrett, 1973). Thus, beta-adrenoceptors may co-exist in tissues, and perhaps subserve the same physiological functions (Carlsson et al., 1972).

Support for this hypothesis has come from pharmacological studies of lung tissue (Furchgott and Wakade, 1975; Zaagsma et al., 1978; and O'Donnell and Wanstall, 1979a) and from direct binding studies of a number of central and peripheral tissues (Barnett et al., 1979; Nahorski 1978; Minneman et al., 1979b; Hedberg et al., 1980). The technique of radiolabelled ligand binding has been particularly productive since the preparations which were used allowed optimum accessibility of drugs for receptor sites. Problems of differential rates of drug equilibration with receptor and non-receptor phases in intact tissue preparations may have contributed to the early findings of apparent multiple beta-adrenoceptor heterogeneity.

The importance of the physicochemical properties of drugs and their bio-availability at the beta-adrenoceptor have been stressed by a number of workers (Coleman and Somerville, 1977; Leclerc et al., 1981). Indeed Leclerc et al. (1981) have questioned the concept of dual beta-adrenoceptors since their binding studies failed to show the expected beta-adrenoceptor selectivity of a number of antagonists.

The present study has used radiolabelled ligand binding techniques to study the ³H-DHA binding sites in homogenates of rat lung. The specific ³H-DHA binding satisfied all the criteria expected for binding to a beta-adrenoceptor. Thus the binding sites were saturable, of high affinity, displayed stereoselectivity, and exhibited a pharmacological specificity which was typical of a beta-adrenoceptor (Ahlquist, 1948).
Kinetic analysis of the binding gave identical results to those determined by equilibrium measurements.

The relative potencies of a series of agonists in displacing $^3$H-DHA binding were indicative of a beta$_2$-adrenoceptor classification as proposed by Lands et al., (1967a). Further support for this classification came from the results of testing beta-adrenoceptor antagonists which have been reported to show considerable beta$_1$- or beta$_2$-selectivities in intact preparations. Thus, the overall affinities of these drugs for the specific binding sites of rat lung membranes correlated well with their affinities determined for intact preparations of guinea pig trachea, which contained a predominance of beta$_2$-adrenoceptors (Furchgott and Wakade, 1975).

In confirmation of previous findings (Rugg et al., 1978), the use of selective beta-adrenoceptor agents resulted in displacement curves which did not analyse as binding to a homogeneous class of sites according to mass action law. Hofstee and Hill plots of these data were curvilinear, which suggested negative co-operative interactions or heterogeneous binding sites for these drugs.

The former explanation could be excluded for the following reasons:

(i) Only agents which demonstrated selectivity for beta$_1$- or beta$_2$-adrenoceptors showed this behaviour, and the degree of curvilinearity of the Hofstee plot correlated with the agents' selectivity.

(ii) For membrane preparations which contained homogeneous beta-adrenoceptor populations, selective agents produced linear Hofstee plots, with Hill slope factors close to unity (See Chapter 4).

The presence of heterogeneous binding sites in the membranes, equivalent to beta$_1$ and beta$_2$-adrenoceptors was suggested by the following findings:-
(i) When displacement curves for selective drugs were analysed by iterative curve fitting to a two site non-interacting model, the fraction of high and low affinity sites determined using beta\textsubscript{1}-selective drugs was the exact inverse of that determined by beta\textsubscript{2}-selective drugs.

(ii) The affinity constants of selective drugs for the beta\textsubscript{2}-adrenoceptor component of rat lung correlated extremely well with their affinities determined for the homogeneous beta\textsubscript{2}-adrenoceptor of rat erythrocyte, $r = 0.99$, slope = 1.00 (See Chapter 4).

(iii) The ratio of affinities of drugs for the high and low affinity binding sites of rat lung correlated well with their known selectivities determined with intact tissue preparations of beta\textsubscript{1} and beta\textsubscript{2}-adrenoceptors. Thus, the recently synthesised beta\textsubscript{2}-adrenoceptor antagonist ICI 118,551 exhibited 60 fold beta\textsubscript{2}-adrenoceptor selectivity using rat lung membranes which was similar to that obtained with isolated pharmacological preparations. (Bilski et al, 1980; O'Donnell and Wanstall, 1980). Similarly betaxolol showed approximately 100 fold beta\textsubscript{1}-adrenoceptor selectivity using rat lung membranes as compared to a 'cardioselectivity' ratio of 220 using \textit{in vitro} tissue preparations (Boudot et al, 1979).

(iv) Occupation of a proportion of the beta-adrenoceptor binding sites of rat lung membranes by an antagonist selective for beta\textsubscript{1}-adrenoceptors transformed a system with heterogeneous binding sites into a system which was almost homogeneous. This finding suggested furthermore, that beta\textsubscript{1} and beta\textsubscript{2}-adrenoceptors co-existed in rat lung membranes in a non-interacting manner.
This study utilised a number of methods to analyse inhibition data, based on graphical and computer-assisted methods. Previous studies of the beta-adrenoceptor binding sites in rat lung membranes used Hofstee analysis of inhibition curves, which suggested the presence of 75% beta$_2$- and 25% beta$_1$-adrenoceptors (Rugg et al., 1978). The pitfalls associated with this method of analysis have been discussed previously (Hancock et al., 1979; Richardson, 1979; Minneman and Molinoff, 1980), and the errors involved in its use have been evaluated in this study.

Results obtained for model systems, and confirmed by analysing experimental data of beta$_1$-selective agents, suggested that Hofstee analysis over-estimated the proportion of minor (beta$_1$) site by approximately 15%. Iterative curve fitting of inhibition data gave values for the ratio of beta$_1$/beta$_2$ sites of 20:80. Minneman et al. (1979b) have used the radioligand $^{125}$I-HYP, and confirmed the presence of both adrenoceptor subtypes in rat lung. These workers employed iterative curve fitting of Hofstee plots to analyse inhibition data, which suggested the presence of approximately 85% beta$_2$, and 15% beta$_1$-adrenoceptors.

Clearly, the most accurate determinations of the proportions of sites in a heterogeneous binding system have been obtained by computer modelling of the original ligand binding data. A number of programmes have been developed which analysed per cent inhibition of radioligand binding and provided best fit estimates of the data to model systems. Birdsall et al., 1978; Richardson 1979; U. Pritchard et al., 1980).

More recently, an improved method of computer modelling of untransformed binding data, based on mass action law principles, was introduced which incorporated the binding characteristics of both radioligand and competitor (Hancock, 1979; Munson and Rodbard, 1980). This analytical procedure, which was derived from the mathematical theory proposed by Feldman (1972), did not require the application of the Cheng and Prusoff equation to determine the inhibition constants; it could be applied
in the case of a selective radioligand; and it allowed for depletion of radioligand. The problem of radioligand depletion, has been reported to have profound effects on the delineation of receptor-heterogeneity in heterogeneous binding systems (Wells et al., 1980).
CHAPTER 4

Beta-adrenoceptors of Rat Erythrocytes and Reticulocytes -
A Comparison with Heterogeneous Rat Lung Beta-Adrenoceptors
4:1 Introduction

Direct receptor labelling techniques have successfully demonstrated the coexistence of beta₁ and beta₂-adrenoceptors in a number of tissues. (Rugg et al, 1978; Nahorski, 1978; Nahorski et al, 1979a). This study was undertaken to determine whether beta-adrenoceptor subtypes could coexist in a single population of cells, and rat erythrocytes were selected as a convenient source of mammalian single-cell types which contained beta-adrenoceptors (Sheppard and Burghardt, 1969).

Rat erythrocytes and its precursor cell type, reticulocytes, have been useful model cells for studying beta-adrenoceptor-effector systems (Palm et al, 1973). Indeed, comparative studies of erythrocytes and reticulocytes have provided useful information on the coupling of beta-adrenoceptor to adenylate cyclase (Charness et al, 1976; Bilezikian et al, 1977 a, b; Beckman and Hollenberg, 1979; Limbird et al, 1980a). Thus, maturation of the rat reticulocyte has been shown to result in diminished beta-adrenoceptor binding sites; a marked loss of guanine nucleotide and catecholamine sensitive adenylate cyclase activities; and a parallel loss of guanine-nucleotide regulatory protein. These observations suggested that reticulocyte maturation was associated with an uncoupling of the beta-adrenoceptor from adenylate cyclase.

The beta-adrenoceptor of these cells has previously been classified using adenylate cyclase measurements (Bilezikian et al, 1977, a) and ligand binding techniques (Charness et al, 1976; Beckman and Hollenberg, 1979) as a beta₂-adrenoceptor. However, this classification differed from that suggested by Gauger et al (1975), with evidence not altogether dissimilar to that above, but lacking the use of highly selective antagonists. This apparent discrepancy could have been due to heterogeneous beta-adrenoceptor binding sites on these cells. Indeed,
during the course of these studies a preliminary report was published which suggested the presence of both beta-adrenoceptors on reticulocytes in the ratio beta_2/beta_1 of approximately 3 (Kaiser et al., 1979).

The study to be described used highly selective beta-adrenoceptor antagonists to determine the degree of beta-adrenoceptor heterogeneity of the specific $^3$H-DHA binding sites on membrane preparations of rat erythrocytes and reticulocytes. These investigations were aimed at characterising and comparing the beta-adrenoceptors on both cell types, and correlating the results with those determined previously for the heterogeneous sites on rat lung membranes.

4:2 Results

4:2:1 $^3$H-DHA binding to rat erythrocyte and reticulocyte membranes:

Specific $^3$H-DHA binding to red cell membranes from control (erythrocytes) and phenylhydrazine treated rats (reticulocytes) was saturable (Figure 4:1). Scatchard analysis of specific binding data revealed a homogeneous population of binding sites with an equilibrium dissociation constant ($K_D$) of 0.24 (+ 0.03) nM (n=5) for rat erythrocyte membranes, and 0.25 (+ 0.02) nM (n=3) for rat reticulocyte membranes.

Figure 4:1 shows that, although the affinities of $^3$H-DHA for the binding sites of both preparations were similar, the total number of specific binding sites was three to four fold higher in reticulocytes. The mean $B_{\text{max}}$ was 760 (+9) fmoles/mg protein for reticulocytes and 195 (+8) fmoles/mg protein for erythrocytes (n=5). Analysis of these saturation data by the method of Hill revealed the absence of cooperative interactions (Hill coefficients equalled 0.98 (+0.03) erythrocytes; and 0.97 (+0.04) reticulocytes).
Specific binding of $^3$H-DHA to rat erythrocytes (△) and reticulocytes (▲) membranes.

Cell membranes were incubated with increasing concentrations of $^3$H-DHA for 30 min at 22°C, and specific binding was determined as previously described. The data shown were representative of five such experiments performed in triplicate. (A) The specific binding data plotted directly. (B) Scatchard analysis of data presented in (A). From these data, values of $B_{\text{max}}$ and $K_D$ were: erythrocyte $K_D = 0.26$ nM, $B_{\text{max}} = 187$ fmoles/mg protein; reticulocyte $K_D = 0.23$ nM, $B_{\text{max}} = 748$ fmoles/mg protein. (C) Hill plot of the same data. Hill coefficients ($n_H$) were calculated from the slope of the lines: erythrocyte $n_H = 0.98$, $r = 0.998$; reticulocyte $n_H = 0.97$, $r = 0.997$. 

Figure 4:1
4:2:2 Kinetics

Specific binding of $^3$H-DHA to rat erythrocytes and reticulocytes was rapid and reversible. Using $^3$H-DHA concentrations of 1.2-1.6nM, $t_1$ values (time for half maximal binding) for association were 1.5 ($\pm$0.2) min for erythrocytes, and 1.3 ($\pm$0.2) min for reticulocytes at 22°C. Association and dissociation rate constants for $^3$H-DHA were calculated using methods described in Appendix 4. The second order association rate constant ($k_+1$) and dissociation rate constant ($k_-1$), calculated for rat erythrocyte membranes at 22°C were: 0.22 ($\pm$ 0.03) nM$^{-1}$ min$^{-1}$; and 0.047 ($\pm$ 0.010) min$^{-1}$ respectively, (n=3). The equivalent values for rat reticulocyte membranes were: 0.197 ($\pm$ 0.014) nM$^{-1}$ min$^{-1}$; and 0.068 ($\pm$ 0.003) min$^{-1}$, (n=3). The dissociation constants calculated from the fraction of $k_-1/k_+1$ were 0.21 nM (erythrocytes) and 0.34 nM (reticulocytes).

4:2:3 Pharmacological Specificity

(A) Agonists

Beta-adrenoceptor agonists were compared for their ability to compete with specific $^3$H-DHA binding to membranes of rat erythrocytes and reticulocytes. Figure 4:2 shows the inhibition curves produced by the full agonists isoprenaline, adrenaline and noradrenaline and the partial agonist salbutamol (see 4:2:4). From these data it was apparent that the affinities of the agonists for both receptor preparations were similar, and their order of potencies was suggestive of a beta$_2$-adrenoceptor (see also Table 4:1).

Both preparations produced shallow agonist displacement curves, with Hill slope factors ($n_H$) < 1. However, the inhibition curves produced by the catecholamine agonists with rat erythrocyte membranes had significantly greater slope factors than similar curves obtained for rat reticulocyte membranes ($p <0.05$). Thus, isoprenaline demonstrated a slope factor $n_H = 0.78 (+0.02)$ for erythrocytes; and $n_H = 0.53 (+0.02)$ for reticulocytes.
Figure 4:2

Inhibition of specific $^3$H-DHA binding to rat erythrocyte (A) and rat reticulocyte (B) membranes by agonists: (-)-isoprenaline (○); (-)-adrenaline (▲); (±)-salbutamol (▲); and (-)-noradrenaline (■). $^3$H-DHA, 1.5 nM, and the agonist under investigation were incubated for 30 min at 22°, and specific binding assessed as described previously. The data represented the mean (± SEM) of 3-7 experiments performed in duplicate.
Table 4:1

$K_i$ values of adrenergic agents for the specific $^3$H-DHA binding sites on rat erythrocytes, reticulocytes and lung membranes.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Rat erythrocyte</th>
<th>Rat reticulocyte</th>
<th>Rat lung</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Agonists</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(-)-Isoprenaline</td>
<td>33 (± 3)</td>
<td>34 (± 3)</td>
<td>27 (± 3)</td>
</tr>
<tr>
<td>(-)-Noradrenaline</td>
<td>2,900 (± 320)</td>
<td>2,000 (± 230)</td>
<td>1,430 (± 180)</td>
</tr>
<tr>
<td>(-)-Adrenaline</td>
<td>220 (± 14)</td>
<td>145 (± 13)</td>
<td>250 (± 25)</td>
</tr>
<tr>
<td>(±)-Salbutamol</td>
<td>360 (± 26)</td>
<td>370 (± 30)</td>
<td>660 (± 130)</td>
</tr>
<tr>
<td><strong>Antagonists</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(+)-Propranolol</td>
<td>29 (± 2)</td>
<td>-</td>
<td>55 (± 2)</td>
</tr>
<tr>
<td>(-)-Propranolol</td>
<td>0.44 (± 0.01)</td>
<td>-</td>
<td>0.69 (± 0.05)</td>
</tr>
<tr>
<td>(-)-Timolol</td>
<td>0.39 (± 0.01)</td>
<td>0.40 (± 0.03)</td>
<td>0.46 (± 0.04)</td>
</tr>
<tr>
<td>(-)-Alprenolol</td>
<td>0.37 (± 0.02)</td>
<td>-</td>
<td>0.54 (± 0.04)</td>
</tr>
</tbody>
</table>

IC$_{50}$ values were determined graphically and $K_i$ values calculated from the Cheng & Prusoff equation. The values represented the mean (± SEM) of 3-8 experiments performed in duplicate.
(B) **Antagonists**

The affinities of non-selective antagonists for the specific $^3$H-DHA binding sites on rat erythrocytes were compared to those obtained using membranes of rat reticulocytes and lung (Table 4:1). The binding sites on rat erythrocyte membranes, like those of rat lung, were clearly stereoselective for the (-) isomer of propranolol. Furthermore, the good agreement of inhibition constants which was obtained for these preparations suggested that the beta-adrenoceptors of rat erythrocyte, reticulocyte and lung were similar.

Previous studies indicated that highly selective beta-adrenoceptor agents produced displacement curves with rat lung membranes which were flattened and indicative of heterogeneous binding sites (Chapter 3). In marked contrast, the competition curves obtained for these agents with rat erythrocyte and reticulocyte membranes suggested a homogeneous receptor population with binding governed by mass action law.

Thus, Figure 4:3 shows inhibition data obtained for the beta$_1$-selective antagonist atenolol using rat erythrocyte and rat lung membranes. Linear Hofstee and Hill plots, with a slope factor for atenolol which approached unity ($n_H=0.97 \pm 0.02$, $n=6$), indicated that the erythrocyte membranes contained homogeneous binding sites for this drug.

The Hill slope factors and inhibition constants of highly selective beta-adrenoceptor agents have been calculated using rat erythrocyte and reticulocyte membranes (Table 4:2). These inhibition constants were compared with those derived for the beta$_2$-adrenoceptor component of rat lung by iterative curve fitting (Chapter 3). For preparations of rat erythrocytes and reticulocytes, Hill slope factors of all selective agents approached unity. This was strong evidence that the beta-adrenoceptor binding sites on these cells were homogeneous. Furthermore, the pKi's (-log$_{10}$ inhibition constants) of selective agents obtained with rat erythrocytes and reticulocytes correlated almost perfectly with those obtained for beta$_2$-adrenoceptor component of rat lung (reticulocyte
Figure 4:3
Inhibition of specific $^3$H-DHA binding to rat lung (△) and rat erythrocyte (▲) membranes by (±)-atenolol. Atenolol was incubated with membranes in the presence of 1.5 nM $^3$H-DHA and specific binding was assayed as previously described. The inhibition data have been plotted directly (A), and analysed by Hofstee (B) and Hill (C) plots. These data represented the mean (± SEM) of nine (rat lung) and six (rat erythrocyte) experiments performed in duplicate.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Rat reticulocyte</th>
<th>Rat erythrocyte</th>
<th>Rat lung</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_i$ (nM)</td>
<td>$n_H$</td>
<td>$K_i$ (nM)</td>
</tr>
<tr>
<td><strong>$\beta_1$-selective</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atenolol</td>
<td>5,200 (± 200)</td>
<td>1.03 (± 0.02)</td>
<td>3,500 (± 240)</td>
</tr>
<tr>
<td>Practolol</td>
<td>37,000 (± 3,400)</td>
<td>1.02 (± 0.01)</td>
<td>27,400 (± 1,000)</td>
</tr>
<tr>
<td>Metoprolol</td>
<td>380 (± 29)</td>
<td>0.97 (± 0.03)</td>
<td>273 (± 7)</td>
</tr>
<tr>
<td>Betaxolol</td>
<td>202 (± 12)</td>
<td>1.02 (± 0.01)</td>
<td>-</td>
</tr>
<tr>
<td><strong>$\beta_2$-selective</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proterol</td>
<td>26.2 (± 1.8)</td>
<td>0.88 (± 0.02)</td>
<td>32 (± 2.7)</td>
</tr>
<tr>
<td>ICI 118.551</td>
<td>1.10 (± 0.04)</td>
<td>1.05 (± 0.03)</td>
<td>0.96 (± 0.03)</td>
</tr>
</tbody>
</table>

Hill slope factors were determined for rat erythrocyte and reticulocyte preparations by Hill analysis of the inhibition data. Results represented the mean (± SEM) of 3–8 experiments performed in duplicate. Inhibition data for rat lung sites were obtained from averaged inhibition curves of 3–9 experiments (see Table 3:3).
These findings argued strongly for a beta<sub>2</sub>-adrenoceptor classification for the specific <sup>3</sup>H-DHA binding sites on rat erythrocyte and reticulocyte membranes.

**Adenylate cyclase activities of rat erythrocyte and reticulocyte membranes**

Freshly prepared rat erythrocyte and reticulocyte membranes were assayed for basal and catecholamine stimulated adenylate cyclase activities. Results presented in Figure 4:4 clearly showed the profoundly different levels of catecholamine stimulated enzyme activities in these membranes. Thus, the maximal isoprenaline stimulated activity of reticulocyte membranes was some sixteen times greater than that of erythrocyte membranes. In contrast, basal activities, measured in the absence of agonist, were only four to five fold higher in reticulocyte membranes. Assays performed in the absence or presence of GTP gave similar results.

The abilities of agonists to stimulate adenylate cyclase of these preparations were compared (Figure 4:4). Adrenaline was a full agonist in reticulocyte and erythrocyte membranes, whereas noradrenaline appeared to exert only 80% of the maximal isoprenaline stimulated activity. This finding probably reflected the low potency of noradrenaline in beta<sub>2</sub>-adrenoceptor preparations (Lefkowitz, 1975a; Dickinson unpublished) rather than partial agonist activity. The non-catecholamine agents salbutamol and procaterol appeared to be partial agonists since adenylate cyclase activities stimulated by 10<sup>-4</sup>M drug concentrations were significantly (p<0.05) less than the corresponding isoprenaline maximum. Furthermore, the degree of partial agonist activity for both drugs was approximately two fold higher in membranes from rat reticulocytes. This finding suggested that the partial agonists induced a greater coupling of reticulocyte beta-adrenoceptors to adenylate cyclase.
Figure 4
Stimulation of adenylate cyclase of rat reticulocyte (A) and rat erythrocyte (B) by agonists (-)-isoprenaline (Iso), (-)-adrenaline (Adr), (-)-noradrenaline (NA), (±)-salbutamol (Sal) and (±)-procaterol (Pro). Freshly prepared membranes of rat erythrocytes and reticulocytes were assayed in the absence (basal activity) and presence of $10^{-4}$ M agonists and $10^{-5}$ M GTP. Incubations were conducted at $37^\circ$C for 5 min and adenylate cyclase activity was determined as previously described. The results represented the mean (± SEM) of three determinations.
4:3 Discussion

The results of this study confirmed the presence on rat erythrocytes and reticulocytes of $^3$H-DHA binding sites which have all the characteristics of beta-adrenoceptors. The maximal number of specific binding sites for $^3$H-DHA was increased in reticulocyte membranes about three fold as compared to erythrocytes, whereas the affinity of $^3$H-DHA for these sites was unchanged. These findings were similar to those reported previously (Charness et al., 1976; Kaiser et al., 1978a; Limbird et al., 1980a) differing only in the increase in the maximum number of binding sites. However, since receptor density has been shown to be dependent on the degree of reticulocytosis (Kaiser et al. 1978, b), the higher values obtained above probably reflected the greater degree of reticulocytosis induced by my dosing regime.

The characteristics of the beta-adrenoceptor binding sites on erythrocytes were in almost all respects identical to those on reticulocytes. The kinetics of specific $^3$H-DHA binding to both preparations were not statistically different. In addition, the affinities of a series of agonists and antagonists for the beta-adrenoceptors on these cells were very similar, which suggested that the beta-adrenoceptor binding sites were identical. Other workers, using $^{125}$I-HYP binding (Bilezikian et al., 1977, a; Beckman and Hollenberg, 1979); $^3$H-DHA binding (Kaiser et al., 1978, b); and adenylate cyclase measurements (Beckman et al., 1979) have reached similar conclusions.

The major differences between membranes prepared from these cell types lies in the coupling of their beta-adrenoceptors to adenylate cyclase. Thus, rat reticulocyte membranes possessed a greatly enhanced catecholamine stimulated adenylate cyclase, which was approximately sixteen times greater than that of rat erythrocyte membranes. Basal adenylate cyclase activity, and the maximal number of specific $^3$H-DHA binding sites, were increased only three to four fold. Guanine nucleotide modulation of agonist affinities, which was
demonstrable for reticulocyte membranes under appropriate conditions (see Chapter 7), was absent in erythrocyte membranes. These observations indicated that maturation of rat erythrocytes was associated with decreased catecholamine and guanine nucleotide responsiveness.

The molecular mechanisms responsible for these effects have been investigated in a recent study (Limbird et al, 1980a). These authors used a number of techniques to probe the component interactions involved in beta-adrenoceptor-cyclase coupling in these cell types. Their results showed that maturation of the rat erythrocyte was associated with losses of guanine nucleotide regulatory proteins (N-protein). However, these losses paralleled those of beta-adrenoceptor number, which suggested that the stoichiometry of the (receptor-Nprotein-cyclase)-complex may have been similar in erythrocytes and reticulocytes. Therefore, the unresponsiveness observed in erythrocyte preparations was suggested to result from inefficiencies in the communication of the components involved in coupling beta-adrenoceptor to adenylate cyclase.

Further evidence that the erythrocyte beta-adrenoceptor-cyclase system was less efficiently coupled came from the studies with full and partial agonists. Thus, measurements of adenylate cyclase suggested that the non-catecholamine sympathomimetic agents salbutamol and procaterol were weaker partial agonists in membranes prepared from erythrocytes than reticulocytes. Furthermore, results of binding studies indicated that the slopes of the displacement curves obtained for full agonists were significantly greater using erythrocyte membranes than preparations of reticulocytes. This observation could be interpreted, in the light of the recent model of Lefkowitz and colleagues (See Chapter 7), to indicate that agonist interaction with reticulocyte beta-adrenoceptors resulted in the induction of greater proportions of a high affinity state of the beta-adrenoceptor. Since it is this state of the receptor which has been suggested to couple to adenylate cyclase
(Stadel et al, 1980), full agonists would be postulated to produce a more effective stimulation of reticulocyte adenylate cyclase than of erythrocyte cyclase.

The main aim of this study was to characterise the beta-adrenoceptors of these cell types in an effort to determine whether beta-adrenoceptor subtypes could co-exist in a single population of cells. The relative potencies of a series of agonists in displacing $^3$H-DHA binding to rat reticulocyte and erythrocyte membranes were identical with those determined using rat lung membranes. These findings indicated a beta$_2$-adrenoceptor classification as proposed by Lands (1967). The results of testing selective adrenoceptor antagonists in these systems confirmed this conclusion. Thus, the affinities of selective agents for beta-adrenoceptors of rat erythrocyte and reticulocyte membranes correlated extremely well with their affinities at the beta$_2$-adrenoceptor binding sites of rat lung membranes.

In contrast to the heterogeneous beta-adrenoceptor binding sites of rat lung membranes, rat reticulocyte and erythrocyte membranes possessed a homogeneous population of beta-adrenoceptors. This conclusion, which was based on the use of selective beta-adrenoceptor antagonists to delineate beta-adrenoceptor heterogeneity, differed from that of Kaiser et al., (1979). These workers suggested the presence of both beta$_1$ and beta$_2$-adrenoceptors on rat reticulocytes, with the latter subtype predominating. However, these results may have been due to their use of poorly selective agonists to quantify beta-adrenoceptor heterogeneity.

The data provided by the present study indicated that a single population of cells contained a homogeneous class of beta-adrenoceptors. The co-presence of beta-adrenoceptor sub-types in membrane preparations of rat lung could, therefore, reflect the cellular heterogeneity of this tissue. Alternatively the beta-adrenoceptors might co-exist on the same cell type, and perhaps subserve the same physiological function.
Support for the concept of beta-adrenoceptor heterogeneity on a single cell type has been provided recently by biochemical studies of primary cultures of glial cells and derived sub clones. (Ebersolt et al, 1981; Homburger et al, 1981). Pharmacological studies of chronotropic responses in cat heart (Carlsson et al, 1972; O'Donnell and Wanstall 1979b); and relaxation of guinea pig trachea (Furchgott and Wakade, 1975; Zaagsma et al, 1976; O'Donnell and Wanstall, 1979, a) have provided additional evidence which indicated that beta_1 and beta_2-adrenoceptors were both located on pharmacologically responsive cell types. Furthermore, investigations of the ontogeny of rat lung beta-adrenoceptors showed a constant proportion of beta-adrenoceptor sub-types from foetus to adult (Whitsett et al, 1981; Ashton et al, 1981). In view of the marked proliferation of cell types which occurred during the post-natal phase, the constant proportion of these subtypes suggested that they occurred on the same cell type.
CHAPTER 5

Radiolabelled Ligand Binding to Membrane Preparations of Beta₁-Adrenoceptors
5:1 Introduction

Beta\textsubscript{1}-adrenoceptors have been implicated in the regulation of fat cell lipolysis (Lands et al., 1967a; Loakpradit and Lockwood, 1977) and cardiac inotropic and chronotropic responses (Lands et al., 1967a; Wasserman and Levy, 1974). However, pharmacological studies of cat heart (Carlsson et al., 1972; O’Donnell and Wanstall 1979 b) have suggested the presence of functionally active beta\textsubscript{1} and beta\textsubscript{2}-adrenoceptors in cardiac tissue. Support for this concept of beta-adrenoceptor heterogeneity in tissues has been provided by direct binding studies of membrane preparations of brain (Nahorski, 1978) and lung (Rugg et al., 1978; see also Chapter 3).

The present study was designed to characterise the binding sites on membranes from beta\textsubscript{1}-containing tissues; to investigate the degree of beta-adrenoceptor heterogeneity of these preparations; and to determine whether the pharmacological specificity of beta\textsubscript{1} (and beta\textsubscript{2})-adrenoceptors was conserved between tissue and species. For these studies, tissue preparations of rat heart, rat cerebral cortex and rabbit lung were used as the source of beta\textsubscript{1}-adrenoceptors.

Rat heart had previously been shown to contain beta\textsubscript{1}-adrenoceptors (Burges and Blackburn, 1972), although work with cat heart suggested the presence of both subtypes which had a differential distribution within the heart (Carlsson et al., 1972, 1977). Thus, the population of beta-adrenoceptors in ventricular myocardium, which modulated inotropic responses, were postulated to contain a higher proportion of beta\textsubscript{1}-adrenoceptors. Rat ventricular tissue has therefore been used as a potential source of relatively pure beta\textsubscript{1}-adrenoceptors.

Radiolabelled ligand binding studies have provided evidence for a differential localisation of beta-adrenoceptors in mammalian brain (Bylund and Snyder, 1976). Cerebral cortex has been reported to contain
high proportions of beta<sub>1</sub>-adrenoceptors, whereas cerebellum contained mostly beta<sub>2</sub>-adrenoceptors (Nahorski and Willcocks, 1978). The presence of both beta<sub>1</sub> and beta<sub>2</sub> adrenoceptors in cerebral cortex has permitted a comparison of central beta-adrenoceptors with those in peripheral tissues.

Bronchodilation was originally proposed as a beta<sub>2</sub>-adrenoceptor mediated response (Lands et al 1967a). Results from a number of studies have confirmed the predominance of beta<sub>2</sub>-adrenoceptors in lung tissue of rat (Burges and Blackburn, 1972); guinea pig (Buckner and Patil, 1971); calf (Ariens, 1967); human (Hedges and Turner, 1969); and cat (Apperley et al, 1976). In marked contrast rabbit lung has been reported to contain a predominance of beta<sub>1</sub>-adrenoceptors (Coleman and Somerville, 1977). The present study has extended the previous comparison of the beta-adrenoceptors of rat and rabbit lung (Rugg et al 1978).

5:2 Results

5:2:1 ³H-DHA binding to membrane preparations of rat myocardium, rat cerebral cortex and rabbit lung

Binding of ³H-DHA to membrane preparations of rat heart, rat cerebral cortex and rabbit lung was measured in the absence and presence of 200uM(-) isoprenaline to define non-specific binding. Specific binding to all membranes was saturable (Figure 5:1), and the equilibrium dissociation constants (K<sub>D</sub>) for ³H-DHA were not significantly different - rat myocardium, K<sub>D</sub> = 0.53 (+0.03), n=8; rat cerebral cortex K<sub>D</sub> = 0.59 (+0.09), n=3; and rabbit lung K<sub>D</sub> = 0.59 (+0.05), n=3. However, the maximal number of specific ³H-DHA binding sites (B<sub>max</sub>) differed markedly for these preparations - rat myocardium, B<sub>max</sub> = 35 (+4) fmoles/mg protein; rat cerebral cortex, B<sub>max</sub> = 118 (+12) fmoles/mg protein; rabbit lung, B<sub>max</sub> = 390 (+20) fmoles/mg protein.
Figure 5:1

Specific binding of $^3$H-DHA to rabbit lung (■), rat cerebral cortex (□), and rat myocardial (▲) membranes. Membranes were incubated with increasing concentrations of $^3$H-DHA for 30 min at 22°C, and specific binding was assessed as described previously. A (i), B (i) - the data plotted directly.

A (ii), B (ii) - Scatchard analysis of the same data. The results shown were representative of three (rabbit lung), three (rat cerebral cortex), and eight (rat heart) experiments performed in triplicate.
The specific $^3$H-DHA binding sites on membranes from potassium chloride extracted rat myocardium saturated at $^3$H-DHA concentrations < 4 nM. Scatchard analysis of these data was linear and provided estimates of $K_D$ which correlated with those determined for brain and lung membranes (Figure 5:1). However, at higher concentrations of radioligand, specific binding to most preparations of rat heart increased further, in an apparently linear fashion (data not shown). Scatchard plots of binding data which included the higher ligand concentrations were curvilinear. This finding suggested that the radioligand was binding to a second, isoprenaline displaceable, site which had high capacity and lower affinity for $^3$H-DHA.

This low affinity $^3$H-DHA binding site clearly corresponded to the non-stereoselective site observed concurrently in rat myocardial membranes by Winek and Bha'lla (1979). Membrane preparations of lung tissue (rat and rabbit), erythrocytes (rat, frog and chick), rat cerebral cortex, or rat epididymal fat did not appear to possess this second $^3$H-DHA site (data not shown). However, saturation data of frog myocardium showed similar characteristics to those described for rat heart, indicating that such sites were common to cardiac tissue from different species. Recently, Mauger and Worcel (1979) have described a low affinity site for $^3$H-DHA in a muscle cell line.

5:2:2 Kinetics of $^3$H-DHA binding to rabbit lung and rat cerebral cortex membranes

Specific $^3$H-DHA binding to rabbit lung membranes at 22° was rapid and reversible, with times for half maximal association and dissociation of 1 minute and 3.9 minutes respectively (Figure 5:2). These results showed a significant difference between the kinetics of
Figure 5:2
Kinetic analysis of $^3$H-DHA binding to rabbit lung membranes. (A) Membrane preparations were incubated at 22°C with 1.1 nM $^3$H-DHA and specific binding was measured as a function of time (△). After 30 min, 200 μM (-)-isoprenaline was added and the decrease in specific binding was measured at timed intervals (△). (B) The first order plot of the dissociation reaction. Where $K_1 = 0.164\text{min}^{-1}$ is the dissociation rate constant. (C) The pseudo first order plot of the association reaction. Where $K_{OBS}$ is the observed pseudo first order association rate constant, and $K_{+1} = 0.631\text{nM}^{-1}\text{min}^{-1}$ is the second order association rate constant. The binding site concentration for these experiments was 0.050 nM. These data were representative of five experiments performed in duplicate.
specific $^3$H-DHA binding to rabbit lung and those determined under identical conditions for rat lung (See Chapter 3). Thus, rates of association and dissociation of $^3$H-DHA binding to rabbit lung membranes were considerably faster than to preparations of rat lung.

The kinetic parameters of specific $^3$H-DHA binding to rabbit lung and rat cerebral cortex membranes were compared with values obtained previously for membranes of the beta$_2$-adrenoceptor-containing tissues rat lung and rat reticulocytes (Table 5:1). Results indicated that $^3$H-DHA bound to rabbit lung and rat cerebral cortex membranes two to three times faster than to rat lung or rat reticulocyte membranes. Similarly, the time taken for half maximal dissociation of $^3$H-DHA from rabbit lung (4.4min) or rat cerebral cortex (4.3min) membranes was two to three times less than that from the beta$_2$-adrenoceptors containing membranes ($\sim$ 10min). The equilibrium dissociation constants for $^3$H-DHA binding to these preparations, calculated as the fraction of the dissociation and association rate constants, were similar.

5:2:3 Pharmacological specificity of $^3$H-DHA binding to rabbit lung, rat cerebral cortex and rat myocardial membranes

(A) Agonists

The ability of adrenoceptor agonists to displace specific $^3$H-DHA binding to rabbit lung membranes was determined (Figure 5:3). The order of potency isoprenaline > noradrenaline = adrenaline > salbutamol was indicative of an overall beta$_1$-adrenoceptor classification for the specific $^3$H-DHA binding sites (Lands et al., 1967a; Daly and Levy, 1979). The inhibition constants of these agonists were calculated and compared with values obtained for rat cerebral cortex membranes (courtesy Dr S.R. Nahorski). Table 5:2 shows the excellent correlation obtained, which suggested that the beta-adrenoceptors of these preparations were similar.
Table 5:1

The kinetic parameters of specific $^3$H-DHA binding to membrane preparations of rabbit lung, rat cerebral cortex, rat lung and rat reticulocyte.

<table>
<thead>
<tr>
<th>Kinetic determination</th>
<th>Rabbit lung</th>
<th>Rat cerebral cortex</th>
<th>Rat reticulocyte</th>
<th>Rat lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{+1}$ (nM$^{-1}$, min$^{-1}$)</td>
<td>0.572 ($\pm$ 0.05)</td>
<td>0.40</td>
<td>0.197 ($\pm$ 0.014)</td>
<td>0.262 ($\pm$ 0.012)</td>
</tr>
<tr>
<td>$K_{-1}$ (min$^{-1}$)</td>
<td>0.157 ($\pm$ 0.003)</td>
<td>0.162</td>
<td>0.068 ($\pm$ 0.003)</td>
<td>0.069 ($\pm$ 0.003)</td>
</tr>
<tr>
<td>$K_D$, $K_{-1}$/$K_{+1}$, nM</td>
<td>0.27</td>
<td>0.40</td>
<td>0.35</td>
<td>0.26</td>
</tr>
<tr>
<td>$t_{1/2}$ (diss) (min)</td>
<td>4.4</td>
<td>4.3</td>
<td>10.2</td>
<td>10.0</td>
</tr>
</tbody>
</table>

Association ($K_{+1}$) and dissociation ($K_{-1}$) rate constants were calculated as described in Appendix 4. Equilibrium dissociation constants ($K_D$) were calculated from the fraction $K_{-1}/K_{+1}$, and $t_{1/2}$ (diss) (time for half maximal dissociation of specific $^3$H-DHA binding) was calculated from the formula $t_{1/2} = 0.693/K_{-1}$. Results represented the mean of five determinations for all preparations except rat cerebral cortex, where $n = 2$. 
Figure 5:3
Inhibition of specific $^3$H-DHA binding to rabbit lung membranes by (-)-isoprenaline (□); (-)-noradrenaline (▲); (-)-adrenaline (△); and (±)-salbutamol (■). $^3$H-DHA, 1.5 nM, and the agonists under investigation were incubated with membranes for 30 min at 22°C in Tris-HCl buffer, pH 7.8, and specific binding was measured as described previously.

The data represented the mean (± SEM) of 3-8 experiments performed in duplicate.
Table 5:2 Inhibition constants ($K_i$) of beta-adrenoceptor agonists for $^3$H-DHA binding sites on rabbit lung and rat cerebral cortex membranes

<table>
<thead>
<tr>
<th>Agonist</th>
<th>$K_i$ (nM)</th>
<th>Rabbit lung</th>
<th>Rat cerebral cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-) isoprenaline</td>
<td>23 (+2)</td>
<td></td>
<td>107 (+9)</td>
</tr>
<tr>
<td>(-) noradrenaline</td>
<td>370 (+35)</td>
<td></td>
<td>407 (+71)</td>
</tr>
<tr>
<td>(-) adrenaline</td>
<td>570 (+38)</td>
<td></td>
<td>540 (+51)</td>
</tr>
<tr>
<td>(+) salbutamol</td>
<td>2060 (+330)</td>
<td></td>
<td>1600 (+200)</td>
</tr>
</tbody>
</table>

Results represented the mean (+SEM) of 3-8 experimental determinations performed in duplicate. $K_i$ values for rat cerebral cortex membranes were kindly supplied by Dr S.R. Nahorski.
The use of agonist affinities to characterise beta-adrenoceptors was somewhat hazardous, however, since agonist binding can be modulated in some systems, and not others, by ions and guanine nucleotides. Such problems were highlighted by the data obtained for salbutamol. The inhibition constants which were observed in the absence of GTP indicated that salbutamol was selective for the beta_2-adrenoceptors of rat lung over those of rabbit lung membranes (compare Tables 5:2 with 3:1). However, when assayed in the presence of GTP, the affinities of salbutamol for rat and rabbit lung beta-adrenoceptors were identical (data not shown). This latter finding implied that the observed pharmacological beta_2-adrenoceptor selectivity of salbutamol may not be expressed by selective affinity at beta_2-adrenoceptors.

(B) Antagonists

The affinities of antagonists for beta-adrenoceptor binding sites have been reported to be unaffected by endogenous modulators (Williams and Lefkowitz, 1978). Therefore, these drugs provided better tools for the characterisation of the binding sites on membrane preparations of rat heart, rat cerebral cortex, and rabbit lung. Inhibition constants of adrenoceptor drugs were calculated from competition experiments and the values compared with those obtained previously for preparations of rat lung (Table 5:3).

The beta-adrenoceptor antagonists (-) timolol and (-) propranolol possessed affinities for the membrane preparations, which differed by less than two fold. This result confirmed the classification of these antagonists as 'non-selective'. Binding to the beta-adrenoceptors of rat heart, rat cerebral cortex and rabbit lung membranes was clearly stereoselective as demonstrated by stereoselectivity indices (pK_i(-) isomer-pK_i (+) isomer) for propranolol which ranged from 1.93 - 2.28.
Table 5.3
Inhibition constants of adrenoceptor drugs for $^3$H-DHA binding sites on rat heart, rat cerebral cortex, rabbit lung and rat lung membranes.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Rat heart</th>
<th>Rat cerebral cortex</th>
<th>Rabbit lung</th>
<th>Rat lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-Timolol</td>
<td>0.57 ± 0.07</td>
<td>0.78 ± 0.04</td>
<td>ND</td>
<td>0.46 ± 0.01</td>
</tr>
<tr>
<td>(-)-Propranolol</td>
<td>1.10 ± 0.15</td>
<td>0.60 ± 0.10</td>
<td>0.90 ± 0.06</td>
<td>0.74 ± 0.05</td>
</tr>
<tr>
<td>(+)-Propranolol</td>
<td>208 ± 40</td>
<td>96 ± 8</td>
<td>77 ± 6</td>
<td>55 ± 2.3</td>
</tr>
<tr>
<td>Phentolamine</td>
<td>ND</td>
<td>ND</td>
<td>76,000 ± 5,500</td>
<td>72,000 ± 6,000</td>
</tr>
</tbody>
</table>

Inhibition constants were calculated as described previously (Table 5.2). Results represented the mean (± SEM) of at least three experiments performed in duplicate. The $K_i$ values for rat cerebral cortex membranes were kindly supplied by Dr. S. R. Nahorski.
The potent alpha-adrenoceptor antagonist phentolamine was a weak inhibitor of $^3$H-DHA binding to rabbit lung membranes, with an affinity equivalent to that determined for membranes of rat lung. This finding indicated that phentolamine, a drug often used in high concentration to lower non-specific binding of radioligand (Minneman et al, 1979c), was non-selective for either beta$_1$ or beta$_2$-adrenoceptors.

Beta-adrenoceptor antagonists which have shown selectivity for either beta$_1$ or beta$_2$-adrenoceptors by pharmacological evaluations, were tested as inhibitors of specific $^3$H-DHA binding to rat heart, rat cerebral cortex and rabbit lung membranes. These experiments were conducted in order to further characterise the binding sites on these membranes and to delineate the degree of beta-adrenoceptor heterogeneity present. Inhibition curves of the beta$_1$-selective antagonist betaxolol, and the beta$_2$-selective antagonist ICI 118,551 were obtained for rat heart and rat cerebral cortex membranes (Figure 5:4) and rabbit lung and rat reticulocyte membranes (Figure 5:5). The latter preparation has previously been shown to contain a homogeneous population of beta$_2$-adrenoceptors (Chapter 4).

Heterogeneous binding sites were suggested for rat heart, rat cerebral cortex and rabbit lung membranes by inhibition curves of ICI 118,551 and betaxolol, which showed distinct inflections. Mean curves were subjected to iterative curve fitting to a two site model, and the IC$_{50}$ values of drugs for these sites, and proportions of sites were calculated (Figure 5:4, Figure 5:5).

From the data presented in Figure 5:4 it was estimated that both rat heart, and rat cerebral cortex membranes contained 30-35% of a site having high affinity for ICI 118,551 (beta$_2$-adrenoceptors) and 65-70% of a site having high affinity for betaxolol (beta$_1$-adrenoceptors).
Figure 5:4
Inhibition curves of ICI 118.551 (open symbols) and betaxolol (closed symbols) obtained with (A) rat cerebral cortex and (B) rat heart membranes. Curves were analysed by iterative curve-fitting to a two-site model, and the proportion of sites (%) and IC<sub>50</sub> values (nM) of drugs for these sites were calculated. Results represented mean curves of 3-5 determinations performed in duplicate.
Figure 5:5

Inhibition curves of ICI 118.551 (open symbols) and betaxolol (closed symbols) obtained with (A) rabbit lung and (B) rat reticulocyte membranes. The experimental protocol was identical to that described in Figure 5:4. The proportion of sites and IC\textsubscript{50} values (nM) for rabbit lung membranes were obtained by iterative curve fitting of mean inhibition curves (n = 3-5). The data shown for rat reticulocyte represented the mean of 3 or 5
The affinities of these drugs for the beta_1 or beta_2-adrenoceptors of either preparation were in good agreement.

The data obtained for rabbit lung membranes (Figure 5:5) indicated a large proportion (80%) of sites having high affinity for betaxolol (beta_1-adrenoceptors), and a minor proportion of sites having high affinity for ICI 118,551. In contrast, the inhibition curves obtained for these antagonists using rat reticulocyte membranes were indicative of a homogeneous population of beta_2-adrenoceptors with binding governed by mass action law. The IC_{50} values of betaxolol and ICI 118,551 in this pure beta_2-adrenoceptor system correlated well with their affinities at the beta_2-adrenoceptor binding sites on membranes of rat heart, rat cerebral cortex and rabbit lung. This finding suggested that the beta_2-adrenoceptor binding sites of these preparations were similar.

In order to evaluate the degree of similarity of beta_1 and beta_2-adrenoceptor binding sites in tissues, the affinities of selective drugs for the beta-adrenoceptor subtypes of a number of tissues were compared. Table 5:4 shows the inhibition constants determined for membrane preparations which contained a predominance of beta_1-adrenoceptors (rat heart, rat brain and rabbit lung); a predominance of beta_2-adrenoceptors (rat lung) and homogeneous beta_2-adrenoceptors (rat reticulocytes).

The affinities of drugs for the beta_1 or beta_2-adrenoceptor sites of these different tissue preparations were in good agreement, and were not influenced by the relative proportion of subtypes in the preparation. In general, the inhibition constants of drugs for a particular subtype differed by < three fold. According to Furchgott's guidelines (Furchgott, 1972), this finding indicated considerable uniformity of both beta_1 and beta_2-adrenoceptors between tissue and species.
Table 5.4

Inhibition constants of adrenoceptor agents for beta1 and beta2 adrenoceptor binding sites.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Beta1-adrenoceptor sites</th>
<th>Inhibition constants (nM)</th>
<th>Beta2-adrenoceptor sites</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rat heart</td>
<td>Rat cerebral cortex</td>
<td>Rabbit lung</td>
<td>Rat lung</td>
</tr>
<tr>
<td>Atenolol</td>
<td>ND</td>
<td>*67</td>
<td>180</td>
<td>40</td>
</tr>
<tr>
<td>Betaxolol</td>
<td>4</td>
<td>3.8</td>
<td>3.7</td>
<td>1.9</td>
</tr>
<tr>
<td>Practolol</td>
<td>ND</td>
<td>*310</td>
<td>ND</td>
<td>341</td>
</tr>
<tr>
<td>Metoprolol</td>
<td>15</td>
<td>ND</td>
<td>ND</td>
<td>21</td>
</tr>
<tr>
<td>ICI 118.551</td>
<td>214</td>
<td>420</td>
<td>63</td>
<td>107</td>
</tr>
<tr>
<td>Procaterol</td>
<td>2,060</td>
<td>*2,600</td>
<td>2,800</td>
<td>4,100</td>
</tr>
</tbody>
</table>

Mean inhibition curves of 3-9 determinations were analysed by iterative curve-fitting to a two-site model and IC50 values and proportions of each beta-adrenoceptor sites were estimated. Analysis of individual curves gave values in excellent agreement, with SEM's <15%. The proportion of beta1 adrenoceptor sites were: rat heart 72 (±3%), rat cerebral cortex 65 (±3%), and rabbit lung 79 (±1%). Inhibition constants for drugs at beta1 and beta2 adrenoceptors were calculated by the method of Cheng & Prusoff (1973). The inhibition constants of drugs for the homogeneous binding sites on rat reticulocyte membranes were calculated as previously described (Chapter 4). * Data kindly supplied by Dr. S.R. Nahorski. ND = not determined.
5:3 Discussion

The concept of dual beta-adrenoceptors having strict organ-specific locations (Lands et al, 1967) has provided the impetus for experiments aimed at delineating and classifying beta-adrenoceptors in tissues. Properly controlled biochemical (Burges and Blackburn, 1972; Lefkowitz, 1975a) and pharmacological (Daly and Levy, 1979) experiments have provided evidence for the existence of beta_1 and beta_2-adrenoceptors in tissues equivalent to those proposed by Lands et al. However, there still exists some controversy as to the exact number of beta-adrenoceptor sub-types (Boissier et al, 1971, Harms, 1977). Indeed, the beta_1 and beta_2-adrenoceptor classification of Lands has been suggested to represent only two extremes of a variable spectrum of different isoreceptors (Brittain et al, 1970).

The major aim of the present work was to determine whether the pharmacological properties of the beta-adrenoceptor subtypes were conserved between tissue and species. Preliminary evidence that the characteristics of beta-adrenoceptor subtypes from different tissues were similar, was provided by the early binding studies from these laboratories (Barnett et al, 1978; Rugg et al, 1978; Nahorski, 1978). The binding parameters of rat lung, rat erythrocyte and reticulocyte membranes (Chapters 3 and 4) provided further indications for the uniformity of rat beta_2-adrenoceptors. The present study characterised, more fully, the beta-adrenoceptor binding sites on rat heart, rat cerebral cortex and rabbit lung membranes.

The properties of the specific \(^{3}H\)-DHA binding sites on these preparations were in almost all respects identical. Thus, the equilibrium dissociation constant for the radioligand, the kinetics of radioligand association and dissociation, and the affinities of adrenoceptor agonists and non-selective beta-adrenoceptor antagonists were all in good agreement. Furthermore, the affinities of agonists
and selective adrenoceptor antagonists for the beta-adrenoceptor binding sites on these preparations were indicative of an overall beta₁-adrenoceptor classification.

The differences between the tissue preparations lay in the numbers of specific ³H-DHA binding sites, and the degree of beta-adrenoceptor heterogeneity present. Rabbit lung contained the highest number of binding sites and the highest proportion of beta₁-adrenoceptors (80%). In contrast, rat myocardial membranes exhibited low numbers of beta-adrenoceptor binding sites, which were estimated to contain about 70% beta₁-adrenoceptors.

The use of selective beta-adrenoceptor agents has provided a means of characterising beta₁ and beta₂-adrenoceptor binding sites in membrane preparations from different tissues and species. Results of these comparative studies suggested that the properties of mammalian beta₁ and beta₂-adrenoceptors were highly conserved. These findings provided support for the concept that there were only two beta-adrenoceptors in mammalian tissue (Lands et al, 1967a,b).

Concurrent investigations by Minneman and colleagues have provided additional evidence to support the above conclusions. These workers used the radioligand ¹²⁵I-HYP, and demonstrated that the pharmacological specificities of beta₁ and beta₂-adrenoceptors were identical in a range of tissues from different mammalian species (Minneman et al 1979b, 1979c).

These observations have led to the general conclusion that the major properties of beta₁ or beta₂-adrenoceptor binding sites of mammalian tissues were similar. However, the question of whether the accessory binding sites of beta-adrenoceptor subtypes from different tissues were identical remained open. The agents used in this study had a wide range of potencies and structures which allowed extensive
mapping of the binding sites. It remained possible, however, that these agents had structural features which disallowed the detection of small differences in the accessory binding sites of the beta-adrenoceptors examined. Such differences in beta₁-adrenoceptors of guinea pig and human atria have apparently been observed using beta-adrenoceptor antagonists related to tolamolol (Harms, 1976).

More serious objections to the concept of only two mammalian beta-adrenoceptors has come from the studies of rat adipose tissue. The beta-adrenoceptors in this tissue, which mediated lipolysis, have been classified as beta₁-sites (Lands et al, 1967a). This classification has been questioned, since the affinities of a number of beta-adrenoceptor antagonists in this system were not correlated with their affinities at rat atrial beta₁-adrenoceptors (Harms et al, 1974). Furthermore, the stereoselectivity indices determined for beta-blockers at rat adipocyte beta-adrenoceptors were significantly less than values for rat atrial (beta₁) or diaphragm (beta₂)-adrenoceptors (Harms et al, 1977). These observations have led to the suggestion that the beta-adrenoceptors of rat adipose tissue represented a third subtype.

However, the measurement of agonist induced fat cell lipolysis, and its attenuation by drugs was complicated by the necessary presence of BSA in the medium, and the nature of the tissue. Thus, adsorption of drugs by BSA or partitioning of lipophilic drugs into fat vacuoles may have contributed to these anomalous affinities. Although the former problem has been taken into consideration in recent work (De Vente et al, 1980), the characterisation of these adipocyte beta-adrenoceptors must await the application of binding techniques.

Radio-labelled ligand binding techniques have provided an excellent means of characterising beta-adrenoceptor binding sites in tissue preparations. It was encouraging, therefore, that the pharmacological properties of these beta₁ and beta₂-sites correlated
extremely well with their properties as determined by classical pharmacological techniques, although their location and function in tissues were unresolved. Evidence that radioligands labelled pharmacologically responsive sites has been suggested by binding studies of cat (Hedberg et al, 1980) and rabbit (Brodde and Leifert, 1981) atria, where beta_1 and beta_2-adrenoceptors were demonstrated. The chronotropic responses of cat atria (Carlsson et al, 1972; O'Donnell and Wanstall 1969,b) and inotropic responses of rabbit atria (Wagner et al, 1981) have been shown to be mediated by both beta_1 and beta_2-adrenoceptors.

In contrast to these results, the chronotropic responses of guinea pig (Zaagsma et al 1979; O'Donnell and Wanstall, 1979b; Kaumann et al 1978); and rat (Bryan et al, 1981) atria have been reported to be mediated exclusively through beta_1-adrenoceptors. In apparent contradiction to these data, radioligand binding experiments demonstrated beta_1 and beta_2-adrenoceptors in guinea pig atria (Hedberg et al 1980) and rat heart (Minneman et al 1979c; this study). These observations suggested that the radioligand was labelling beta_2-adrenoceptors in these preparations which were unassociated with contractile responses.

These findings implied the need for caution in extrapolating results of binding experiments in an attempt to define the physiological function of beta-adrenoceptor subtypes in tissues. Clearly these binding sites may be unassociated with pharmacological responses, or functionally uncoupled from the effector system. Thus, the proportions of beta_2-adrenoceptor sites in membrane preparations of rat (Barnett et al 1978; this study), guinea pig (Engel 1980) and rabbit (Rugg et al, 1978; this study) lung gave little indication of the sensitivity of these lung preparations to relaxation by catecholamines (McDougal et al, 1953; Jamieson et al, 1962; Doidge et al, 1980).
CHAPTER 6

Characterisation of the Beta-Adrenoceptor Binding Sites of Avian and Amphibian Erythrocytes
6:1 Introduction

The dual beta-adrenoceptor hypothesis of Lands et al (1967) was based on experiments performed on tissue from mammalian species. This classification of beta-adrenoceptors was subsequently extended to Amphibia and Aves (Lands et al, 1969). Considerable support for the concept of only two beta-adrenoceptors within the Mammalia has been provided by pharmacological (see review by Daly and Levy, 1979), and biochemical (Nahorski, 1978; Minneman et al, 1979c; 1980) evaluations.

However, few comprehensive studies have been performed which were aimed at the classification of beta-adrenoceptors from tissue of non-mammalian origin. Those that have been reported generally used the relative potencies of agonists to define these beta-adrenoceptors (Lands et al, 1969; Lefkowitz, 1975a; Brown et al, 1976a; Stene-Larson and Helle, 1978a; Daly and Levy, 1979) and good correlations between mammalian and non-mammalian beta-adrenoceptors were observed.

Consequently avian and amphibian erythrocytes have been used by a number of workers as model systems for beta₁ and beta₂-adrenoceptors respectively (Vauquelin et al, 1979; Pike and Lefkowitz, 1979). Extensive studies of structure-activity relationships have been described for these preparations using adenylate cyclase and binding measurements. (Mukherjee et al, 1976; Bilezikian et al, 1978).

The present studies were designed to characterise, more fully, the beta-adrenoceptor binding sites on avian (chick) and amphibian (frog) erythrocytes using beta-adrenoceptor antagonists. The properties of these binding sites have been compared with those determined previously for mammalian beta₁-adrenoceptor (Chapter 5), and beta₂-adrenoceptors (Chapters 3 and 4).
6:2 Results

6:2:1 Binding of $^{3}$H-DHA to membrane preparations of chick and frog erythrocytes

Specific binding of $^{3}$H-DHA to chick and frog erythrocytes was saturable and of high affinity, with equilibrium dissociation constants of $0.76 \pm 0.03$ nM ($n=6$), and $0.28 \pm 0.02$ nM ($n=5$) respectively. The maximal number of binding sites were $440 \pm 35$ fmoles/mg protein (chick erythrocytes) and $83 \pm 10$ fmoles/mg protein (frog erythrocytes). However, the density of binding sites obtained for these preparations was dependent on the purity of the membrane preparation. Thus, removal of 'nuclear' material associated with frog erythrocyte membranes resulted in a ten fold enrichment of specific $^{3}$H-DHA binding sites (see Methods).

The Hill coefficients of $^{3}$H-DHA binding to chick $1.03 \pm 0.02$ and frog $0.99 \pm 0.03$ erythrocytes were not significantly different from 1, suggesting the absence of co-operative interactions.

6:2:2 Kinetic characteristics of $^{3}$H-DHA binding to chick erythrocyte membranes

The kinetics of $^{3}$H-DHA binding to chick erythrocyte membranes at $22^\circ$ were both extremely rapid, and indicative of a second order reaction between ligand and receptor (Figure 6:1). The mean association and dissociation rate constants were: $1.24 \pm 0.15$ nM$^{-1}$ min$^{-1}$, and $0.61 \pm 0.04$ min$^{-1}$ respectively ($n=3$). Dissociation of $^{3}$H-DHA from its specific binding sites proceeded at $22^\circ$ with a $t_1$ of 1.1 min. The equilibrium dissociation constant, calculated as the fraction of the rate constants, was $0.49$ nM in good agreement with that determined from saturation experiments.

These results indicated that the radioligand associated and dissociated from binding sites on avian erythrocytes at markedly different rates to those determined for mammalian beta$_1$ or beta$_2$-adrenoceptors (see
Kinetics of association and dissociation of $^3$H-DHA to chick erythrocyte membranes at 22°C. The upper figures show the association kinetics with the pseudo first-order plot of the association reaction. The lower figures show the dissociation kinetics and the first-order plot of the dissociation reaction. $^3$H-DHA was present at 1.4 nM, and 0.10 nM was bound at equilibrium. These data were representative of three experiments performed in duplicate.
Table 5:1). Thus, rates of association and dissociation were two to four times faster than for mammalian beta\textsubscript{1}-adrenoceptors, and five to nine times faster than for beta\textsubscript{2}-adrenoceptors.

6:2:3 Pharmacological characteristics of the specific binding sites on chick and frog erythrocytes

The binding of $^{3}$H-DHA to avian and amphibian erythrocytes had the characteristics of an interaction with beta-adrenoeceptors. Thus, binding was stereoselective, and displaceable by agonist and antagonist drugs with potencies expected for binding to a beta-adrenoceptor. Data presented in Table 6:1 compares the inhibition constants of beta-adrenoceptor agonists and non-selective antagonists for the binding sites on chick and frog erythrocytes with values obtained for mammalian beta\textsubscript{1}-(rabbit lung) and beta\textsubscript{2}-(rat erythrocyte) adrenoceptors.

From these data it was apparent that propranolol exhibited stereoselectivity indices for chick (2.1) and frog (1.9) erythrocytes which were similar to the indices obtained for mammalian beta\textsubscript{1} and beta\textsubscript{2}-adrenoceptors (see also Chapter 5). Inhibition constants of the non-selective antagonists (-) timolol and (-) propranolol were within two fold of the values obtained for beta\textsubscript{1} and beta\textsubscript{2}-adrenoceptors. These results suggested considerable pharmacological uniformity between the beta-adrenoceptor binding sites of these preparations.

The agonist displacement curves obtained for chick and frog erythrocytes differed in form and pharmacological characteristics (Figure 6:2). Thus, the catecholamines generated steep displacement curves with chick erythrocyte membranes, and noradrenaline had an affinity $\geq$ adrenaline. The relative potencies of agonists in displacing $^{3}$H-DHA binding to chick erythrocyte membranes correlated extremely well with their potencies for rabbit lung beta-adrenoceptors (Table 6:1).
Table 6:1
Inhibition constants of beta-adrenoceptor agonists and non-selective antagonists for membranes of frog, chick and rat erythrocytes and rabbit lung.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Frog erythrocyte</th>
<th>Chick erythrocyte</th>
<th>Rat erythrocyte (\beta_2)</th>
<th>Rabbit lung (\beta_1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(−)-Propranolol</td>
<td>0.68 (± 0.05)</td>
<td>0.75 (± 0.01)</td>
<td>0.44 (± 0.01)</td>
<td>0.90 (± 0.06)</td>
</tr>
<tr>
<td>(+)-Propranolol</td>
<td>49 (± 0.9)</td>
<td>95 (± 9)</td>
<td>29 (± 1.5)</td>
<td>77 (± 6)</td>
</tr>
<tr>
<td>(−)-Timolol</td>
<td>0.29 (± 0.03)</td>
<td>0.41 (± 0.06)</td>
<td>0.39 (± 0.01)</td>
<td>ND</td>
</tr>
<tr>
<td>(−)-Isoprenaline</td>
<td>77 (± 8)</td>
<td>23 (± 2)</td>
<td>33 (± 3)</td>
<td>23 (± 2)</td>
</tr>
<tr>
<td>(−)-Noradrenaline</td>
<td>16,500 (± 1,200)</td>
<td>120 (± 8)</td>
<td>3,000 (± 300)</td>
<td>370 (± 35)</td>
</tr>
<tr>
<td>(−)-Adrenaline</td>
<td>1,200 (± 105)</td>
<td>420 (± 36)</td>
<td>220 (± 14)</td>
<td>570 (± 38)</td>
</tr>
<tr>
<td>(±)-Salbutamol</td>
<td>670 (± 70)</td>
<td>3,260 (± 300)</td>
<td>360 (± 26)</td>
<td>2,060 (± 330)</td>
</tr>
<tr>
<td>(±)-Isoetharine</td>
<td>6,700 (± 800)</td>
<td>9,410 (± 850)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Inhibition constants of drugs were calculated from IC\(_{50}\) values as previously described. Data represent the mean (± SEM) of 3-8 determinations performed in duplicate.
Figure 6:2
Inhibition of specific $^3$H-DHA binding to chick erythrocyte membranes (A) and frog erythrocyte membranes (B) by agonists (-)-isoprenaline ($\circ$), (-)-noradrenaline ($\Delta$), (-)-adrenaline ($\triangle$) and (±)-salbutamol ($\bullet$). Specific $^3$H-DHA binding to the membranes was determined under standard assay conditions at $22^\circ$C, using 1.5 nM $^3$H-DHA. The data represented the mean ($\pm$ SEM) of 3-6 determinations performed in duplicate.
These findings suggested a beta₁-adrenoceptor classification for the binding sites on avian erythrocytes, in confirmation of previous characterisations (Brown et al, 1976a).

The catecholamine displacement curves obtained for frog erythrocyte membranes were shallow, and noradrenaline had an affinity which was > 10 fold weaker than adrenaline (Figure 6:2). This suggested that the binding sites on frog erythrocytes were beta₂-adrenoceptors as classified by Lands et al (1967).

In order to confirm these classifications, further detailed characterisation of the binding sites was performed using selective beta-adrenoceptor agents. Selective drugs generated displacement curves with chick and frog erythrocyte membranes which had Hill slope factors which were not significantly different from one. This finding suggested that the binding sites, like those found previously on rat erythrocytes, corresponded to a homogeneous population of beta-adrenoceptors.

The results of examining selective beta₁ or beta₂-adrenoceptor agents in these systems are presented in Table 6:2 and Figures 6:3, 6:4 and 6:5. The inhibition constants of drugs for chick, frog and rat erythrocyte beta-adrenoceptors have been compared with their mean inhibition constants obtained for mammalian beta₁ and beta₂-adrenoceptors.

A correlation of -log (inhibition constants) of drugs determined for rat erythrocyte beta-adrenoceptors with their mean -log (inhibition constants) for the beta₂-adrenoceptors of four mammalian tissues is shown in Figure 6:3. The line drawn is one with a slope of 1, i.e. a perfect correlation between the two sets of data. The excellence of the fit to this line (slope of data = 0.96 r = 0.995) suggested, as previously noted, that the beta-adrenoceptors on mammalian erythrocytes were identical to the beta₂ adrenoceptors from a number of mammalian
Table 6:2

Inhibition constants of selective drugs for beta-adrenoceptors of rat, frog and chick erythrocytes, and their mean inhibition constants for mammalian beta1- and beta2-adrenoceptors.

<table>
<thead>
<tr>
<th>Beta-adrenoceptor agent</th>
<th>Rat erythrocyte</th>
<th>Frog erythrocyte</th>
<th>Chick erythrocyte</th>
<th>Mammalian Betal-adrenoceptors</th>
<th>Mammalian Bet2-adrenoceptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atenolol</td>
<td>3,470 (± 240)</td>
<td>2,800 (± 190)</td>
<td>930 (± 22)</td>
<td>96 (± 42)</td>
<td>7,050 (± 1,900)</td>
</tr>
<tr>
<td>Practolol</td>
<td>27,400 (± 1,000)</td>
<td>5,220 (± 370)</td>
<td>2,200 (± 300)</td>
<td>326</td>
<td>37,200 (± 5,300)</td>
</tr>
<tr>
<td>Betaxolol</td>
<td>*202 (± 12)</td>
<td>182 (± 4)</td>
<td>46.5 (± 5)</td>
<td>3.4 (± 0.5)</td>
<td>245 (± 26)</td>
</tr>
<tr>
<td>Metoprolol</td>
<td>273 (± 7)</td>
<td>365 (± 4)</td>
<td>220 (± 20)</td>
<td>18</td>
<td>350 (± 65)</td>
</tr>
<tr>
<td>ICI 118.551</td>
<td>0.96 (± 0.03)</td>
<td>9.4 (± 0.3)</td>
<td>38.3 (± 4)</td>
<td>201 (± 80)</td>
<td>1.71 (± 0.36)</td>
</tr>
<tr>
<td>Procateler</td>
<td>31.8 (± 2.7)</td>
<td>370 (± 40)</td>
<td>7,500 (± 1,000)</td>
<td>2,900 (± 430)</td>
<td>32 (± 4.5)</td>
</tr>
<tr>
<td>H35/25</td>
<td>128 (± 2)</td>
<td>540 (± 30)</td>
<td>ND</td>
<td>ND</td>
<td>230</td>
</tr>
</tbody>
</table>

IC50 values of drugs for erythrocyte preparations were determined from Hill plots, and inhibition constants were derived as previously described. Results represented the mean (± SEM) of 3-8 determinations. Mean inhibition constants of drugs for mammalian beta1- or beta2-adrenoceptors were taken from Table 5:4. *Data obtained using rat reticulocyte membranes.
Correlation between -log inhibition constants (pK_i) of selective drugs for beta-adrenoceptor sites of rat erythrocytes and their mean inhibition constants for the beta_1-adrenoceptors of rat lung, rat cerebral cortex, rat heart and rabbit lung. Inhibition constants were calculated for rat erythrocyte beta-adrenoceptors as described previously, and values represented the mean (± SEM) of 3-7 determinations. Inhibition constants of drugs for beta_2-adrenoceptors of rat and rabbit tissue preparations were derived from mean curves of 3-8 determinations as described in Table 5:4.
tissues. Comparison of the data with mammalian beta1-adrenoceptors (not shown) gave a very poor correlation (slope of data -0.08, r = 0.116).

The goodness of fit of the previous data provided a yardstick by which to judge the following correlations. Figure 6:4 compares values of (-log inhibition constants) obtained for drugs using chick erythrocyte membranes with equivalent values obtained at mammalian beta1 and beta2-adrenoceptors. Clearly both correlations were poor, which suggested that avian beta-adrenoceptors were different from mammalian beta1 and beta2-adrenoceptors. The inhibition constants of beta1-selective antagonists for chick erythrocyte beta-adrenoceptors were all significantly greater than those obtained at mammalian beta1-adrenoceptors (Table 6:2). Furthermore, metoprolol had an affinity which indicated a beta2-adrenoceptor classification for the binding sites on chick erythrocytes.

Figure 6:5 compares the pharmacological characteristics of the beta-adrenoceptor binding sites on frog erythrocytes with mammalian beta1 and beta2-adrenoceptor sites. The higher correlation coefficient and slope of the data for the correlation with mammalian beta2-adrenoceptors indicated that the binding sites on frog erythrocytes were closer to a beta2-adrenoceptor classification. However, the anomalous affinities of three compounds suggested that the frog erythrocyte beta-adrenoceptors may have distinct differences to mammalian beta2-adrenoceptors.

6:3 Discussion

Beta-adrenoceptors of avian and amphibian erythrocytes have been used extensively as models for beta1 and beta2-adrenoceptors respectively, and a great deal of useful information has been published on the beta-adrenoceptor -adenylate cyclase coupling in these systems. (Levitzki and Helmreich, 1979; Lefkowitz and Hoffman, 1980). It was critical, therefore, to delineate uniformity between these beta-adrenoceptors and those of mammalian tissues. This study characterised
Figure 6:4
Correlation between -log (inhibition constants) (pKᵢ) of selective drugs for beta-adrenoceptor binding sites of chick erythrocytes and their pKᵢ's for mammalian beta₂-adrenoceptors (upper) and beta₁-adrenoceptors (lower).

Inhibition constants for chick erythrocyte beta-adrenoceptors were calculated as described previously. The values shown represented the mean (± SEM) of 3-6 determinations. Inhibition constants of drugs for beta₁- and beta₂-adrenoceptors of 2-5 mammalian tissue preparations were derived as shown in Table 5:4, using the curves of 3-8 determinations. Mean values (± SEM) for inhibition constants at both beta₁- and beta₂-adrenoceptors were then calculated.
Figure 6:5
Correlation between pK_i of selective drugs for beta-adrenoceptor binding sites of frog erythrocytes and their mean pK_i for beta_2-adrenoceptors (upper) or beta_1-adrenoceptors (lower). Inhibition constants were calculated as described previously, and the data shown represented the mean (± SEM) of at least 3 determinations in each case.
the $^3$H-DHA binding sites on chick and frog erythrocytes and compared their pharmacological characteristics with those found previously for mammalian beta$_1$ and beta$_2$-adrenoceptors.

The binding sites on these non-mammalian erythrocytes displayed all the characteristics expected for a beta-adrenoceptor. Thus, specific binding of radioligand was saturable, reversible, stereo-selective and inhibited by beta-adrenoceptor agents with potencies expected for interaction with a beta-adrenoceptor. There was, however, no evidence of negative co-operativity between the beta-adrenoceptors as was previously reported for frog erythrocytes (Limbird and Lefkowitz, 1976). Recently, other workers have failed to confirm negative co-operativity in this system (Pollet et al, 1979).

The relative affinities of the endogenous catecholamines noradrenaline and adrenaline for the binding sites on chick and frog erythrocytes suggested beta$_1$ and beta$_2$-adrenoceptor classifications, as previously reported (Lefkowitz 1975a; Brown et al, 1976a). Moreover, the relative affinities of a number of natural and synthetic agonists for the beta-adrenoceptor binding sites on chick erythrocytes was similar to their potency ratios determined for inotropic responses of chicken atria (Lands et al 1969, Daly and Levy, 1979). Although there were hazards associated with the use of agonist affinities to characterise receptors (Chapter 5), these findings suggested similarities between the beta-adrenoceptors of chicken atria and erythrocytes.

The affinities of non-selective beta-adrenoceptor antagonists for the binding sites of chick and frog erythrocytes were virtually identical to those determined for mammalian beta-adrenoceptors which suggested that certain characteristics of the binding sites were similar in all tissues. However, the affinities of a number of selective antagonists in these systems deviated markedly from those
expected for mammalian beta₁ and beta₂-adrenoceptors. Thus, although the overall pharmacological pattern in chick erythrocytes more closely resembled mammalian beta₁ rather than beta₂-sites, the beta₁-selective antagonists exhibited affinities which were all at least seven fold weaker than those for mammalian beta₁-adrenoceptors. These differences were considerably greater than the three fold differences in affinities which Furchgott (1972) has suggested would be reasonable evidence for the existence of receptor heterogeneity.

The beta₁-selective antagonists differed from the non-selective agents in having para substitutions on the aromatic nucleus. It seems reasonable to conclude, therefore, that the accessory binding sites which interacted with these groups may be lacking in chick erythrocyte beta-adrenoceptors. Gibson et al (1979) have compared the affinities of a number of ortho, meta, and para substituted beta-blockers for the beta₁-adrenoceptor binding sites of rat and guinea pig heart, with those of turkey erythrocytes. They showed that para-substitution was associated with decreased affinities and ortho or meta substitutions increased affinities of drugs for the beta-adrenoceptors of turkey erythrocytes. These findings indicated differences in the accessory binding sites of mammalian beta₁-adrenoceptors and turkey erythrocyte beta-adrenoceptors.

In addition to these pharmacological distinctions, there were major differences between radioligand binding to beta-adrenoceptors of chick erythrocytes and mammalian beta₁-adrenoceptors. The association and dissociation rates for ³H-DHA binding to both beta₁ and beta₂-adrenoceptors of mammalian tissue preparations has been shown to be relatively constant (Chapter 5). It was significant, therefore, that the association and dissociation rates of radioligand binding to avian erythrocytes were considerably higher than the equivalent rates of mammalian beta₁-adrenoceptors. Other workers, using the radio-
labelled ligand $^{125}$I-HYP have reported binding kinetics to turkey erythrocytes which were considerably enhanced in comparison to mammalian beta$_1$-adrenoceptors (Brown et al, 1976a; Minneman et al, 1980). These kinetic differences have been suggested to relate to differences in the entropy of activation for the beta-adrenoceptors of avian erythrocytes (Minneman et al, 1980).

The previous observations added to the growing list of unusual characteristics of avian erythrocyte beta-adrenoceptors. Thus, the efficacies of agonists at turkey erythrocyte beta-adrenoceptors and their efficacies at mammalian beta$_1$- (or beta$_2$)-adrenoceptors were not correlated (Minneman et al, 1980). Turkey erythrocytes did not 'down-regulate' their beta-adrenoceptor number in response to incubation with agonists (Hanski and Levitzki 1978; Hoffman et al, 1979). Furthermore, agonist affinities for avian beta-adrenoceptors were not modulated by guanine nucleotides under standard assay conditions (Bilezikian et al, 1978; see also Chapter 7).

The beta-adrenoceptors of frog erythrocytes possessed pharmacological characteristics which were closer to those of mammalian beta$_2$-sites, although significant deviations were again observed. In this case, the beta$_2$-selective agents were significantly weaker than expected for a mammalian beta$_2$-adrenoceptor site. Since these drugs possessed substitutions at the alpha-carbon of the side chain, it seemed likely that the accessory binding sites which interacted with this group in mammalian beta$_2$-adrenoceptors differed from those of frog erythrocytes.

The possibility that the pharmacological properties of these non-mammalian beta-adrenoceptors related to the co-presence of beta$_1$ and beta$_2$-adrenoceptors could be eliminated since all the antagonists tested generated displacement curves with slope factors close to unity, which indicated homogeneity of the labelled sites. It also seemed unlikely that the differences observed in beta-adrenoceptors of chick and frog erythrocytes reflected the atypical nature of the erythrocyte
preparations, since rat erythrocyte beta-adrenoceptors had identical properties to those of other mammalian beta₂-adrenoceptors. Moreover, the atypical beta-adrenoceptors of chick erythrocytes were not related to the juvenile nature of the animals used, since similar pharmacological properties have been described for turkey erythrocytes (Minneman et al., 1980). In view of these observations, it seemed most likely that the differential characteristics related to phylogenetic differences between the amphibians, avia, and mammals. Before true phylogenetic differences can be proposed, it will be important to establish whether the pharmacological properties of these atypical beta-adrenoceptors are conserved between tissues of amphibian and aves. In support of this concept, preliminary studies of the beta-adrenoceptor binding sites in frog heart have indicated similar pharmacological properties to those on frog erythrocytes (Nahorski et al. unpublished).

It is not yet clear whether these non-mammalian sites should be classed as further beta-adrenoceptor subtypes. Clearly the affinities of a number of non-selective agents for these sites, together with the relative affinities of the endogenous catecholamines adrenaline and noradrenaline, suggested that the major binding characteristics of these sites as beta-adrenoceptors were conserved. The differences in accessory binding sites which were delineated by 'pharmacological' agents provided support for the concept of 'isoreceptors' as proposed by Brittain et al. (1970).
CHAPTER 7

Agonist interaction with beta-adrenoceptors
7.1 Introduction

Rapid progress has been made in recent years in the elucidation of the mechanism by which agonist occupation of beta-adrenoceptors is translated into a biochemical response (activation of adenylate cyclase). A number of models have been proposed to account for the processes involved in receptor-effector coupling which inevitably reflected the characteristics of the system from which they were derived (see Tolkovsky and Levitzki 1978; Hollenberg 1978; Ariens 1979; Rodbell 1980; DeLean et al 1980; Swillens and Dumont 1980). A testable model will be developed along lines drawn by DeLean and co-workers, and Swillens and Dumont which accounts for many of the experimental findings of beta-adrenoceptor-effector coupling. This model excludes the involvement of (presently) hypothetical components (Levitzki and Atlas, 1981).

At present the beta-adrenoceptor-effector system appears to consist of three components, (i) a receptor, (ii) a guanine nucleotide regulatory protein, N protein, and (iii) adenylate cyclase. A number of alternative approaches have been made by different laboratories to investigate the functioning of these components. These have included (i) radiolabelled ligand binding techniques coupled with adenylate cyclase measurements (Hoffman and Lefkowitz, 1980), (ii) purification and separation of the components (Pfeuffer and Helmreich, 1975; Limbird and Lefkowitz 1977), (iii) measurements of the thermodynamic parameters for binding and cyclase activation (Weiland et al, 1979), (iv) studies aimed at correlating the effects of perturbing agents on properties of the membrane system (Bakardjieva et al, 1979), (v) studies of genetically manipulated cell lines possessing deletions of certain components (Ross et al, 1977), (vi) cell fusion techniques (Orly and Schramm, 1976) (vii) reconstitution studies of membranes (Eimerl et al, 1980) or phospholipid vesicles (Fleming and Ross, 1980) and (viii) target size analysis of the interacting components (Houslay et al, 1977).
Results of such studies have led to a description of the events leading to adenylate cyclase activation as follows: a beta-adrenoceptor agent (A) binds to receptor (R) and, depending on its intrinsic activity induces a conformational change in the receptor, which becomes more susceptible to alkylation by NEM (Vauquelin et al. 1980 a and b). Activation of the receptor (R*) allows for a productive association of guanine nucleotide regulatory protein (Nprotein) and formation of A-R-Nprotein complex. This ternary complex accumulates and represents the 'high affinity' state of the receptor. Activation of the Nprotein follows an exchange of bound GDP for GTP. The binding of GTP results in a destabilisation of the complex and a dissociation of agonist-receptor from activated Nprotein. The association of Nprotein with inactive adenylate cyclase (E) results in enzyme activation (E*), and catalytic production of CAMP. Once the adenylate cyclase is activated, GTP is hydrolysed to GDP by an active GTP-ase which resides on the Nprotein. The Nprotein can then rejoin the cycle of reactions. The evidence in favour of this scheme has been presented in a number of recent reviews (Stadel et al., 1981a, Limbird 1981).

The above components are thought to be vectorially placed in a lipid environment, the physical properties of which can profoundly influence receptor-effector coupling (Ross and Gilman, 1980). In addition the system is probably under control by cytoskeletal elements (Insel and Kennedy, 1978). At present there is some controversy as to the rate-limiting step in this system (Levitzki 1980). The reaction has been suggested to be the dissociation of GDP bound to the A-R-Np complex (Swillens et al., 1979) for turkey erythrocyte membranes, and the introduction of GTP into the Nprotein in a reconstituted system (Citri and Schramm 1980). Iyengar et al. (1980) have postulated that an 'isomerisation' of adenylate cyclase, which follows occupation of Nprotein by Gpp(NH)p, represents the rate limiting step.
Prolonged occupation of receptors by agonists have been shown to result in desensitisation of the agonist-induced responsiveness. The molecular mechanisms involved in this response remain to be determined, but they include an impaired ability of receptors to form high affinity A-R-N-protein complexes (Stadel et al, 1981b). Alternatively receptors may be 'down-regulated' (Su et al, 1979), a procedure which may involve covalent modification of receptors (Levitzki and Atlas, 1981) or internalisation of receptors (Chuang et al, 1980). Recently 8-Br.cyclic AMP has been suggested to reproduce isoprenaline-induced desensitisation in turkey erythrocyte membranes, by an effect on the N-protein. (Stadel et al, 1981b). This implied that the N-protein may also be under negative feedback control by the product of adenylate cyclase, perhaps indirectly through a cAMP-dependent phosphorylation reaction.

A diagramatic illustration of the events between receptor occupation and adenylate cyclase stimulation is shown below:-

A diagramatic illustration of the events between receptor occupation and adenylate cyclase stimulation is shown below:-

A = beta-adrenoceptor agonist
R = beta-adrenoceptor
\(N_{GDP}\) = GDP-loaded N-protein
\(N_{GTP}\) = active GTP-loaded N-protein
E = adenylate cyclase

Asterisks denote activated moiety.
\(\Rightarrow\) possible rate limiting step.
The studies to be described will use radiolabelled ligand binding techniques to investigate the applicability of this scheme to the beta-adrenoceptor preparation used previously. In particular, agonist binding to beta-adrenoceptors in the absence and presence of GTP will be studied, since this approach has been particularly fruitful for the investigation of agonist interaction with frog erythrocyte beta-adrenoceptors. The influence of temperature, ions and membrane perturbing agents will also be examined.

7:2 Results

7:2:1 Agonist binding to beta-adrenoceptors and its modulation by guanine nucleotides

(A) Measurement of GTP-sensitive agonist binding under standard conditions

Agonist binding to beta-adrenoceptors was assessed by measuring the competition between agonist and $^3$H-DHA for the specific sites on membranes. This indirect technique has been shown to produce identical results to those obtained using radio-labelled agonists directly (Lefkowitz and Hamp, 1977). The effects of guanine nucleotides upon isoprenaline binding to beta-adrenoceptors of mammalian and non-mammalian tissue preparations were determined using standard assay conditions as described in 'Methods'. These experiments were therefore conducted in 50mM-Tris HCl pH 7.8 buffer at 22°.

Isoprenaline displacement of $^3$H-DHA binding to rat lung membranes was measured in the absence and presence of increasing concentrations of GTP (Figure 7:1). The inhibition curve obtained in the absence of nucleotide exhibited heterotropic binding with a slope factor, $n_H \approx 0.6$. The presence of GTP $10^{-7}$-$10^{-3}$M, resulted in a gradual decrease in the overall affinity of isoprenaline, and a graded increase in the slope of the curves. The maximum shift in the agonist curve was obtained with 100µM GTP and the concentration of GTP causing half maximal effect was approximately 8µM which was similar to the value reported for the fat cell beta-adrenoceptor (Malbon, 1980). These results could be explained
Figure 7:1
Effect of GTP on isoprenaline binding to rat lung beta-adrenoceptors.
Isoprenaline binding to rat lung membranes was measured in the absence and presence of increasing concentrations of GTP (10⁻⁷-10⁻³), employing standard assay conditions. Results were the mean of 2-5 determinations performed in duplicate.
by assuming the existence of high and low affinity states of the beta_{2}-adrenoceptor which were converted to a homogeneous low affinity state by concentrations of GTP > 100\mu M. Curve fitting the data to a two 'state' model indicated that the ratio of the affinities of isoprenaline for the putative high and low affinity states was progressively decreased with increasing GTP concentrations (data not shown). The term receptor 'state' is used, rather than site, because the beta-adrenoceptors bound agonists with a variety of apparent affinities.

The ability of related purine nucleotides to decrease the affinity of isoprenaline for the beta-adrenoceptor on rat lung membranes was also examined. Table 7:1 shows that the non-hydrolysable analogue guanylylimidodiphosphate (Gpp(NH)p) was the most potent nucleotide tested. Gpp(NH)p has been reported to be more potent than GTP in causing stimulation of adenylate cyclase, and decreasing agonist binding affinities in turkey erythrocytes, (Spiegel and Aurbach, 1974); frog erythrocytes (Lefkowitz et al, 1976); and rat fat cells (Malbon, 1980). The order of potencies of the nucleotides in altering isoprenaline binding to rat lung membranes, Gpp(NH)p > GTP > ATP ≈ GMP, was similar to that obtained using frog erythrocyte membranes (Williams and Lefkowitz, 1977).

<table>
<thead>
<tr>
<th>Nucleotide (μM)</th>
<th>Shift of IC_{50} for isoprenaline fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gpp(NH)p</td>
<td>2.2</td>
</tr>
<tr>
<td>10</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
</tr>
<tr>
<td>GTP</td>
<td>0.5</td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td></td>
</tr>
<tr>
<td>GMP</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Table 7:1 Effect of purine nucleotides upon the displacement of \(^3\)H DMA binding to rat lung membranes by (-) isoprenaline
The effect of GTP on isoprenaline binding to beta_1- and beta_2-adrenoceptors was compared. Figure 7:2 shows data obtained for the beta-adrenoceptor preparations - frog erythrocytes, rat erythrocytes, and rat reticulocytes. The binding of isoprenaline to frog erythrocyte beta-adrenoceptors was markedly influenced by GTP, and these data modelled to a two-receptor state system in the absence of GTP consisting of 46% of receptors in the high affinity state, and to a one-state system in the presence of GTP. The IC_{50} of isoprenaline in the presence of GTP (1.5x10^{-7}M) was close to that for the low affinity receptor state (1.4x10^{-7}M). The isoprenaline displacement curves obtained with rat reticulocyte and rat erythrocyte membranes were not markedly affected by GTP. (Figure 7:2 B and C). Similar findings were observed for the beta_1-adrenoceptor preparations of chick erythrocytes and rabbit lung (Figure 7:3). Thus, GTP caused a small parallel shift in the isoprenaline curves of rat reticulocytes and rabbit lung membranes but the nucleotide did not alter the isoprenaline curves of rat or chick erythrocyte membranes.

The absence of GTP-induced alterations in agonist binding characteristics in these systems did not support the universal applicability of the above receptor model. However, the inability of GTP to modulate isoprenaline binding in these 'non-reactive' systems could have been due to:-(i) hydrolysis of GTP by active GTPases, (ii) loss of essential endogenous factors, (iii) the absence of essential cations, (iv) differential temperature requirements, (v) tight binding of GDP. Experiments were therefore conducted to test for these possibilities.

(B) Use of the non-hydrolysable analogue Gpp(NH)p

The replacement of GTP by Gpp(NH)p was examined under standard assay conditions of temperature and ionic conditions. Isoprenaline binding in the presence of 100μM Gpp(NH)p was determined for membrane
Isoprenaline displacement of specific $^3$H-DHA binding to (A) frog erythrocyte, (B) rat reticulocyte and (C) rat erythrocyte membranes in the absence (solid symbols) and presence (open symbols) of GTP (100 μM). Specific binding was measured under standard assay conditions (see Methods) at 22°C. $^3$H-DHA was present for all experiments at a concentration of 1-1.5 nM. Results were the mean of 3-5 experiments performed in duplicate and SEM's were <10%.
Figure 7:3
Inhibition of specific $^3$H-DHA binding to (A) chick erythrocyte and (B) rabbit lung membranes by isoprenaline, in the absence (solid symbols) and presence (open symbols) of 100 μM GTP. Experiments were conducted at 22°C under standard incubation conditions. Results were the mean (± SEM) of six (chick erythrocyte) and seven (rabbit lung) determinations performed in duplicate.
preparations of rat erythrocytes, rat reticulocytes and chick erythrocytes. These data were indistinguishable from those obtained using GTP (data not shown), which suggested that hydrolysis of GTP was not involved in the 'non-reactivity' of these systems.

(C) The influence of endogenous factors

The membrane preparations used in the previous studies were well washed, which may have resulted in the loss of endogenous factors such as those reported to be present in lysates of rat reticulocytes (Omrani et al, 1980). The degree of membrane purification has been reported to influence the agonist binding data obtained with frog erythrocytes (Kent et al, 1980). Therefore isoprenaline displacement curves were determined for washed rat reticulocyte membranes to which dialysed rat lung supernatant (obtained after the first centrifugation at 50,000g, see Methods) or rat reticulocyte lysate had been added. The effect of GTP upon isoprenaline binding was identical in the absence and presence of these supernatants (data not shown).

(D) The effect of Mg\(^{2+}\) on isoprenaline binding

Isoprenaline displacement of \(^3\)H-DHA binding to membrane preparations of rat lung, rat erythrocytes and reticulocytes, and chick erythrocytes was determined in the absence and presence of 5mM Mg\(^{2+}\), using standard assay conditions. The inclusion of this cation failed to have any significant effect on isoprenaline binding to these beta-adrenoceptors, either on the overall affinity, or the slope factor of the displacement curves (data not shown). Experiments performed with 10mM MgCl\(_2\), 1mM EDTA using the above membrane preparations (and including rabbit lung membranes) gave similar results.

The total Mg content of membrane preparations, in which isoprenaline affinities were modulated by GTP (rat lung) or unaffected (chick, rat erythrocytes) were determined by atomic absorption measurements (courtesy
Results showed that rat lung membranes (7nmols total Mg /mg protein) were not enriched in this cation relative to chick (13.5nmols Mg /mg protein) or rat (4nmols/mg protein) erythrocyte membranes.

The dual influence of temperature and Mg$^{2+}$

Isoprenaline displacement of $^3$H-DHA binding to membrane preparations of rat reticulocytes, rat erythrocytes, rabbit lung, and chick erythrocytes was measured at 37° in buffers containing 10mM MgCl$_2$ 1mM EDTA. Figure 7:4 shows the results obtained in the absence and presence of 100μM GTP. Under these conditions GTP modulated the binding of isoprenaline to all membrane preparations with the exception of rat erythrocytes. Isoprenaline binding in the presence of GTP was indicative of binding to a uniform state of the receptor according to mass action law. The importance of both incubation temperature, and the presence of Mg /EDTA for GTP modulation of agonist binding was demonstrated by comparing agonist binding data at 37° in the absence and presence of Mg/EDTA. Under such conditions, GTP produced a 1.7 and 2.8 fold increase in the $IC_{50}$ of isoprenaline for rat reticulocyte beta-adrenoceptors respectively. Similar observations were made using rabbit lung membranes. Therefore, in these receptor systems full modulation of agonist affinity by GTP was obtained only when incubations were performed at physiological temperatures, and in the presence of Mg /EDTA. In contrast, agonist binding data to rat lung membranes was unaffected by temperature and Mg /EDTA.

The modulation of agonist binding to avian erythrocyte beta-adrenoceptors by guanine nucleotides had an absolute requirement for the presence of Mg /EDTA, and incubation temperatures $>30^{\circ}$ (Figure 7:5). Thus, isoprenaline binding was unaffected by GTP, in the absence of Mg /EDTA at 22°, 30° (Figure 7:5A) or 37° (Weiland et al., 1979). In the presence of Mg /EDTA, GTP decreased the affinity of isoprenaline at 30°, but the nucleotide was ineffective at 22° (Figure 7:5B).
Modulation of isoprenaline binding to rat reticulocyte (A), rat erythrocyte (B), rabbit lung (C) and chick erythrocyte (D) by GTP in the presence of Mg\(^{2+}\) EDTA. Membranes were incubated in Tris buffers containing 10 mM MgCl\(_2\), 1 mM EDTA for 15-20 min. at 37°C in the absence (closed symbols) and presence (open symbols) of 100 µM GTP, and isoprenaline displacement of specific \(^3\)H-DHA binding was determined. Results were the mean of 3-5 experiments performed in duplicate (SEM's were <10%).
Figure 7:5
The effect of temperature and Mg$^{2+}$-EDTA on the ability of GTP to modulate isoprenaline binding to chick erythrocyte membranes. Fig. A: Membranes were incubated at 22°C (▲,●) for 30 min., or 30°C (■,□) for 20 min. under standard assay conditions in the absence (closed symbols) and presence (open symbols) of GTP. Fig. B: Assays were performed in the presence of 10 mM MgCl$_2$/1 mM EDTA. Results were the mean of 3-6 experiments performed in duplicate. SEM's were <10%.
The effect of temperature and Mg-EDTA on the ability of GTP to modulate isoprenaline binding to beta_1 and beta_2-adrenoceptors of rabbit lung

The membrane preparations of rabbit (and rat) lung contained a heterogeneous population of beta-adrenoceptor binding sites. In these systems, agonists had the potential for binding to putative high and low affinity states of both subtypes, which would make interpretation of GTP effects particularly difficult. Therefore, experiments were conducted which explored agonist binding to beta_1 and beta_2-adrenoceptor sites, which had been 'isolated' by the use of selective antagonists.

Figure 7:6 (A and B) shows isoprenaline binding to control rabbit lung membranes and to membranes where a large proportion of beta_1 or beta_2-adrenoceptors had been occupied with atenolol and ICI 118,551 respectively. These experiments were conducted at 22°C in Tris-buffer. The isoprenaline displacement curve obtained with control membranes, where both beta_1 and beta_2-adrenoceptors were accessible, had a slope factor of 0.78. GTP (100μM) shifted this curve to higher isoprenaline concentration, without affecting the slope factor. Isoprenaline binding to the 'isolated' beta_1-adrenoceptors was characterised by a steeper displacement curve, n_H = 0.90 which was not markedly affected by GTP (Figure 7:7,B). In contrast, the curve of isoprenaline binding to the 'isolated' beta_2-adrenoceptors was shallow, and GTP shifted and steepened this curve to the right (Figure 7:6,B). Under these experimental conditions, the overall affinity of isoprenaline for the beta_1-adrenoceptors of this preparation was 5-fold greater than its affinity for the beta_2-adrenoceptors.

These results suggested that at 22°C, under standard assay conditions, isoprenaline binding to the beta_2-adrenoceptors induced high affinity agonist-receptor complexes which were converted to a uniform low affinity state of the receptor by GTP. Isoprenaline binding to beta_1-adrenoceptors
Figure 7:6
Modulation of isoprenaline binding to rabbit lung beta1 and beta2 adrenoceptors by GTP at different temperatures. Figs. (A) and (C): Isoprenaline displacement of $^3$H-DHA ($\approx 1.5$ nM) binding was measured at 22°C (Fig. A) or 37°C (Fig. C) under standard assay conditions, in the absence (closed symbols) and presence (open symbols) of 100 $\mu$M GTP. Figs. (B) and (D): Incubations were performed at 22°C (B) or 37°C (D) under identical conditions, except for the inclusion of 50 nM ICI 118.551 or 75 $\mu$M atenolol to occupy 90% of the beta2 and beta1 adrenoceptors respectively. Results were the mean of 3-5 experiments performed in duplicate. SEM's were <10%.
was characterised by a high proportion of GTP-insensitive, sites. Therefore, the isoprenaline displacement curves of control membranes probably represented three isoprenaline binding states in the absence (high and low affinity states of beta-adrenoceptors, low affinity state of beta_1-adrenoceptors) and two binding states (low affinity beta_2, low affinity beta_1) in the presence of GTP.

Similar experiments were conducted at 37°C (Figure 7:6 C and D). Under these conditions, isoprenaline binding to control rabbit lung membranes was more GTP-sensitive, which suggested that a much higher proportion of these sites had formed high affinity agonist-receptor complexes. In support of this conclusion, isoprenaline binding to the 'isolated' beta_1-adrenoceptors, which comprised 80% of the total receptors, was markedly influenced by GTP (Figure 7:6,D). The isoprenaline binding curves of control rabbit lung membranes at 37°C could be postulated to consist of high and low affinity binding states for both beta_1 and beta_2-adrenoceptors. If the affinities of isoprenaline for these states were similar for beta_1 and beta_2-adrenoceptors, the control membranes would possess two affinity states for isoprenaline in the absence and one affinity state in the presence of GTP. In support of this postulate, isoprenaline displacement of ^3H-DHA binding to control membranes was indicative of binding to a single binding state of the receptor.

The influence of membrane perturbing agents - cis vaccenic acid

Chick erythrocytes and rabbit lung membranes were treated with the fatty acid cis-vaccenic acid. Isoprenaline binding to treated and control membranes was determined at 22°C in the absence and presence of Gpp(NH)p, in MgCl_2-EDTA containing buffers (Figure 7:7). The major effect of cis-vaccenic acid treatment in both membrane systems was an increased ability of Gpp(NH)p to modulate isoprenaline affinity. Thus,
Figure 7.7
The effect of cis vaccenic acid treatment on Gpp(NH)p-sensitive isoprenaline binding to chick erythrocyte (A) and rabbit lung (B) membranes. Membranes were preincubated with 1 mM cis vaccenic acid in EtOH at 4°C for 30 min. Control membranes were treated with EtOH (1% v/v). Isoprenaline displacement of ³H-DHA binding was measured at 22°C in the absence (closed symbols) and presence (open symbols) of 100 µM Gpp(NH)p in Mg/EDTA buffers. Results represented the mean of at least 3 determinations performed in duplicate. SEM's were <10%.
Gpp(NH)p barely influenced isoprenaline binding to control chick erythrocyte beta-adrenoceptors, whereas the nucleotide caused a five-fold increase in the IC$_{50}$ of isoprenaline for treated membranes. Similar, quantitatively smaller results were obtained for rabbit lung membranes. These effects were not related to the presence of cis vaccenic acid in the subsequent assay, nor to major alterations in the affinity of the radioligand for the treated membranes. The $K_D$ of $^3$H-DHA for the beta-adrenoceptors of control and treated membranes equalled 1.2nM and 2.0nM respectively. There was a relatively selective effect of cis-vaccenic acid treatment on the isoprenaline binding data obtained in the absence of guanine nucleotides. This suggested that fatty acid treatment, perhaps by fluidising the membrane lipids, was allowing more high affinity states of the receptor to be induced by isoprenaline. However, treatment of rabbit lung membranes appeared to result in a parallel shift in the agonist displacement curve which suggested that the proportions of high and low affinity states of these receptors had not altered.

Similar experiments were performed using chick erythrocyte membranes at 37$^\circ$ (data not shown). The ability of guanine nucleotides to modulate isoprenaline binding at this temperature was similar for control and treated membranes. The effect of treatment was a three-fold increase in the affinity of isoprenaline, measured in the absence or presence of GTP.

Rat erythrocyte membranes, which failed to demonstrate GTP modulation of isoprenaline binding under optimum ion and temperature conditions, were treated with cis vaccenic acid. Isoprenaline binding to these membranes was identical in the absence and presence of GTP, although the IC$_{50}$ of isoprenaline in this system was 2-3-fold more potent than previous values obtained with non-treated membranes.
Membrane preparations of rat and rabbit lung, chick and rat erythrocytes were treated with cis vaccenic acid in the absence of Mg/EDTA. Under these conditions fatty acid treatment resulted in a considerable loss of specific $^3$H-DHA binding sites (data not shown). This was also accompanied by large increases in non-specific binding (as defined by 200μM (-) isoprenaline), which were especially marked for chick erythrocyte membranes.

7:2:2 Partial agonist and antagonist binding to beta-adrenoceptors and its modulation by GTP

(A) The relationship of GTP-sensitive binding to intrinsic activity

The ability of GTP to modulate full agonist (isoprenaline) partial agonist (salbutamol) and antagonist (propranolol) binding to rat lung membranes was determined using standard assay conditions. (Figure 7:8). Clearly GTP had no effect on propranolol binding, whereas the binding of salbutamol and isoprenaline was modulated by this nucleotide. In the presence of GTP, all curves were indicative of binding to a homogeneous state of the receptors according to mass-action law. The difference between the full and partial agonist was the proportion of high affinity binding state generated, and the ratio of the affinities of the drugs for the high and low affinity states of the receptor (isoprenaline 46% high affinity state, ratio $K_L/K_H = 50$; salbutamol 34% high affinity state, $K_L/K_H = 19$).

The concept (Lefkowitz et al, 1976) that GTP sensitive agonist binding was directly correlated with a drug's efficacy was examined. The intrinsic activities of agonists upon adenylate cyclase, were compared with the ability of GTP to modulate binding of these agents. Table 7:2 shows the intrinsic activities of agonists relative to the isoprenaline maxima) on beta$_1$-adrenoceptors of chick erythrocytes, and beta$_2$-adrenoceptors of rat reticulocytes and rat lung. These data indicated
Figure 7:8
The effect of GTP upon full agonist, partial agonist and antagonist binding to rat lung membranes. Rat lung membranes were incubated under standard assay conditions in 50 mM Tris-HCl, pH 7.8. (-)-Propranolol (A), (±)-salbutamol (B) and (-)-isoprenaline (C) inhibition of $^3$H-DHA binding to rat lung membranes was assessed in the absence (closed symbols) and presence (open symbols) of 100 μM GTP. Results represented the mean curves of 3-8 determinations performed in duplicate. SEM's were <10%.
clear differences between the ability of salbutamol and procaterol to activate the beta_2-adrenoceptors of rat lung and reticulocytes, where they were partial agonists, and the beta_1-adrenoceptors of chick erythrocytes, where they had negligible activity. Binding studies performed under optimal conditions for the measurement of GTP-sensitive binding, showed that neither salbutamol nor procaterol binding to chick erythrocyte beta-adrenoceptors was modulated by GTP (Figure 7:9).

**TABLE 7:2 Intrinsic activities of agonists on adenylate cyclase of chick erythrocytes, rat erythrocytes and rat lung**

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Intrinsic activity (% of Isoprenaline maxima).</th>
<th>Chick</th>
<th>Rat reticulocytes</th>
<th>Rat lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoprenaline</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>99 (±6)</td>
<td>90 (±6)</td>
<td>60 (±5)</td>
<td></td>
</tr>
<tr>
<td>Salbutamol</td>
<td>5 (±3)</td>
<td>62 (±4)</td>
<td>56 (±5)</td>
<td></td>
</tr>
<tr>
<td>Adrenaline</td>
<td>96 (±4)</td>
<td>101 (±5)</td>
<td>88 (±10)</td>
<td></td>
</tr>
<tr>
<td>Procaterol</td>
<td>3 (±2)</td>
<td>72 (±5)</td>
<td>64 (±3)</td>
<td></td>
</tr>
</tbody>
</table>

These results provided support for the concept that GTP-sensitive binding correlated with the intrinsic activity of the agonist.

However, the apparent partial agonist activity of noradrenaline at rat lung beta-adrenoceptors was not reflected by a diminished capacity of GTP to modulate noradrenaline binding (data not shown). Noradrenaline has been reported to be a partial agonist at the beta_2-adrenoceptors of frog erythrocytes (Pike and Lefkowitz, 1980), although full agonist activity has been described for this agent at rat lung beta-adrenoceptors (Minneman *et al.*, 1979a). The possibility that the above result related
Figure 7:9
Effect of GTP upon agonist binding to chick erythrocyte membranes. Chick erythrocytes were incubated in Mg-EDTA buffers in the absence (closed symbols) and presence (open symbols) of 100 μM GTP. Specific $^3$H-DHA binding was assessed in the presence of increasing concentrations of (−)-isoprenaline (A), (±)-salbutamol (B) and (±)-procaterol (C), as previously described. Incubations were performed for 15 min. at 37°C. The results were the mean of at least three determinations. SEM's were <10%.
to noradrenaline stimulation of inhibitory alpha₂-adrenoceptors was tested by performing adenylate cyclase measurements in the presence of phentolamine (1μM). Similar noradrenaline-stimulated adenylate cyclase activities were found in the absence or presence of this alpha-adrenoceptor antagonist (data not shown), which argued against this possibility. A further apparent anomaly was provided by the data obtained for procaterol, which was a partial agonist at rat lung beta₂-adrenoceptors although the binding data was not markedly affected by GTP (data not shown). The measurements of agonist stimulated adenylate cyclase activity in rat lung membranes were complicated by the heterogeneous receptor system, and the methodological difficulties of a system having very high basal enzyme activity. Clearly future studies aimed at relating intrinsic activity of drugs to GTP-sensitive binding would be best performed with homogeneous receptor systems.

(B) Differential efficacy at beta-adrenoceptor subtypes as a mechanism for the in vivo selectivity of partial agonists

Salbutamol exerted partial agonist activity at beta₂-adrenoceptors of rat lung and rat reticulocytes but it was inactive at beta₁-adrenoceptors of chick erythrocytes (Table 7:2) and rat heart (Burges and Blackburn, 1972; Minneman et al., 1979a). Binding studies indicated that this agent exerted no selective affinity for beta₂-adrenoceptors (Rugg et al., 1978; Minneman et al., 1979a). These findings suggested that the in vivo beta₂-adrenoceptor selectivity of this drug was perhaps related to selective efficacy at beta₂-adrenoceptors. This concept was studied using membrane preparations of rat and rabbit lung. The affinity of salbutamol for these beta-adrenoceptors was examined in the absence and presence of 100μM GTP, using standard incubation conditions.

Figure 7:10 shows that GTP modulated salbutamol binding to rat lung
Figure 7:10
Modulation of salbutamol binding to rat lung (A) and rabbit lung (B) membranes by GTP. Rat and rabbit lung membranes were incubated with increasing concentrations of salbutamol in the absence (closed symbols) and presence (open symbols) of 100 µM GTP. Incubations were performed at 22°C for 30 min., and specific ³H-DHA binding was determined using standard assay conditions. Results shown were the mean (± SEM) of 3 determinations performed in duplicate.
beta$_2$-adrenoceptors, but the nucleotide was without effect at rabbit lung beta$_1$-adrenoceptors. In the presence of GTP, the affinity of salbutamol for these receptor systems was identical.

The effect of GTP on salbutamol binding to beta$_1$ and beta$_2$-adrenoceptors of rabbit lung membranes was determined at 37°C in the presence of Mg/EDTA (Figure 7:11). Under these conditions, salbutamol binding to control membranes (Figure 7:11,A) was modulated by GTP, which resulted in a small shift in the overall IC$_{50}$ of salbutamol from 5.5-7.5μM. Salbutamol binding to the beta$_1$ or beta$_2$-adrenoceptors was also examined by prior occupation of one subtype by selective antagonists. Salbutamol binding to the beta$_2$-adrenoceptors of rabbit lung was similar to that seen with rat lung membranes (compare Figure 7:11,C with Figure 7:10,A), which suggested that a proportion of the receptors were in a high affinity, GTP-sensitive state. In contrast, salbutamol binding to rabbit lung beta$_1$-adrenoceptors was little influenced by GTP. These results confirmed the non-selective affinity of salbutamol for beta$_1$ and beta$_2$-adrenoceptors, and suggested that the ability of this partial agonist to induce high affinity states of beta$_2$-adrenoceptors was considerably greater than its respective capacity at beta$_1$-adrenoceptors.

Salbutamol displacement of $^3$H-DHA binding to the homogeneous beta$_1$-adrenoceptor system in guinea pig myocardial membranes was indicative of binding to a homogeneous low-affinity binding state of the receptor (data not shown). This finding provided further indications for the poor efficacy of salbutamol at beta$_1$-adrenoceptors.

7:2:3 The effect of temperature upon agonist and antagonist binding to beta-adrenoceptors

(A) Agonists

The effect of temperature upon isoprenaline binding to beta$_1$ and beta$_2$ adrenoceptors was investigated using standard assay conditions. (Figure 7:12). Results showed that increasing the incubation temperature...
The effect of GTP upon salbutamol binding to beta_1 and beta_2-adrenoceptors of rabbit lung. Fig. A: Salbutamol displacement of specific ^3H-DHA binding was measured at 37°C for 15 min. in the absence (closed symbols) and presence (open symbols) of 100 μM GTP in buffers containing MgCl_2/EDTA. Figs. B and C: Identical incubations were performed in the presence of 50 nM ICI 118,551 (B) or 75 μM atenolol (C). Results represented mean curves of two experiments performed in duplicate.
Figure 7:12
Effect of temperature upon isoprenaline displacement of $^3$H-DHA binding to (A) chick erythrocyte, (B) rabbit lung, (C) rat lung and (D) rat reticulocyte membranes. Incubations were performed under standard assay conditions for 30 min. at $22^\circ$C (■), 20 min. at $30^\circ$C (▲) and 15 min. at $37^\circ$C (▲). IC$_{50}$ values were obtained and $K_i$ values were calculated from the equation of Cheng & Prusoff. The equilibrium dissociation constants used for this correction were determined at the appropriate temperatures. Results represented the mean of at least 3 determinations for each condition. SEM's were <10%.
from 22°-37° had profound effects on the affinity of isoprenaline for chick erythrocyte beta-adrenoceptors (Figure 7:12, A). The inhibition constants of isoprenaline at 37° were 8-fold higher than values obtained at room temperature. Similar temperature dependencies were observed using incubation conditions which contained Mg /EDTA (data not shown). This temperature induced shift of agonist affinity was less marked in the other preparations, being 2.5, 2.0 and 1.5 fold for rabbit lung, rat reticulocyte and lung membranes respectively.

These decreases in the affinity of isoprenaline did not appear to result from alterations in the pH of the Tris-HCl incubation buffer since the inhibition constant of isoprenaline measured at pH 7.1 was identical (data not shown). Moreover, these changes were not a reflection of major alterations in the affinity of ^H-DHA for beta-adrenoceptors at higher temperatures. Dissociation constants of the radioligand at 37° were similar to values obtained at 22° (see Table 7:3). These findings suggested that the alterations in agonist binding were a function of the temperature changes.

(B) Antagonists

The binding of radiolabelled beta-adrenoceptor antagonist ^H-DHA was examined at different temperatures. Table 7:3 shows the results obtained for a number of beta_1 and beta_2-adrenoceptor preparations. The dissociation constants of ^H-DHA for all preparations were little influenced by raising the temperature from 22°-37°. The slight decrease in the affinities of ^H-DHA probably reflected the greater influence of temperature on the dissociation rate constants (previous experiments had shown that ^H-DHA dissociated from rat lung and erythrocyte beta-adrenoceptors 5-6 times more rapidly at 37° than 22°).
**TABLE 7.3** The effect of temperature on the equilibrium dissociation constant of $^3$H-DHA

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Equilibrium dissociation constant (nM)</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$22^\circ$</td>
</tr>
<tr>
<td>Rat lung</td>
<td>0.35</td>
<td>5</td>
</tr>
<tr>
<td>Rat reticulocyte</td>
<td>0.25</td>
<td>5</td>
</tr>
<tr>
<td>Chick erythrocyte</td>
<td>0.73</td>
<td>7</td>
</tr>
<tr>
<td>Rabbit lung</td>
<td>0.59</td>
<td>3</td>
</tr>
</tbody>
</table>

Experiments were conducted under standard incubation conditions. Results were the mean of the stated number of experiments performed in triplicate. SEM's were <10%.

(C) **Thermodynamic parameters of antagonist and agonist binding to beta$_1$ and beta$_2$-adrenoceptors**

Data obtained for antagonist and agonist binding to beta-adrenoceptors at different temperatures were analysed using methods described by Weiland et al (1980).
Table 7:4 shows the thermodynamic parameters of $^3$H-DHA binding to beta$_1$ and beta$_2$-adrenoceptor preparations. The values shown were calculated for binding at 37°. Estimations of the change in enthalpy of $^3$H-DHA binding assumed a linear van't Hoff plot. This was experimentally verified for the binding of $^3$H-DHA to chick erythrocyte membranes over the temperature range 22°-37°. Binding of $^3$H-DHA was relatively insensitive to temperature changes over the range studied (22°-37°) which resulted in small or negligible changes in enthalpy (Table 7:4, column 3). Specific $^3$H-DHA binding to these beta-adrenoceptors was probably driven by a large, thermodynamically favourable, increase in entropy (Table 7:4, column 4).

The thermodynamic parameters of isoprenaline binding to beta$_1$ and beta$_2$-adrenoceptors were analysed (Table 7:5). Values were calculated from the overall isoprenaline displacement curves, in the absence of GTP, which therefore represented the mean of both the putative high and low affinity binding states. Experiments were performed over a temperature range 22°-37°. The binding of isoprenaline to beta-adrenoceptors of rat erythrocytes, reticulocytes and lung was associated with decreases in enthalpy and favourable increases in entropy. Therefore, agonist binding to these beta$_2$-adrenoceptor preparations was probably driven (like $^3$H-DHA binding) by increases in entropy. In contrast, isoprenaline binding to rabbit lung and chick erythrocyte beta-adrenoceptors resulted in considerable decreases in enthalpy and entropy. Thus, agonist binding to these beta$_1$ adrenoceptors appeared to be driven by the decrease in enthalpy.
TABLE 7:4 Thermodynamic parameters of $^3$H-DHA binding to beta-adrenoceptors

<table>
<thead>
<tr>
<th>Tissue</th>
<th>$\frac{K_D}{K_D}^{37^\circ}$</th>
<th>$2 \Delta G^0$ K cals/mol</th>
<th>$3 \Delta H^0$ K cals/mol</th>
<th>$4 \Delta S^0$ entropy units</th>
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</thead>
<tbody>
<tr>
<td>Rat lung</td>
<td>1.2</td>
<td>-13.32</td>
<td>-2.11</td>
<td>+36.2</td>
</tr>
<tr>
<td>Rat reticulocyte</td>
<td>1.0</td>
<td>-13.64</td>
<td>0</td>
<td>+44</td>
</tr>
<tr>
<td>Chick erythrocyte</td>
<td>1.2</td>
<td>-12.89</td>
<td>-1.75</td>
<td>+35.9</td>
</tr>
<tr>
<td>Rabbit lung</td>
<td>1.1</td>
<td>-13.04</td>
<td>-3.02</td>
<td>+32.3</td>
</tr>
</tbody>
</table>

1. Ratio of the equilibrium dissociation constants for $^3$H-DHA were obtained under standard conditions.

2. The standard Gibbs free energy change ($\Delta G^0$) of $^3$H-DHA binding was calculated from the equation $\Delta G^0 = -RT \ln K_A$ where $R$ was the gas constant (1.99 cal. mol$^{-1}$deg$^{-1}$), $K_A$ was the equilibrium association constant (= 1/$K_D$); and $T$ the temperature in K. Values were calculated for $37^\circ$ under standard assay conditions.

3. The change in enthalpy upon $^3$H-DHA binding ($\Delta H^0$) was calculated from the equation: $\ln K_A = -\Delta H^0/RT + \Delta S^0/R$ where $\Delta S^0$ was the standard entropy change. Thus, the slope of a van't Hoff plot ($\ln K_a$ vs $\frac{1}{T}$) equalled $-\Delta H^0/R$.

4. The standard entropy change $\Delta S^0$ was calculated from the equation $\Delta S^0 = \Delta H^0 - T \Delta S^0$. 
TABLE 7:5 Thermodynamic parameters of isoprenaline binding to beta₁ and beta₂-adrenoceptors

<table>
<thead>
<tr>
<th>Tissue</th>
<th>1 Kᵢ 37° Kᵢ 22°</th>
<th>2 ΔG° Kcals/mol</th>
<th>3 ΔH° Kcals/mol</th>
<th>4 ΔS° (Entropy units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat reticulocyte</td>
<td>1.9 -10.3 -7.6</td>
<td>+8.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat erythrocyte</td>
<td>1.9 -9.9 -7.4</td>
<td>+8.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat lung</td>
<td>1.4 -10.3 -3.7</td>
<td>+21.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit lung</td>
<td>2.5 -10.2 -10.9</td>
<td>-2.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chick erythrocyte</td>
<td>8.3 -9.5 -21.9</td>
<td>-40</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. Ratio of the inhibition constants obtained for isoprenaline under standard conditions. These represented overall inhibition constants for both putative high and low affinity states of the receptor.

2. The standard Gibbs free energy change (ΔG°) of isoprenaline binding was calculated from the equation ΔG° = -RT ln Kᵢ where Kᵢ = (I/Kᵢ). All thermodynamic parameters were calculated for 37°, under standard assay conditions.

3. The change in enthalpy upon isoprenaline binding (ΔH°) was calculated from the slope of the van't Hoff plot which equalled - ΔH°/R.

4. The standard entropy change (ΔS°) was calculated as described previously (Table 7:4).
7:3 Discussion

Avian and amphibian erythrocytes have been most extensively studied as model systems for elucidating the molecular mechanisms of beta-adrenoceptor-effector coupling (for reviews see Levitzki and Helmreich, 1979; Hoffman and Lefkowitz, 1980). One major difference of agonist interaction with frog and avian erythrocyte beta-adrenoceptors was the ability of GTP to modulate agonist binding in the former system to a homogeneous low affinity binding state (Williams and Lefkowitz, 1977). In contrast, agonist binding curves obtained with turkey erythrocyte membranes have been reported to be steep and not influenced by guanine nucleotides (Brown et al., 1976b; Tolkovsky and Levitzki, 1978; Weiland et al., 1979). Agonist binding to frog erythrocytes was best described by a model, where agonists induced high affinity states of the receptor which interconverted under the influence of guanine nucleotides to a homogeneous low affinity state (Kent et al., 1980; DeLean et al., 1980). Results of the present investigations confirmed the applicability of this model for agonist binding to rat lung where increasing concentrations of GTP caused graded decreases in heterotropic binding. Recently, Heidenreich et al., (1980) have demonstrated that the binding of the agonist, hydroxybenzylioprenaline (HBI) to rat lung membranes resulted in curvilinear Scatchard plots, which were progressively linearised by increasing concentrations of Gpp(NH)p. These findings argued against any model based on non-interconverting states of the receptor.

The present study showed that under standard assay conditions, guanine nucleotides were unable to influence the proportions of isoprenaline-induced high affinity binding state in membranes of rat reticulocytes, rabbit lung or chick erythrocytes. A number of other workers have also reported GTP insensitive agonist binding to beta-adrenoceptors of rat reticulocytes and turkey erythrocytes (Brown...

Recently, however, GTP-modulation of agonist binding has been observed in these systems (Lad et al, 1980a; Stadel et al, 1980; Limbird et al, 1980a; Simpson et al 1980). The reason for these discrepancies was originally related to differences in the membrane preparations (Stadel et al, 1980), or tightly bound GDP (Lad et al, 1980a). The results of the present study and concurrent investigations by Shane et al (1981) have indicated the importance of Mg-EDTA for GTP sensitive agonist binding to be delineated. In addition, incubations at, or near, physiological temperatures was found to be critical for full GTP modulation of agonist binding (Simpson and Pfeuffer, 1980). Under these conditions, agonist binding to the above beta-adrenoceptor preparations was well fitted to a two binding state model in the absence of GTP, and a one state model in the presence of GTP.

The importance of Mg$^{2+}$ for the induction of high affinity agonist-receptor complexes has been described for beta-adrenoceptors of frog erythrocytes (Williams et al, 1978); mouse lymphoma S49 cells; rat glioma cells (Bird and Maguire 1978); and rat reticulocytes (Shane et al, 1981). This cation also had an important regulatory influence upon agonist binding to oxytocin receptors (Pearlmutter et al, 1979); prostaglandin receptors (Williams et al, 1978); platelet alpha$_2$ receptors (Tsai and Lefkowitz 1979); and rat heart muscarinic receptors (Hulme et al, 1981a). However, the present studies indicated that agonist binding to beta-adrenoceptor preparations, measured in the absence of guanine nucleotides, was not significantly influenced by Mg$^{2+}$ alone, or in combination with EDTA. These findings suggested that, perhaps, the Mg$^{2+}$-stimulated binding was attenuated on cooling the incubation mixture prior to filtration, as was shown for $^3$H-clonidine binding to rat brain alpha$_2$-adrenoceptors (Glossmann et al, 1980). Alternatively (and more likely) exogenous Mg$^{2+}$ may not have been
required in these systems for high affinity agonist binding to be expressed. It was significant that isoprenaline binding to frog erythrocyte beta-adrenoceptors, measured at 22°C and in the absence of added Mg²⁺, resulted in the formation of a significant proportion of high affinity, GTP-sensitive, binding state. Others have reported agonist competition curves obtained in the absence of Mg²⁺ which were steep and GTP-insensitive (Hoffman and Lefkowitz, 1980). However, the frog erythrocyte membranes which were used by these workers were prepared in EDTA-containing buffers (Williams et al, 1978) and were probably cation-depleted.

The beta-adrenoceptor preparations used for the present studies probably possessed endogenous cations, which allowed functional association of receptor and Nprotein to occur. The major effect of Mg²⁺/EDTA in these systems was to enhance the inhibition of agonist binding by GTP. Similar findings have also been made for muscarinic agonist binding to rat heart and brain muscarinic receptors (Wei and Sulakhe, 1980; Hulme et al. 1981b). These effects of Mg²⁺/EDTA may have related to (i) an occupation of cation binding sites which allosterically enhanced the affinity of GTP for the Nprotein, (ii) the production of Mg-GTP, which was a more effective substrate for the Nprotein or (iii) an enhanced rate of, agonist stimulated, dissociation of GDP from the Nprotein (Cassel and Selinger, 1978). The presence of Mg²⁺/EDTA was required for full expression of GTP modulation of agonist binding to chick erythrocytes, rat reticulocytes and rabbit lung beta-adrenoceptors, but not for the beta₂-adrenoceptor preparations of frog erythrocytes and rat lung. These differential dependencies may have reflected: (i) different degrees of occupation of the cation binding sites by endogenous ions, (ii) the relative tightness of GDP-binding to the Nproteins or (iii) structural differences in the subunits of the Nproteins, which possessed different regulatory characteristics.
The importance of fulfilling the divalent cation requirement has been demonstrated for the alpha_2-adrenoceptor of brain membranes. In this system, divalent cations increased the affinity of Gpp(NH)p for the clonidine-receptor complex about 100-fold (Glossmann and Presek 1979). These observations implied a role for a cation site which could influence the subsequent binding of guanine nucleotides. High affinity GDP-binding to the Nprotein almost certainly contributed to the above effects in avian erythrocytes, since membranes from this source are usually isolated with tightly bound Mg^{2+} and GDP (Cech et al, 1980). However, this phenomenon was not shared by liver or fat cell membranes (Lad et al, 1980b) and it may not be generally applicable.

Recently, the Nprotein from membranes of rabbit liver and turkey erythrocyte have been reported to exhibit different subunit structures (Northup et al, 1980). The absence of a 52,000 Mr subunit from avian erythrocytes Nprotein and a lower proportion of this subunit is rat erythrocytes (Larner and Ross, 1981) may have related to the insensitivity of these preparations to guanine nucleotides. In support of this postulate, alteration in the stoichiometry of the 52,000/45,000 Mr subunits of rabbit hepatic Nprotein resulted in different regulatory responses to nucleotides and cations (Sternweis et al, unpublished observations). In the light of these findings, the Nprotein subunit composition may be a major determinant of the guanine nucleotide responsiveness of the above systems.

The only membrane system in which agonist binding was unaffected by guanine nucleotides under optimum ion and temperature conditions, was that of rat erythrocytes, a finding which confirmed previous work of Bilezikian et al (1977b) The maturation of rat reticulocytes into erythrocytes has been shown to result in functional uncoupling of the receptor-effector system, and a reduced ability of the beta-adrenoceptors to form high affinity states. Thus, Limbird et al (1980a) have described isoprenaline competition curves obtained with rat
erythrocyte membranes, which were steep, with Hill slope factors which approximated unity. In the present study, equivalent Hill slope factors were 0.8-0.9, which suggested that a small degree of heterotropic binding was still apparent, even in the presence of GTP. In view of these findings, it was of interest that the agonist HBI dissociated from membranes of rat erythrocytes and reticulocytes at three different rates (Limbird et al, 1980a). Clearly further experimental work will be required before postulating intermediate GTP-insensitive, affinity states of these beta-adrenoceptors. Kinetic studies of radiolabelled agonist binding to beta-adrenoceptors under different ionic and temperature conditions will certainly aid our understanding of these mechanisms.

The present study has examined the effects of temperature on agonist binding in the absence and presence of guanine nucleotides. In most systems (with the exception of rat lung membranes) the inhibition of agonist binding by GTP was enhanced by elevated incubation temperatures. Guanine nucleotide modulation of agonist binding to chick erythrocytes, like that to pigeon and turkey erythrocytes (Simpson and Pfeuffer, 1980) was totally dependent on incubation temperatures $\geq 30^\circ$. These findings have been interpreted by others as indicating the need for a 'fluid' membrane for the induction of high affinity agonist-receptor complexes. Thus, cis vaccenic acid insertion into avian erythrocytes, to increase membrane fluidity, resulted in an increased rate of adenylate cyclase activation, and a greater formation of agonist-induced high affinity state. Agonist binding to these treated membranes was modulated by guanine nucleotides at room temperature (Orly and Schramm, 1975; Briggs and Lefkowitz, 1980).

The present study confirmed the ability of cis vaccenic acid to enhance the 'GTP shift' of agonist binding to chick erythrocyte beta-adrenoceptors at $22^\circ$, although the induction of greater proportions of
high affinity agonist state was not apparent. The major effect of cis-vaccenic acid treatment was an overall increase in the affinity of agonists for beta-adrenoceptors. This result was similar to the observations of agonist binding following solubilisation of beta-adrenoceptors (See Chapter 8), although in the present case, GTP-sensitive binding was retained and enhanced. These agonist affinity increases contrasted markedly with the decreased affinities which were observed by increasing the incubation temperature. Moreover, cis-vaccenic acid treated chick erythrocyte membranes also underwent temperature-induced alterations of agonist affinities. These findings suggested that fatty acid insertion into membranes was not a priori associated with fluidity changes, but perhaps was associated with reduced lipid-protein interactions.

Results of the present work suggested that Mg\(^{2+}\) was important for retention of the 'order' of the beta-adrenoceptor system. Thus, cis-vaccenic acid treatment of membranes in Mg\(^{2+}\)-free buffers resulted in the 'loss' of specific \(^3\)H-DHA binding sites into the fluidised membranes. Similar observations have also been made with Chang liver cells where fluidity increases, above the transition temperature, were associated with disordering of the system and losses of beta-adrenoceptors (Bakardjieva et al, 1979). These results suggested that cations may contribute to the maintenance of the integrity of the beta-adrenoceptor system, perhaps by interacting with acidic phospholipids. Indeed, the acidic lipids cerebroside sulphate and cardiolipin may be intimately associated with opiate (Lee and Smith, 1980) and dopamine (Boyan-Salyers and Clement Cormier 1981) receptors respectively.

The affinities of agonists for chick erythrocyte and rabbit lung beta\(_1\)-adrenoceptors decreased with increasing temperature, whereas antagonist affinities were unaffected. These results confirmed previous work on avian erythrocyte beta-adrenoceptors (Pike and Lefkowitz 1978;
Weiland et al., 1979). In contrast, the agonist affinities for beta$_2$-adrenoceptors of frog erythrocytes (Pike and Lefkowitz, 1978) and rat lung and reticulocyte were little influenced by temperature changes. These findings suggested that agonist binding to beta$_1$ and beta$_2$-adrenoceptors had differential temperature sensitivities. This conclusion was not substantiated by the work of Weiland et al., (1980), however, who showed no fundamental differences between the thermodynamic parameters of agonist binding to beta$_1$ and beta$_2$-adrenoceptors of a number of mammalian tissues. Moreover, the quantitative effects of temperature on isoprenaline binding to beta-adrenoceptors of chick and turkey erythrocytes (Briggs and Lefkowitz, 1980) was markedly greater than values obtained for turkey erythrocytes by Weiland et al., (1979) and Simpson et al., (1980).

The explanation for these differences probably lies in the hypotonic incubation conditions employed in the former studies, which used $^3$H-DHA, compared to the latter $^{125}$I-HYP binding studies, where NaCl (90-150mM) was present to decrease N.S.B. Sodium chloride has been shown to have pronounced effects on the affinities of agonists for beta-adrenoceptors (U'Pritchard et al., 1978), opiate receptors (Port and Snyder, 1975), alpha$_2$-adrenoceptors (Glossmann and Presek, 1979) and muscarinic receptors (Birdsall et al., 1979b). In addition, the ionic strength of the incubation medium has been shown to influence muscarinic antagonist binding to rat myocardial receptors (Hulme et al., 1981a) and to protect particulate and soluble receptors from thermal inactivation (Chapter 8). These observations implied that the presence of Na$^+$ could have profoundly influenced the effects of temperature on agonist binding.

Isoprenaline binding to beta$_1$-adrenoceptors, under hypotonic conditions, had thermodynamic parameters which were characterised by decreases in entropy and enthalpy, whereas binding to beta$_2$-adrenoceptors
resulted in increases in entropy and decreases in enthalpy. The former thermodynamic parameters for agonist binding have been interpreted by others as indicating agonist induced conformational changes in the receptor, perhaps related to increased ionic bonding and ordering of receptor and lipids (Weiland et al, 1980). The binding of agonists to beta\(^2\)-adrenoceptors (like those of antagonists) was driven by increases in entropy perhaps related to displacement of water molecules from regions of the receptor, or from binding groups on the ligand. These proposals suggested that agonist binding to beta\(^1\)-adrenoceptors would be more 'productive' in coupling receptor occupation to effector response. Since the converse seemed to be the case, there was clearly a need for caution before ascribing molecular mechanisms from measurements of gross thermodynamic changes associated with binding to membrane bound sites (see Franklin, 1980).

The preceding studies have examined the effects of ions and temperature on agonist interaction with beta\(^1\) and beta\(^2\)-adrenoceptors. The results suggested that the beta\(^1\)-adrenoceptor preparations of chick erythorcytes and rabbit lung were more sensitive to changes in these parameters, which implied that there were different receptor-effector coupling efficiencies between beta\(^1\) and beta\(^2\)-adrenoceptors. The studies of rabbit lung beta\(^1\) and beta\(^2\) adrenoceptors also suggested that these receptors were differentially coupled to adenylate cyclase under appropriate ionic and temperature conditions. Thus, at 22\(^{\circ}\) in the absence of Mg\(^{2+}\), beta\(^1\)-adrenoceptors existed in a uniform low affinity, GTP-insensitive (uncoupled) state, whereas the beta\(^2\)-adrenoceptors were capable of forming high affinity states of the receptor, which may relate to adenylate cyclase activation (Stadel et al, 1980).

One drawback to the above conclusion was the results obtained with the rat reticulocyte beta-adrenoceptor-effector system. This system had ion and temperature requirements for GTP-modulation of agonist binding
which were unlike those of frog erythrocyte and rat lung, and more akin to a beta$_1$-adrenoceptor preparation. Clearly, it will be important to establish whether the above differences related to fundamental alterations in the composition of the Nprotein subunits of these systems, or to the relative affinities of modulators for the components.

The concept of drug selectivity being exerted by differential efficacy at beta-adrenoceptor subtypes has been explored with the partial agonist salbutamol, which has been shown to exhibit beta$_2$-adrenoceptor selectivity in vivo (Daly and Levy, 1979). Results of the present study confirmed earlier observations, which demonstrated that salbutamol exerted no selective affinity for beta$_2$-adrenoceptors (Nahorski, 1978; Minneman et al 1979, a). Salbutamol binding to rat and rabbit lung beta$_2$-adrenoceptors was modulated by GTP which suggested that salbutamol could induce high affinity forms of the receptor and thereby couple receptor occupation to effector activation. Under optimal ion and temperature conditions, salbutamol binding to beta$_1$-adrenoceptors was GTP-insensitive, which suggested poor receptor-effector coupling in these systems. In support of this proposal, salbutamol had no efficacy at the beta$_1$-adrenoceptors of chick erythrocytes (this study) or rat heart (Minneman et al 1979a), and acted as an antagonist at the beta$_1$-adrenoceptor linked adenylate cyclase of rat cerebral cortex membranes (Dolphin et al, 1979).

Moreover, salbutamol has been reported to stimulate adenylate cyclase in rat lung membranes to a greater extent than rabbit lung membranes (Nahorski 1979), and exert greater partial agonist activity at beta$_2$-adrenoceptors of isolated rat lung cells than beta$_1$-adrenoceptors of isolated rat cardiac cells (Hazeki and Ui, 1980). These observations suggested that the in vivo selectivity of salbutamol was correlated with (i) its ability to more effectively couple beta$_2$-adrenoceptors to adenylate cyclase activation, and (ii) the stimulus-response relationships of the tissues involved (Kenakin and Beek, 1980).
CHAPTER 8

Solubilisation of beta₁ and beta₂-adrenoceptors from mammalian lung
8:1 Introduction

Results of the previous work have been interpreted in the light of the dual beta-adrenoceptor concept, with the subtypes behaving as independent and separate entities. There were however other schools of thought which regarded beta-adrenoceptors as macromolecules, which interconverted to alpha-adrenoceptors under appropriate conditions (Nickerson & Kunos, 1977). Alternatively beta-adrenoceptors have been regarded as single entities, the characteristics of which were directed by the lipid environment, ions, hormones, pH or temperature (Ahlquist, 1977).

The interconversion hypothesis which proposed that alpha and beta-adrenoceptors represented different allosteric configurations of the same active site, was based on evidence that amphibian heart adrenoceptors converted from beta- to alpha with decreased temperatures (Kunos et al, 1968). This concept has subsequently been refuted by a number of workers (Martinez & McNeill 1977, Stene-Larsen & Helle, 1978b), and physical separation of the receptors has been described (Wood et al, 1979), which indicated that alpha and beta-adrenoceptor binding sites did not reside on the same macromolecule. The possibility that beta-adrenoceptor subtypes were also interconvertible has been suggested by Lacombe et al (1976). Thus, malignant transformation of normal rat liver into Zajdela hepatoma was reported to be accompanied by a transformation of beta₂- to beta₁-adrenoceptors. This finding was interpreted as indicating that beta-adrenoceptors constituted a single entity, which was susceptible to reversible modification to either subtype. Other workers have supported the "single receptor" concept and suggested that the existence of beta-adrenoceptor subtypes reflected the metabolic state of the tissue (Schumann et al, 1972).

The present study has attempted to solubilise beta₁- and beta₂-adrenoceptors in order to see whether beta₁ and beta₂-subclasses could be identified in preparations freed of the constraints of the intact
membrane. Physical separation and characterisation of beta-adrenoceptor subtypes would be the ultimate proof of their existence as separate entities. At the onset of these studies beta-adrenoceptors of the non-mammalian tissues turkey and frog erythrocytes had been successfully solubilised in active form using the plant glycoside digitonin (Caron & Lefkowitz, 1976; Vauquelin et al, 1977). Soluble beta-adrenoceptors from mammalian tissues: S49 lymphoma cells (Haga et al, 1978), canine myocardium (Wrenn and Haber, 1979) and guinea pig lung (Kleinstein and Glossman 1978) had also been described using the detergents Lubrol PX, deoxycholate, and digitonin respectively. The wide range of detergents which were used, suggested that the beta-adrenoceptors of these tissues had different lipid microenvironments, since solubilisation of integral membrane proteins depended on replacement of hydrophobic receptor protein-lipid interactions with detergent-protein interactions (see reviews by Helenius and Simons, 1975 and Lindstrom 1978). Major differences in stabilities between soluble beta-adrenoceptors from mammalian and non-mammalian tissues had also been reported. Thus, Triton X100-solubilised beta-adrenoceptors of canine heart and liver were extremely labile (Stauss et al 1978) in contrast to the stable preparations from frog erythrocytes (Caron and Lefkowitz 1976).

Solubilisation of membrane proteins required (i) a suitable, rich, source of receptors, (ii) an effective means of solubilising the receptor and (iii) a means of assaying the soluble protein. The work to be described used rat and rabbit lung membranes, which were amongst the richest and most accessible sources of mammalian beta_2-adrenoceptors and beta_1-adrenoceptors respectively. These membrane preparations had both beta_1 and beta_2-adrenoceptors present in the proportions 80:20 (rabbit lung) and 20:80 (rat lung). Soluble beta-adrenoceptors were assayed initially using a PEG-precipitation technique as described in 'Methods'. However, the presence of the detergent digitonin inhibited
the PEG-precipitation of soluble receptor protein, and resulted in non-linear protein dilution curves (see 2:4:2:Biii). Therefore, a technique of adsorbing free radiolabelled ligand to BSA-covered charcoal was developed for the assay of soluble beta-adrenoceptors (see Methods), which was simpler than PEG precipitation, and which was not affected by the presence of detergent. The results to be described in the following chapter were obtained using this technique.

**8:2 Methods**

**8:2:1 Digitonin solubilisation of beta-adrenoceptors**

Lungs were removed from male Wistar rats (150-200g) or New Zealand white rabbits and dissected free of major bronchi. Alternatively, lung tissue was used which had been previously stored frozen at -50°C. Tissues were homogenised in 10 vols of 50mM Tris-HCl pH 7.6 using a Polytron homogeniser (3 x 5 sec bursts, setting 5). The homogenate was passed through a double layer of cheese cloth and centrifuged at 500g for 10 min to remove fibrous tissue. The supernatant was centrifuged at 50,000g for 20 min and the resulting pellet washed three times with 50mM Tris-HCl pH 7.8. The final pellet was taken up in ice-cold Tris buffer to a protein concentration of 10mg/ml.

Solubilisation of membrane-bound beta-adrenoceptors was performed at 4°C for 30 min in a medium containing 0.5% digitonin (Sigma); 50mM Tris-HCl pH 7.8; 100mM NaCl; using a digitonin:protein ratio of 3:1. Gentle agitation was performed during this time, and following solubilisation the membranes were centrifuged at 50,000g for 1h. The supernatant which lay above the pellet was carefully pipetted from the centrifuge tube. This straw-coloured supernatant served as the source of soluble preparation, and it was used directly, or frozen and stored at -50°C until required. Freezing did not appear to affect the binding characteristics of the soluble preparations.
8:3 Results
8:3:1 Solubilisation of beta-adrenoeceptors - methodological considerations

(A) Selection of detergent and optimisation of solubilisation conditions

A number of detergents were examined for their abilities to solubilise beta-adrenoeceptor binding sites from rabbit lung. These agents were selected for their previous successful use in solubilising a wide range of membrane bound proteins in active form. Table 8:1 shows the yield of protein and specific $^3$H-DHA binding sites obtained. Clearly most detergents were effective in solubilising membrane protein - deoxycholate and Triton X100 solubilised 61% and 48% respectively. However, the yields of $^3$H-DHA binding sites which were obtained with these non-ionic detergents were poor, as was that using octylglycoside. High salt (KCl) concentrations was totally ineffective in solubilising beta-adrenoeceptors.

The plant glycoside digitonin was uniquely successful in producing significant yields of soluble specific $^3$H-DHA sites, although it was possible that beta-adrenoeceptors were solubilised by the other detergents but not detected because of detergent interferences in the assay. Digitonin-solubilisation of beta-adrenoeceptors from rabbit lung membranes was considerably enhanced by sodium chloride. Thus, IMNaCl/0.5% digitonin solubilised 29% of the specific $^3$H-DHA sites of the membranes, although this was accompanied by a greater solubilisation of non-receptor protein. Therefore, the specific activity of soluble receptors obtained using these conditions were not enhanced over those prepared in NaCl-free buffers. The optimum specific activity of soluble rabbit lung beta-adrenoeceptors was obtained using 100mMNaCl - 0.5% digitonin, conditions which resulted in approximately 20% yield of beta-adrenoeceptors.

This relatively poor yield of soluble beta-adrenoeceptors from rabbit lung membranes could have resulted from co-solubilisation of membrane bound proteases (Michaelson et al, 1974). However the recovery of beta-adrenoeceptors was not enhanced by inclusion of the protease...
Table 8:1
Solubilisation of rabbit lung beta-adrenoceptors by different detergents.

<table>
<thead>
<tr>
<th>Detergent</th>
<th>Protein Yield (%)</th>
<th>Yield specific (^{3}\text{H-DHA binding sites} ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5% Deoxycholate</td>
<td>61</td>
<td>0</td>
</tr>
<tr>
<td>0.5% Triton-X-100</td>
<td>48</td>
<td>9 *</td>
</tr>
<tr>
<td>0.25% Triton-X-100, 0.5% Digitonin</td>
<td>44</td>
<td>9 *</td>
</tr>
<tr>
<td>1 M KCl</td>
<td>N.D.</td>
<td>0</td>
</tr>
<tr>
<td>0.5% Octylglycoside</td>
<td>N.D.</td>
<td>5 *</td>
</tr>
<tr>
<td>0.5% Digitonin (Fischer)</td>
<td>34</td>
<td>13</td>
</tr>
<tr>
<td>0.5% Digitonin (Sigma)</td>
<td>33</td>
<td>12</td>
</tr>
<tr>
<td>0.5% Digitonin, 10 mM NaCl</td>
<td>33.5</td>
<td>16.5</td>
</tr>
<tr>
<td>0.5% Digitonin, 100 mM NaCl</td>
<td>36</td>
<td>19.5</td>
</tr>
<tr>
<td>0.5% Digitonin, 500 mM NaCl</td>
<td>62</td>
<td>18</td>
</tr>
<tr>
<td>0.5% Digitonin, 1 M NaCl</td>
<td>71</td>
<td>23</td>
</tr>
</tbody>
</table>

Membranes were incubated with the stated concentration of solubiliser for 30 min. at 4\(^{\circ}\) in 50 mM Tris-HCl, pH 7.8. Supernatants were assayed for specific \(^{3}\text{H-DHA binding using the charcoal separation technique described in 'Methods'}}. Results were the mean of 2-7 experiments performed in duplicate. *Addition of charcoal caused catalytic oxidation of the isoprenaline used to assase NSB. The values quoted were estimates, using extrapolated values of NSB.
inhibitor phenylmethylsulphonyl fluoride (10μM) during solubilisation (data not shown). The addition of 1mM EDTA, or prior occupation of beta-adrenoceptors with \(^3\)H-DHA also failed to increase the yield of soluble beta-adrenoceptors (data not shown). Estimations were made of the number of beta-adrenoceptors remaining on rabbit lung membranes after digitonin extraction. Results indicated that digitonin treated membranes had only 10% of the original number of beta-adrenoceptors (Bmax = 35(±4)fmoles/mg protein, n = 4), which indicated that the poor yield was not related to incomplete solubilisation. This finding also accounted for the inability of secondary extractions to solubilise further beta-adrenoceptors. Solubilisation of rat lung membranes, in the presence of 100mM NaCl, proceeded with greater yields of specific \(^3\)H-DHA binding sites (40(±5)%, n=7), although recoveries of soluble protein (34(±3), n=7) were similar to those for rabbit lung membranes.

The beta-adrenoceptors of rat erythrocytes were also solubilised in good yield using the protocol described in 8:2:1. In contrast, the beta-adrenoceptors of chick erythrocytes were extremely resistant to digitonin-solubilisation. The yields of soluble membrane protein from this tissue were low (≈ 10%), and soluble \(^3\)H-DHA sites were not detected. Attempts were made to solubilise chick erythrocyte beta-adrenoceptors after prior incubation at 30\(^\circ\) with isoprenaline/MgCl\(_2\), a protocol found effective for the solubilisation of turkey erythrocyte beta-adrenoceptors (Eimerl et al, 1980). However, no specific \(^3\)H-DHA binding sites were located in the detergent extracts (data not shown). In addition, prior treatment of membranes with cis-vaccenic acid also failed to allow the solubilisation of active beta-adrenoceptors, although the yield of soluble membrane protein was enhanced (data not shown).
(B) Time course of digitonin solubilisation

Digitonin-solubilisation of rat and rabbit lung membranes at 4 °C was rapid (Figure 8:1). Maximal yields of soluble $^3$H-DHA binding sites from rabbit lung membranes were obtained after 7 min. Solubilisation of rat lung membranes resulted in a significant yield of beta-adrenoceptor binding sites after 7 min., and there was an increased yield of soluble $^3$H-DHA sites after 20 min. of solubilisation. The pharmacological characteristics of soluble receptors obtained after 60 min. extraction were identical to those obtained after 10 min (data not shown). This indicated that prolonged solubilisation times did not result in greater extraction of beta-adrenoceptor subtypes which were present in minor proportions.

(C) Characterisation of the 'soluble' nature of the digitonin-extracts

The separation of soluble detergent extracts from particulate matter was accomplished by centrifugation at the relatively low centrifugation force of 50,000g for 1h. This protocol was found to be satisfactory since further centrifugation of the supernatants at 100,000g for 2h failed to sediment visible particulate matter, and failed to decrease the number of specific $^3$H-DHA binding sites of the supernatants. The digitonin-extracts of rat and rabbit lung membranes passed through cellulose nitrate filters of 0.2μm pore size without loss of specific $^3$H-DHA binding sites. In addition, the detergent extracts of rat and rabbit lung were included in the volume of sephacryl S200 and S300 columns. Electron microscopy of soluble supernatants was performed using the 'dipping' procedure described by Eimerl et al (1980). Examination of the samples revealed aggregates formed by the glutaraldehyde fixation, but failed to reveal any vesicular, membrane-like structures (data not shown). These cumulative results indicated that the detergent extracts were truly 'soluble'. 
Figure 8:1
Time course of digitonin solubilisation of specific $^3$H-DHA binding sites from rat (A) and rabbit (B) lung membranes. Membranes were solubilised for the indicated times at 4°C using conditions described in section 8:2:1, and soluble protein was assayed for specific $^3$H-DHA binding. Results were the mean of triplicate determinations from single experiments.
8:3:2 General properties of the soluble $^3$H-DHA binding sites

(A) Stability

The NaCl-digitonin solubilised preparations of rat lung were remarkably stable. They could be frozen and thawed; or freeze dried and reconstituted without significant losses of binding capacity. Figure 8:2(A) shows the effect of prolonged storage at 22° on the specific $^3$H-DHA binding sites of soluble rat lung preparations, which had been solubilised in the absence and presence of NaCl (100 mM). Clearly the NaCl-digitonin solubilised preparation was stable for prolonged periods of time (>3h). In contrast, membranes solubilised in the absence of NaCl resulted in soluble preparations which were considerably more thermolabile. The $^3$H-DHA binding sites of these preparations decayed in a pseudo zero order process with a $t_1/2$ of 2-3h at 22° (Figure 8:2:A). The NaCl-digitonin preparation of rabbit lung was more labile at 22° than the equivalent rat lung preparation (Figure 8:2:B), although a proportion of these sites (40-50%) were relatively stable to prolonged incubation at 22°. Occupation of these binding sites with $^3$H-DHA decreased the rate of the thermal inactivation process (Figure 8:2:B).

Experiments were conducted to compare the thermal stabilities of the specific $^3$H-DHA sites on particulate and soluble rat and rabbit lung preparations. Figure 8:3 shows the results of incubating unoccupied receptors for the indicated times at 22°, 37° and 45°. The beta-adrenoceptors on membranes of rat and rabbit lung were stable at 22° for prolonged periods (<10% loss after 3h), and increased temperatures were associated with faster inactivation times (Figure 8:3, B and D). Such losses of beta-adrenoceptors were not related to temperature-dependent pH changes, since experiments performed at pH 7.1 and 7.8 gave identical results. The beta-adrenoceptors of rat and rabbit lung decayed by a pseudo zero order process at 22°, and by a first order
Figure 8:2
Stabilities of soluble rat and rabbit lung preparations to storage at 22°C. (A) Rat lung membranes were solubilised in the absence (●) or presence (○) of 100 mM NaCl, and the resultant extracts were assayed for specific \(^{3}\)H-DHA binding after storage at 22°C for the indicated times. (B) NaCl-digitonin solubilised rabbit lung preparations were stored in the absence (■) and presence (□) of 1.2 nM \(^{3}\)H-DHA (producing \(\geq 60\%\) occupancy) for the indicated times, and then assayed. The results shown were representative experiments performed 2-4 times.
Figure 8:3

Thermal inactivation of specific $^3$H-DHA binding sites on soluble (A) and (C) and particulate (B) and (D) preparations of rat and rabbit lung respectively. Membrane and soluble preparations were incubated at the indicated temperatures for the stated times, cooled to 22°C, and assayed for specific $^3$H-DHA binding. The results shown were representative experiments performed 2-5 times.
process at $37^\circ$ since plots of $\ln$ receptor loss vs. time were linear. At $45^\circ$, losses of receptors were apparently second order as indicated by linear plots of $(\text{receptor loss})^{-1}$ vs. time (data not shown). The beta-adrenoceptors of rat lung membranes were inactivated at considerably faster rates than those of rabbit lung membranes: at $37^\circ$ half maximal receptor loss occurred at 3h and 5h for rat and rabbit lung respectively. This result suggested that membrane-bound beta$_1$-adrenoceptors were more stable to thermal inactivation than beta$_2$-adrenoceptors. In support of this conclusion, only 15% of unoccupied beta$_1$-adrenoceptors on chick erythrocyte membranes were lost after incubation at $37^\circ$ for 4h (data not shown).

The thermal stabilities of NaCl-digitonin solubilised preparations of rat and rabbit lung were compared (Figure 8:3, A and C). As shown previously the binding sites on soluble rat lung preparations were little affected by storage at $22^\circ$, whereas temperatures of $37^\circ$ resulted in rapid loss of specific binding sites (Figure 8:3, A). Inactivation of these soluble binding sites at $37^\circ$ was a second order process, which was 90% complete at 3h. Thus, the stabilities of the $^3$H-DHA binding sites of soluble and particulate rat lung preparations were similar.

The thermal inactivation data obtained for soluble rabbit lung $^3$H-DHA binding sites was complex (Figure 8:3, C). In contrast to the data obtained for membranes, the loss of soluble sites at $22^\circ$ was rapid. At both $22^\circ$ and $37^\circ$, there was an approximately 50% loss of binding sites after 1h and 2h respectively, followed by a slow loss over the proceeding 3-4h. These results suggested that soluble rabbit lung $^3$H-DHA binding sites were more thermolabile at $22^\circ$, than membrane bound sites. It should be borne in mind that the presence of both subtypes, having differential stabilities, in soluble preparations, would provide for a complex overall picture. The pharmacological characteristics of these preparations before and after prolonged storage has been examined in section 8:3:3:E.
Preliminary experiments had established the protective effect of NaCl on the rate of loss of rat lung $^3$H-DHA binding sites. Experiments were conducted to investigate whether NaCl could protect particulate rat lung beta-adrenoceptors from thermal inactivation. Figure 8:4(A) shows the results of exposing rat lung beta-adrenoceptor to temperatures of 45°, in incubation media containing increased concentrations of NaCl. Clearly the presence of NaCl markedly decreased the rate of thermal inactivation of these beta-adrenoceptors. Membranes in the presence of 150mM NaCl lost only 20% of sites after 2h as compared with 75% loss in NaCl-free media. This protective effect was not related to increased tonicity of the medium since replacement of NaCl by colligatively equivalent amounts of sucrose resulted in similar losses of sites to those of Tris-buffer controls (Figure 8:4:A). These decreased rates of receptor loss were not related to Na$^+$ ions per se, since other ions, e.g. MgCl$_2$, could also afford protection. Results from a number of studies indicated that the ionic strength of the medium over the range 0-0.2 was linearly related to this protective effect (Figure 8:4,B). Other experiments conducted at 37° indicated that maximum protection was achieved with ionic strengths $\geq 0.2$ (data not shown).

(B) Effect of sulfhydryl-directed modifying agents

The effects of dithiothreitol (DTT) on $^3$H-DHA binding to soluble and particulate preparations were compared. Figure 8:5(A) shows that preincubation of membranes with 1mM dithiothreitol reduced specific $^3$H-DHA binding to rat and rabbit lung membranes by 70% and 60% respectively. DTT also inhibited $^3$H-DHA binding to soluble receptors (Figure 8:5,B) with 50% inhibition of binding occurring at 0.6mM DTT for both soluble preparations (data not shown). These results suggested that DTT-treatment of particulate preparations resulted in direct modification of the beta-adrenoceptor, rather than altering the
The effect of ionic strength and tonicity upon thermal inactivation of membrane bound beta-adrenoceptors of rat lung. (A) Rat lung membranes were incubated at 45°C for the indicated times in media containing 50 mM Tris-HCl, pH 7.8, and concentrations of NaCl or sucrose as shown. The membranes were cooled to 22°C and specific binding assessed. Results were from a representative experiment performed 2-3 times. (B) Plot of the ionic strength of the incubation medium vs time for 20% loss of beta-adrenoceptors. Ionic strength was calculated using the equation $I = \frac{1}{2} \sum m_i Z_i^2$, where $m_i$ was the molality of the ion and $Z_i$ its charge.
Figure 8:5
Effects of sulfhydryl modifying agents on particulate and soluble preparations of rat and rabbit lung. (A) Membranes were incubated with buffered DTT (1 mM) or Tris buffer for 15 minutes at 22°C, washed four times in Tris-MgCl$_2$-EDTA buffer by centrifugation at 9,000 g for 3 minutes, and assayed for specific $^3$H-DHA binding. (B) Soluble preparations were incubated with buffered NEM or DTT (2 mM) for 15 minutes at 22°C. Aliquots (300 µl) were assayed for $^3$H-DHA binding. Results shown represented the mean (± SEM) of three determinations.
overall properties of the membranes. The relative susceptibilities of rat and rabbit lung beta-adrenoceptors to reduction by DTT were similar, which suggested that disulphide bonds were essential for $^3$H-DHA binding to both these beta$_1$ and beta$_2$-adrenoceptors.

The sulfhydryl group alkylating agent N-ethylmaleimide (NEM) at a final concentration of 1.2mM exerted no inhibition of $^3$H-DHA binding to soluble preparations of rat or rabbit lung (Figure 8:5:B), suggesting that sulfhydryl groups were not critical for $^3$H-DHA binding to these beta$_1$ and beta$_2$-adrenoceptors.

8:3:3 The pharmacological characteristics of soluble $^3$H-DHA binding sites

(A) $^3$H-DHA binding properties

(i) Kinetics

Specific $^3$H-DHA binding to soluble preparation of rat and rabbit lung at 22$^\circ$ was rapid and reversible (Figure 8:6). Association followed pseudo-first order kinetics since the radioligand concentration used (0.9nM) was in great excess of the receptor concentration (0.08nM), and only a small proportion (<10%) of the radioligand was bound at equilibrium. The observed rate constants indicated that binding to soluble rabbit lung proceeded considerably faster than to soluble rat lung. Mean values for association rate constants were $0.202 (\pm 0.06)\text{nM}^{-1}\text{min}^{-1}$ and $0.165 (\pm 0.01)\text{nM}^{-1}\text{min}^{-1}$ (n=3) respectively.

Dissociation of radioligand from soluble protein at 22$^\circ$ was a first order process with $t_\frac{1}{2}$ values for dissociation of 23 min (rat lung) and 11 min (rabbit lung). The mean dissociation rate constants were $0.031 (\pm 0.001)\text{min}^{-1}$ and $0.063 (\pm 0.004)\text{min}^{-1}$ (n=3) for rat and rabbit lung respectively. Therefore the kinetics of $^3$H-DHA binding to soluble preparations of rabbit lung were faster than to rat lung which established that the characteristics of $^3$H-DHA binding to particulate preparations of these tissues, were conserved upon solubilisation. However, solubilisation did result in quantitative differences in the kinetics of radioligand
Figure 8:6

Kinetics of $^3$H-DHA binding to soluble preparations of rat (•) and rabbit (△) lung at 22°C. A (i) Association data plotted directly, $^3$H-DHA concentration was 0.9 nM. (ii) Pseudo first-order plot of the association reaction.

B (i) Following the attainment of equilibrium, dissociation of $^3$H-DHA was measured after the addition of 200 µM (-)-isoprenaline. (ii) First-order plot of the dissociation data. The receptor concentration was 0.08 nM. The results shown were from a representative experiment, which was repeated three times.
binding, since association and dissociation of $^3$H-DHA to soluble protein proceeded 2-3 times slower than to particulate preparations (see Table 8:2). The equilibrium dissociation constants calculated as the fraction of $k_\text{-1}/k_\text{+1}$ were similar for particulate and soluble preparations (Table 8:2).

(ii) **Saturation**

Specific $^3$H-DHA binding to soluble receptor protein represented $> 95\%$ of total binding. It was saturable with characteristics which were similar to those determined for particulate preparations. Table 8:2 compares the specific $^3$H-DHA binding data obtained for particulate and soluble preparations of rat and rabbit lung. The equilibrium dissociation constants of $^3$H-DHA binding to soluble and particulate preparations were not significantly different. Specific binding to all preparations was indicative of a homogeneous population of sites, with binding governed by mass action law.

The maximum number of specific binding sites of NaCl-digitonin solubilised rat lung preparations was similar to that in particulate preparations, since the yield of protein and $^3$H-DHA sites was similar (35-40\%). In contrast, the density of sites in solubilised rabbit lung was less than in particulate preparations. This related to a relatively poor yield of $^3$H-DHA sites (see 8:3:1:A) whereas the yield of solubilised protein was similar to that obtained for rat lung.

(B) **Agonist binding to soluble preparations**

Displacement of specific $^3$H-DHA binding to soluble preparations of rat and rabbit lung by a series of agonists was examined. Figure 8:7 shows that agonist binding to soluble rat lung was characterised by an order of potencies of: isoprenaline > adrenaline > noradrenaline = salbutamol, which was similar to that determined for particulate preparations, and was indicative of an overall beta$_2$-adrenoceptor classification. The order of potencies determined for soluble rabbit
Table 8:2
The characteristics of $^3$H-DHA binding to soluble and particulate preparations of rat and rabbit lung.

<table>
<thead>
<tr>
<th>Agent/Determination</th>
<th>Rat Lung</th>
<th>Rabbit Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soluble</td>
<td>Particulate</td>
</tr>
<tr>
<td>$^3$H-DHA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_D$ (nM)</td>
<td>0.32 (± 0.01)</td>
<td>0.35 (± 0.01)</td>
</tr>
<tr>
<td>$B_{max}$ (fmol/mg prot.)</td>
<td>440 (± 50)</td>
<td>410 (± 20)</td>
</tr>
<tr>
<td>Hill slope ($n_H$)</td>
<td>1.10 (± 0.03)</td>
<td>1.05 (± 0.04)</td>
</tr>
<tr>
<td>*Association rate constant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_{+1}$ (nM$^{-1}$ min$^{-1}$)</td>
<td>0.165 (± 0.01)</td>
<td>0.262 (± 0.01)</td>
</tr>
<tr>
<td>*Dissociation rate constant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_{-1}$ (min$^{-1}$)</td>
<td>0.031 (± 0.001)</td>
<td>0.069 (± 0.004)</td>
</tr>
<tr>
<td>$t_{1/2}$ (diss) (min)</td>
<td>22</td>
<td>10</td>
</tr>
<tr>
<td>$K_D$, $K_{-1}/K_{+1}$ (nM)</td>
<td>0.19</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Results were the mean (± SEM) of 3-6 determinations, performed in duplicate. *Values were obtained at 22°C using 0.9-1.3 nM $^3$H-DHA.
Figure 8:7
Agonist binding to soluble preparations of rat (A) and rabbit (B) lung. Specific $^3$H-DHA binding was determined in the presence of increasing concentrations of (-)-isoprenaline (Δ), (-)-adrenaline (●), (-)-noradrenaline (■), and (±)-salbutamol (□). Results were the mean (± SEM) of 3-7 experiments performed in duplicate.
lung was isoprenaline > noradrenaline = adrenaline > salbutamol, which was again similar to that obtained with membranes, and was suggestive of an overall beta-adrenoceptor classification.

In contrast to the catecholamine displacement curves for particulate rat lung preparations, which were shallow with slope factors < 1, equivalent curves for soluble preparations were steep with slope factors which approached unity. Thus, binding of agonists to soluble preparations of this tissue was indicative of binding to a homogeneous population of sites with binding governed by mass action law. The affinities of the catecholamines for soluble receptors were approximately three to eight fold more potent than for particulate receptors. This 'solubilisation shift' was less pronounced for the partial agonist salbutamol which exhibited only a two-fold increase in affinity (See Table 8:3).

Some of the characteristics of agonist binding to soluble rat lung beta-adrenoceptors were also observed with rabbit lung preparations. Thus, overall catecholamine affinities were greater for soluble than particulate preparations by approximately nine to twelve fold. In contrast, the affinities of salbutamol for soluble and particulate rabbit lung beta-adrenoceptors were not significantly different (See Table 8:3) which may have reflected the lack of efficacy of salbutamol at beta_1-adrenoceptors (See Chapter 7). The competition curves of isoprenaline adrenaline and salbutamol were steep, whereas the curve obtained for noradrenaline was markedly biphasic with an inflection at approximately 60% occupancy. This result suggested the possibility of heterogeneous binding sites perhaps equivalent to beta_1 and beta_2-adrenoceptors in the soluble rabbit lung preparation, since only noradrenaline exhibited subtype-selectivity. The noradrenaline competition curve was analysed by iterative curve fitting to a two-site model. Results indicated the presence of approximately 70% of a site having high affinity for these soluble receptors, and 30% low affinity, with a selectivity index
Table 8:3
Pharmacological specificities of agonist and antagonist binding to soluble and particulate rat and rabbit lung membranes.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Rat Lung</th>
<th>Rabbit Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Particulate</td>
<td>*Soluble</td>
</tr>
<tr>
<td><strong>Agonists</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(-)-Isoprenaline</td>
<td>27 (± 3)</td>
<td>8 (± 1.2)</td>
</tr>
<tr>
<td>(-)-Adrenaline</td>
<td>245 (± 25)</td>
<td>32 (± 5 )</td>
</tr>
<tr>
<td>(-)-Noradrenaline</td>
<td>1,430 (± 180)</td>
<td>470 (± 60)</td>
</tr>
<tr>
<td>(±)-Salbutamol</td>
<td>660 (± 130)</td>
<td>360 (± 52)</td>
</tr>
<tr>
<td><strong>Antagonists</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(-)-Timolol</td>
<td>0.46 (± 0.04)</td>
<td>0.92 (± 0.05)</td>
</tr>
<tr>
<td>(-)-Propranolol</td>
<td>0.69 (± 0.05)</td>
<td>0.86 (± 0.02)</td>
</tr>
<tr>
<td>(+)-Propranolol</td>
<td>55 (± 2)</td>
<td>91 (± 2)</td>
</tr>
</tbody>
</table>

*Soluble receptors were NaCl-digitonin solubilised preparations. Results were the mean (± SEM) of 3-7 experiments performed in duplicate.
between these sites of approximately two hundred-fold.

The above results were all obtained with NaCl-digitonin solubilised preparations. Soluble rat lung beta-adrenoceptors obtained by digitonin solubilisation in the absence of NaCl bound agonists with higher affinities. Thus, the presence of NaCl (100mM) during solubilisation or its subsequent addition, resulted in a 1.6-2-fold decrease in the affinities of all four agonists (data not shown).

The ability of GTP to modulate isoprenaline binding to particulate preparations of rat lung was lost upon solubilisation. Similar results were observed for noradrenaline binding to soluble rabbit lung membranes, where GTP (100μM) failed to affect the biphasic displacement curve of this agonist (data not shown).

(C) Non-selective antagonist binding to soluble receptors

The affinities of the optical isomers of propranolol were slightly weaker in soluble preparations of rat and rabbit lung than in particulate preparations, although the large stereoselectivity index was maintained (Table 8:3). The affinities of timolol for soluble and particulate rat lung beta-adrenoceptors differed by only two-fold. These results suggested that the major characteristics of the beta-adrenoceptors were conserved upon solubilisation, although non-selective antagonists were slightly weaker at soluble beta-adrenoceptors by $2(±0.3)$ fold, $n = 5$. This effect could have been due to (i) the presence of NaCl in the soluble preparations or (ii) slightly altered conformational states of the soluble receptor, which resulted in slightly modified properties. Timolol binding to particulate and soluble preparations of rat lung were examined under similar conditions of NaCl concentration. Results indicated that NaCl (60mM) did not affect the affinity of timolol for particulate receptors. Atenolol binding to particulate preparations of rabbit lung was also unaffected by 60mM NaCl (data not shown).
Moreover, the association and dissociation rate constants of $^3$H-DHA-binding to particulate preparations of rabbit lung were unaffected by 60mM NaCl (data not shown). Sodium chloride did not appear to increase the free $^3$H-DHA concentration in the assay tubes. These findings suggested that antagonists binding to particulate beta-adrenoceptors was unaffected by 60mM NaCl (the highest concentration which would have been present in the assay of soluble receptors). Therefore, the affinity changes observed upon solubilisation were not directly related to the presence of NaCl during the assay of soluble receptors, but may have resulted from slightly altered states of the receptors, perhaps associated with removal of phospholipid constraints on the receptor protein.

(D) Selective-antagonist binding to soluble beta-adrenoceptors

In order to examine whether co-solubilisation of beta$_1$ and beta$_2$-adrenoceptors had occurred, the binding of selective antagonists to soluble preparations was determined. Figure 8:8 shows the results of testing the beta$_1$-selective antagonist atenolol and the beta$_2$-selective antagonist ICI 118,551 in particulate and soluble preparations of rat and rabbit lung. As shown previously, both rat and rabbit lung membrane preparations contained beta$_1$ and beta$_2$-sites. Atenolol and ICI 118,551 generated curves which analysed as: rabbit lung, 80% beta$_1$, 20% beta$_2$; rat lung, 20% beta$_1$, 80% beta$_2$.

In the solubilised rat lung preparations, both atenolol and ICI 118,551 generated curves which approximated to a single class of binding site behaving according to mass action law, and which indicated an almost homogeneous population of beta$_2$-adrenoceptors. This suggested that either (i) there was only one type of beta-adrenoceptor which corresponded to the beta$_2$-subclass, and that an apparent small population of beta$_1$-adrenoceptors was conferred by the constraint of the membrane.
Figure 8:8

Inhibition of specific $^3$H-DHA binding to soluble (filled symbols) and particulate (open symbols) preparations of rabbit (A) and rat (B) lung by ICI 118.551 and atenolol. The curves shown were the mean of 3-7 experiments performed in duplicate. SEM's were <10%. Iterative curve fitting revealed the following parameters for IC$_{50}$ values and proportions of sites: Rabbit Lung: atenolol (particulate) $\beta_1$-site 800 nM 79%, $\beta_2$-site 41,000 nM 21%; (soluble) $\beta_1$-site 2,700 nM 58%, $\beta_2$-site 63,000 nM 42%; ICI 118.551 (particulate) $\beta_1$-site 210 nM 81%, $\beta_2$-site 6 nM 19%; (soluble) $\beta_1$-site 840 nM 60%, $\beta_2$-site 13 nM 40%.

Rat Lung: atenolol (particulate) $\beta_1$-site 210 nM 20%, $\beta_2$-site 31,000 nM 80%; (soluble) $\beta_2$-site 61,000 nM 100%; ICI 118.551 (particulate) $\beta_1$-site 530 nM 17%, $\beta_2$-site 8.5 nM 83%; (soluble) $\beta_1$-site 2,200 nM 6%, $\beta_2$-site 12 nM 94%.
or (ii) there was a selective loss of beta₁-adrenoceptors upon solubilisation of the membranes. The data obtained with rabbit lung, with its greater initial proportion of membrane beta₁-sites, suggested that the latter alternative was correct. Thus, in solubilised rabbit lung, the co-presence of beta₁ and beta₂-sites were clearly seen with both ICI 118,551 and atenolol, although the relative proportions of beta₁-sites was reduced from that seen in the membranes. (Figure 8:8). Similar proportions of subtypes were obtained with both agents (58-60% beta₁-sites), and also with betaxolol and procaterol (data not shown).

These findings allowed the relative yields of the beta-adrenoceptor subtypes to be calculated. Solubilisation of rabbit lung membranes resulted in an overall yield of 16-20% beta-adrenoceptors, of which 50-60% were beta₁-adrenoceptors. Therefore, yields of beta₁ and beta₂-adrenoceptors were approximately 13% and 40% respectively. Equivalent values for solubilisation of rat lung beta₁ and beta₂-adrenoceptors were approximately 10-15% and 40-50% respectively. These yields of beta₁-adrenoceptors were obtained using NaCl-digitonin to solubilise beta-adrenoceptors. Early solubilisations, performed in the absence of NaCl gave lower yields of ³H-DHA sites (≈ 10%), which related to extremely poor yields of beta₁-adrenoceptors. Therefore, the competition curves of selective antagonist binding to such soluble preparations of rat and rabbit lung indicated a homogeneous population of binding sites for the former preparation and the presence of a small (≈ 10%) proportion of beta₁-adrenoceptors for the latter preparation. These soluble rabbit lung beta-adrenoceptors had affinities for adrenaline and noradrenaline which differed by ten-fold, which therefore suggested an overall beta₂-adrenoceptor classification, and which confirmed the low numbers of beta₁-adrenoceptors in these early preparations.
Iterative curve-fitting of the competition curves obtained with NaCl-digitonin solubilised rabbit lung membranes was performed in order to calculate the affinities of drugs for soluble beta₁- and beta₂-adrenoceptors. The analysis of curves obtained with soluble rat lung preparations only gave reliable estimates of the affinities of drugs for beta₂-adrenoceptors since the proportion of beta₁-sites was generally <10%. Table 8:4 shows that solubilisation of beta₁ and beta₂-adrenoceptors resulted in preparations for which selective antagonists had slightly weaker affinities. The 'solubilisation shift' for antagonist affinities at rat and rabbit lung beta₂-adrenoceptors was 2.1(±0.5) and 3.2 (±0.7) respectively (n=4), and 3.7 (±0.5) (n=3) for rabbit lung beta₁-adrenoceptors. In summary, solubilisation of beta-adrenoceptors resulted in preparations which exhibited an approximately three-fold and two-fold decreased affinities for selective and non-selective antagonists respectively, although the major characteristics of the beta-adrenoceptors were conserved in solution.

(E) The pharmacological characteristics of 'aged' soluble rabbit lung preparations

Previous studies had shown that storage of soluble rabbit lung preparations at 22⁰ or 37⁰ resulted in a significant loss in the number of beta-adrenoceptors. Moreover, yields of beta₁-adrenoceptors were considerably less than beta₂-adrenoceptors, a finding which was not related to incomplete solubilisation of membrane-bound beta₁-adrenoceptors. These results suggested that soluble beta₁-adrenoceptors were more labile than beta₂-sites, and that the decrease in receptor number could relate to preferential loss of beta₁-adrenoceptors. Experiments were conducted to analyse the pharmacological characteristics of soluble rabbit lung preparations immediately after solubilisation, and after the number of ³H-DHA sites were reduced following storage for various periods at 22⁰. Figure 8:9 shows the atenolol displacement of specific ³H-DHA binding to
Table 8:4

Inhibition constants of selective beta-adrenoceptor antagonists at beta₁ and beta₂ adrenoceptor sites of soluble and particulate rat and rabbit lung preparations.

<table>
<thead>
<tr>
<th>Selective agent</th>
<th>Rabbit Lung</th>
<th>Rat Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Particulate</td>
<td>Soluble</td>
</tr>
<tr>
<td></td>
<td>β₁ β₂</td>
<td>β₁ β₂</td>
</tr>
<tr>
<td>Atenolol</td>
<td>180 12,700</td>
<td>830 19,500</td>
</tr>
<tr>
<td>Betaxolol</td>
<td>3.7 310</td>
<td>17 1,200</td>
</tr>
<tr>
<td>ICI 118.551</td>
<td>63 0.74</td>
<td>260 4</td>
</tr>
<tr>
<td>Procatelol</td>
<td>2,800 18</td>
<td>ND ND</td>
</tr>
</tbody>
</table>

IC₅₀ values of selective agents at beta₁ and beta₂ adrenoceptor sites were obtained by iterative curve-fitting of competition curves of 3-9 experiments. Inhibition constants were calculated as previously described. *Soluble rat lung preparations had proportions of beta₁-adrenoceptors which were too small to make accurate assessments of affinities of drugs at this site.
Inhibition of specific $^3$H-DHA binding to a soluble preparation of rabbit lung by the beta$_1$-selective antagonist atenolol. Soluble preparations were assayed immediately after solubilisation (▲) or after 1½ h (▲), 5 h (□), or 5½ h (■) incubation at 22°C. Mean curves of 2-4 determinations were analysed by iterative curve-fitting to yield the following parameters: No incubation - beta$_1$-site 1900 nM, 53%, beta$_2$-site 46000 nM, 47%; 1½ h - beta$_1$-site 1500 nM, 36%, beta$_2$-site 62000 nM, 64%; 5 h - beta$_1$-site 2300 nM, 15%, beta$_2$-site 64000 nM, 85%; 5½ h - beta$_2$-site 56000 nM, 100%.
soluble rabbit lung preparations after 1½, 5 and 5½h, after which times losses of beta-adrenoceptors were approximately 35%, 55% and 60%.

Clearly, as the preparation 'aged' and the number of ³H-DHA sites declined, so the atenolol competition curve shifted to the right.

Computer-assisted fitting of the curves demonstrated a preferential loss of beta₁-sites with time, whereas the affinities of atenolol for the beta₁ and beta₂-sites were unchanged.

Similar results to these have been obtained using the beta₂-adrenoceptor selective antagonist ICI 118,551. In this case storage for increased periods of time was associated with a shift of the ICI 118,551 curves to the left. Curve fitting revealed an increased proportion of beta₂ adrenoceptors and a decreased number of beta₁-adrenoceptors with time (data not shown).

Noradrenaline generated displacement curves with soluble rabbit lung preparations which were biphasic and indicative of heterogeneous binding sites for this beta₁-selective agonist (See Figure 8:7), perhaps related to the co-presence of beta₁ and beta₂-adrenoceptors. Experiments were therefore conducted to characterise the agonist binding properties of control soluble rabbit lung preparations, and preparations in which the number of beta₁-adrenoceptors had been reduced following prolonged storage at 22°. Figure 8:10 shows adrenaline and noradrenaline displacement of ³H-DHA binding to control and 'aged' preparations, where the total number of beta-adrenoceptors had been reduced by approximately 45%.

The adrenaline competition curves were identical in both preparations, being steep with Hill slope factors which approached unity. In contrast, noradrenaline binding to control membranes was characterised by inhibition curves which were shallow (n_H=0.4) and which analysed to a two site system having 51% high (IC₅₀=1.2x10⁻⁷M) and 49% low (IC₅₀=1.9x10⁻⁵M) affinity sites for noradrenaline.

After the number of beta₁-adrenoceptors had been reduced by storage, noradrenaline generated a competition curve which was steeper (n_H=0.7), and which analysed as a 2-site system having ≈ 25% high affinity sites.
Figure 8:10
Adrenaline (●, ○) and noradrenaline (▲, △) displacement of specific $^3$H-DHA binding to soluble rabbit lung preparations. Assays were conducted (A) immediately upon thawing the frozen preparation, or (B) after the preparation had been stored at 22°C for 5 h. Results were the mean (± SEM) of 3-4 experiments performed in duplicate.
Therefore, losses of beta-adrenoceptors in these preparations was associated with decreased proportions of the high affinity noradrenaline site (\(\beta_1\)-adrenoceptors). The relative potencies of adrenaline and noradrenaline for these 'aged' preparations was now characteristic of an overall \(\beta_2\)-adrenoceptor classification for the residual sites. It was notable that the affinity of noradrenaline for these \(\beta_2\)-sites (\(K_i=6400\) nM) was approximately ten fold weaker than its value (\(K_i=470\) nM) obtained with soluble rat lung \(\beta_2\)-adrenoceptors.

In conclusion, the above results indicated that both \(\beta_1\)- and \(\beta_2\)-adrenoceptors could be co-solubilised from rabbit lung membranes and that they had differential stabilities to storage at 22°. It seemed likely that the lower recovery of total beta-adrenoceptors from rabbit lung reflected a selective loss of the initial high proportion of \(\beta_1\)-sites in this preparation.

8:4 Discussion

Solubilisation of beta-adrenoceptors from rat and rabbit lung has been attempted using a number of detergents which have been successfully used to solubilise neurotransmitter and hormone receptors. Triton X100, a non ionic detergent which solubilised nicotinic receptors of electrophorus (Prives et al, 1972); GABA and benzodiazepine receptors (Gavish and Snyder 1981); pituitary thyrotropin releasing hormone receptors (Hinkle and Lewis, 1974) and vasopressin receptors (Guillon et al, 1980) did not solubilise beta-adrenoceptors of rabbit lung in active form, although successful solubilisation of canine liver beta-adrenoceptors had been claimed (Strauss et al, 1979). Deoxycholate, a detergent used to solubilise rat brain alpha1-adrenoceptors (Guichenev et al, 1981), and canine myocardial beta-adrenoceptors (Wrenn and Haber, 1979) was also ineffective in solubilising rabbit lung beta-adrenoceptors. This may have reflected detergent interference with the binding assay, since binding of \(^3\)H-propranolol could not be detected in detergent
containing solutions (Wrenn and Haber, 1979). Alternatively, soluble receptor protein could have been quite labile in the concentrations of Doc solutions used (Cori et al, 1973). Octylglycoside, a detergent successfully used to solubilise the D-glucose transporter from red blood cells (Kasahara and Hindoe 1977), also failed to solubilise active beta-adrenoceptors. High salt concentrations, which have been reported to yield 'soluble' preparations of rat brain muscarinic receptors (Carson et al, 1977) and spiroperidol binding sites of calf striatum (Clement-Cormier and Kendrick, 1980) also gave negative results. However, these salt preparations have recently been shown not to conform to the criteria of molecularly dispersed material (Gorrissen et al, 1981).

The plant glycoside digitonin was uniquely effective in solubilising rat and rabbit lung beta-adrenoceptors and allowing their subsequent detection in detergent-containing extracts. The presence of sodium chloride was found to enhance the yield both of soluble protein and beta-adrenoceptors from these tissues, which confirmed similar observations made on digitonin-solubilisation of bovine brain muscarinic receptors (Aronstam et al, 1978).

Sodium chloride-digitonin solubilisation of rat lung and erythrocyte beta_2-adrenoceptors proceeded in good yield, whereas yields of soluble beta_1-adrenoceptors from chick erythrocyte and rabbit lung were negligible and poor respectively. The inability of NaCl-digitonin to solubilise chick erythrocyte beta-adrenoceptors was problematic, since successful solubilisation of turkey erythrocyte beta-adrenoceptors had been reported using a similar solubilisation protocol (Vauquelin et al, 1977; Pike and Lefkowitz 1978). In another report, the presence of isoprenaline during solubilisation of turkey erythrocyte membranes was found to be critical for the production of functionally active soluble beta-adrenoceptors (Eimerl et al, 1980), although such a protocol failed to allow solubilisation of chick erythrocyte beta-adrenoceptors. These results
indicated that either soluble chick erythrocyte beta-adrenoceptors were considerably more labile than those of turkey erythrocytes or that the lipid composition of chick erythrocyte membranes differed enough to disallow the solubilisation of beta-adrenoceptors from this system. The latter explanation was perhaps suggested by the poor yields of soluble membrane protein obtained, although it was noted that the effects of interventions aimed at affecting the lipid environment of chick and turkey erythrocyte membranes (e.g. temperature, fatty acid insertion) were in almost all respects identical (See Chapter 7). Successful digitonin-solubilisation of duck erythrocyte beta-adrenoceptors has been reported after freezing and thawing erythrocyte ghosts, followed by solubilisation at room temperature (Meier and Ruoho, 1978). Clearly, further work will be required in order to optimise the procedure for the solubilisation of chick erythrocyte beta-adrenoceptors.

In addition to the effect of sodium chloride to increase the yield of soluble protein and receptors, it also enhanced the stability of soluble rat and rabbit lung beta-adrenoceptors to prolonged storage at room temperature. Similar findings have been made for soluble beta-adrenoceptors of frog erythrocytes (Caron et al, 1979). Thermal inactivation of membrane-bound beta-adrenoceptors was decreased by sodium chloride, an effect which was directly correlated with the ability of NaCl to increase the ionic strength of the medium. This effect probably resulted from the ability of NaCl to 'salt in' proteins at low ionic strength i.e. to stabilise charged groups on the receptor protein. Under such conditions, the receptor protein was rendered more resistant to thermal denaturation, a process which has been defined as a disordering of the polypeptide chains (Kauzmann, 1959).

The chemical basis of thermal inactivation of proteins is poorly
understood in view of the complexity of the transition states between active and inactive proteins. Membrane-bound rat lung beta-adrenoceptors were denatured by a first order process at 37° but by a second order process to 45°. More complex data have been obtained for the denaturation of haemoglobin by urea. This process proceeded in a highly complicated and co-operative fashion, since rates of inactivation were approximately fifth order in urea (Simko and Kauzmann, 1962). Soluble beta-adrenoceptor preparations of rat and rabbit lung were more thermolabile than membrane-bound receptors, and occupation of soluble beta-adrenoceptors by ligand increased their stabilities to thermal inactivation. Similar results were reported for soluble preparations of canine heart and liver, where occupation of beta-adrenoceptors by 125I-HYP increased the t½ for inactivation at 0° by 35- and 27-fold respectively (Strauss et al, 1979). However, solubilisation of active rat and rabbit lung beta-adrenoceptors was not dependent on prior occupation of membrane-bound receptors with ligand. This finding contrasted with other reports which indicated that occupation of S49 lymphoma cell and turkey erythrocyte beta-adrenoceptors was essential for the solubilisation of active receptor preparations (Haga et al, 1977; Eimerl et al, 1980).

The results of the present study also indicated the importance of disulphide bonds for the binding of 3H-DHA to rat and rabbit lung beta-adrenoceptors. Treatment of membrane bound beta-adrenoceptors by DTT resulted in decreased binding of 3H-DHA, an effect which was not reversible if NEM was included in the washing buffers (unpublished observations). Binding of 3H-DHA to soluble preparations was inhibited by DTT but not by NEM. These results suggested that 3H-DHA binding to beta2- and beta1-adrenoceptors of rat and rabbit lung required an essential disulphide bond. Disulphide bonds have
been implicated in $^3$H-DHA binding to beta-adrenoceptors of turkey erythrocytes (Vauquelin et al, 1979) and rat liver (Guellaen and Hanoune, 1979), although they were apparently not critical for ligand binding to frog erythrocyte beta-adrenoceptors (Mukherjee and Lefkowitz, 1977). Rat liver alpha-adrenoceptors were also reported not to contain critical disulphide bonds (Guellaen and Hanoune, 1979) a finding which provided additional evidence against the interconversion hypothesis (Kunos et al, 1968).

A number of hormone and neurotransmitter receptors appeared to contain essential sulfhydryl and/or disulphide bridge, modification of which markedly influenced agonist and/or antagonist binding. These included:- rat testis interstitial cell gonatrophin receptors (Dufau et al, 1974); and electroplax acetyl choline receptors (Karlin, 1973); calf striated dopamine receptors (Suen et al, 1980); C6 glioma cell beta-adrenoceptors (Lucas et al, 1978); and muscarinic receptors of rat brain (Hedlund and Bartfai 1979) and heart (Wei and Sulakhe, 1980). No generalised model could be drawn from these studies, however, since the effects of modifying agents in these systems differed so markedly, perhaps reflecting the position of these bonds relative to the ligand binding sites. However, these observations do point to a central role of sulfhydryl-disulphide bonds in ligand binding to many receptor systems and their interconversion may be the mechanism by which receptors were desensitised (Levitzki and Atlas, 1981).

The inactivation by DTT of turkey erythrocyte beta-adrenoceptors (Vauquelin et al, 1979) and eel electroplax acetyl choline receptors (Karlin et al, 1976) was attenuated by agonist or antagonist occupation of the receptors, which suggested that the reactive group was closely associated with the ligand binding site. Preliminary experiments using membrane preparations of rat and chick erythrocytes indicated that occupation of these beta-adrenoceptors with isoprenaline afforded some protection against reductive
inactivation by DTT (unpublished observations). This finding suggested that it may be possible to reduce essential disulphide bonds of mammalian beta-adrenoceptors and alkylate them with appropriately designed affinity labels based on beta-adrenoceptor agents. Such techniques have been applied with considerable success to electroplax acetylcholine receptors (Karlin et al., 1976).

The major aim of the present study was to determine the pharmacological characteristics of soluble beta-adrenoceptors from tissue containing beta₁ and beta₂-adrenoceptors. Sodium chloride-digitonin solubilisation of these beta-adrenoceptors resulted in preparations which exhibited most of the characteristics of the membrane-bound receptors. Thus, specific $^3$H-DHA binding to soluble receptors was rapid, reversible and saturable, with equilibrium dissociation constants which were not significantly different from values obtained with membrane-bound beta-adrenoceptors. $^3$H-DHA binding to soluble sites was displaceable by beta-adrenoceptor agents with affinities which indicated binding to a beta-adrenoceptor. The large stereoselective difference of the optical isomers of propranolol observed in membranes was conserved in solution. Moreover, the beta₂ and beta₁-adrenoceptor characteristics of rat and rabbit lung was maintained in solution, as demonstrated by the potency ratios of agonists and selective antagonist affinities.

The heterogeneous beta₁ and beta₂-sites found in particulate preparations of rat lung were less apparent in most soluble preparations, a finding which could be interpreted as evidence against the molecular individuality of beta-adrenoceptor subtypes. However, soluble preparations of rabbit lung exhibited characteristics which strongly suggested the co-presence, in solution, of beta₁ and
beta_2-adrenoceptors. This finding was the first demonstration of the co-solubilisation of beta-adrenoceptor subtypes, and it argued against the concept that the characteristics of beta-adrenoceptors were directed by the lipid environment or the metabolic state of the tissue. However, these results did not unequivocally demonstrate that beta_1 and beta_2 adrenoceptors were separate entities, since they could still have resided on the same macromolecule. Some evidence that soluble beta_1 and beta_2-adrenoceptors were separate entities could be inferred from the results of the stability experiments.

Storage of soluble rabbit lung preparations at 22^oC for prolonged periods of time resulted in an initial, relatively rapid, loss of a proportion of the beta-adrenoceptors, which had the pharmacological characteristics of beta_1-adrenoceptors. The residual sites were pharmacologically equivalent to beta_2-adrenoceptors, and exhibited a stability to storage at 22^oC which was similar to that obtained for rat lung beta_2-adrenoceptors. These data did not support the concept of an interconversion of subtypes from beta_1 to beta_2, but to the preferential loss of beta_1-adrenoceptors. The differential stabilities of rabbit lung beta_1 and beta_2-adrenoceptors suggested that these sites may be separate entities. Support for this concept has come from the work of Venter and colleagues, who showed that autoantibodies present in the serum of patients with allergic rhinitis or asthma inhibited ligand binding to canine or calf lung beta_2-adrenoceptors, but not to heart beta_1-adrenoceptors (Venter et al, 1980).

Few studies have been reported on the solubilisation of mammalian beta-adrenoceptors (Kleinstein and Glossman, 1978; Fraser et al, 1978;
Haga et al., 1978; Wood et al., 1979; Wrenn and Haber, 1979; Schoken et al., 1980) and none of these have accounted for the presence of both beta<sub>1</sub>- and beta<sub>2</sub>-adrenoceptors in most mammalian tissue (Nahorski 1981). In general the pharmacological characteristics of the soluble preparation were similar to those of membrane-bound beta-adrenoceptors, which suggested that the conformational states of the solubilised beta-adrenoceptors were similar to those of membrane-bound receptors. The results of the present work indicated that the major characteristics of beta<sub>1</sub> and beta<sub>2</sub>-adrenoceptors were conserved in solution, although a number of differences were also apparent. Thus, the rates of <sup>3</sup>H-DHA association and dissociation to soluble receptors were slower than to membrane-bound receptors, which may have reflected the importance of the lipid environment to constrain the membrane-bound receptor into a thermodynamically favourable conformation. Once solubilised, rat and rabbit lung beta-adrenoceptors exhibited approximately two to three fold weaker affinities for beta-adrenoceptor antagonists which again suggested that the receptors had undergone slight conformational changes upon solubilisation. Digitonin-extracted preparations of dopamine receptors (Gorissen and Laduron 1979), muscarinic receptors (Beld and Ariens, 1974) and alpha receptors (Wood et al., 1979) have also been reported to show decreased affinities for specific ligands. Transition of receptors from membrane environment to detergent micelle would be expected to result in conformational changes, which could profoundly alter the characteristics of the soluble receptor. Indeed, the ligand binding specificity of purified cholinergic receptor has been shown to differ somewhat from that in the intact membrane (Changeux et al., 1976), and opening a disulphide bridge in C6 glioma cell beta-adrenoceptors, to produce marked conformational alterations, resulted in a 10-25 fold loss in the affinities of the optical isomers of isoprenaline (Lucas et al., 1978).
The major difference between membrane bound and soluble rat and rabbit lung beta-adrenoceptors was a marked increase in affinities shown by agonists for the soluble preparations. Similar findings have been described for digitonin-solubilised beta-adrenoceptors from turkey erythrocytes (Vauquelin et al., 1977; Pike and Lefkowitz, 1978); guinea pig lung (Kleinstein and Glossmann, 1978); rat liver (Wood et al., 1979); and human placenta (Schocken et al., 1980), although apparently not for frog erythrocyte beta-adrenoceptors (Pike and Lefkowitz 1978). These effects could be postulated to have resulted from (i) solubilised receptors being 'locked' into a high affinity state, perhaps associated with Nprotein, or other transducer membrane components or (ii) alterations in the conformational state of the receptors which now became freed from the inhibitory constraints of the membrane lipids.

After affinity chromatography of soluble turkey erythrocyte and guinea pig lung beta-adrenoceptors, the inhibition constants of agonists for these purified receptors were similar to those of membrane-bound receptors (Vauquelin et al., 1977; Bonacker and Glossman, 1981). These findings have been interpreted as indicating that certain membrane components facilitated the affinity changes, and that these were removed from the receptor by affinity chromatography (Bonacker and Glossmann 1981). Nprotein was a possible candidate although solubilisation of Nprotein-coupled receptor has been reported to require prior occupation of receptors by agonists (Limbird et al., 1980b). In addition, guanine nucleotide modulation of agonist binding to soluble beta-adrenoceptors was not apparent (Kleinstein and Glossmann, 1978; this study) which suggested a functional uncoupling of Nprotein and receptor in solution. Reports of guanine nucleotide effects on agonist binding to soluble glucagon (Welton et al., 1977) vasopressin (Guillon et al., 1980) and histamine receptors (Cybulsky et al., 1981) have been described however, which suggested that solubilisation of these systems had not eliminated
interaction between Nprotein and receptor. It was significant in this respect, that digitonin-solubilised rat erythrocyte beta-adrenoceptors, which would not be expected to solubilise as a Nprotein-receptor complex (Limbird et al, 1980 a ) also demonstrated a marked affinity increase for isoprenaline (unpublished observations).

Previous studies of particulate rat and rabbit lung beta-adrenoceptors had established the pharmacological uniformity of the beta-adrenoceptor sub-types of these preparations (see chapters 3 and 5). These results were confirmed for the soluble beta_2-adrenoceptors of rat and rabbit lung. However, a number of observations suggested that soluble beta_2-adrenoceptors of rat and rabbit lung did not possess identical properties. Thus, after thermal inactivation of the beta_1-adrenoceptors of soluble rabbit lung, the residual ^3H-DHA sites had the pharmacological characteristics of beta_2-adrenoceptors, although these receptors were quite stable at 37° in contrast to the labile soluble beta_2-adrenoceptors of rat lung. Moreover, the affinity of noradrenaline for these residual sites was some ten-fold weaker than its affinity for soluble rat lung beta_2-adrenoceptors. Furthermore, ^3H-DHA dissociated from these residual sites with a dissociation rate constant which was greater than that obtained for soluble rat lung beta_2-adrenoceptors (unpublished observations). Although it cannot be ruled out that prolonged storage of the rabbit lung beta_2-adrenoceptors may have contributed to these anomalous properties, these results suggested that the beta_2-adrenoceptor proteins of rabbit and rat lung may exhibit some differences. Physical separation and characterisation of the beta-adrenoceptor subtypes of these preparations is clearly required in order to investigate the molecular individuality of the beta-adrenoceptor subtypes.
CHAPTER 9

Purification of beta-adrenoceptors and their hydrodynamic properties
9:1 Introduction

The definitive evidence that beta-adrenoceptor subtypes were discrete molecular entities required their purification from detergent solubilised preparations. The initial step most commonly employed in the purification of receptors has been the production of suitable affinity adsorbents, which allowed detergent solubilised receptors to be adsorbed to a column and washed free of unbound contaminants. The nicotinic acetylcholine receptor of fish electric organs has served as an archetype for receptor purification, and affinity adsorbents based on acetylcholine analogues (Raftery et al., 1976) and cobra venom toxin (Lindstrom and Patrick, 1974) have been successfully used to effect substantial purification of the receptor. In recent years a number of affinity adsorbents have been developed for the purification of beta-adrenoceptors using columns of agarose-linked catecholamines (Lefkowitz et al., 1972; Venter et al., 1972; Wrenn and Haber 1979) and alprenolol (Vauquelin et al., 1978; Caron et al., 1979), and of these the latter systems have proved more satisfactory (Vauquelin et al., 1978). An alternative approach for the detection and purification of receptors has been the use of antibodies to the receptor. Thus, antibodies to the acetylcholine receptor have been extremely useful as receptor probes in vitro and in vivo (see Lindstrom 1978), and recently monoclonal beta-adrenoceptor antibody affinity columns have been used to purify turkey erythrocyte beta-adrenoceptors to homogeneity (Fraser and Venter, 1980). Preliminary attempts to purify soluble rat lung beta-adrenoceptors using alprenolol-affinity adsorbents will be described in this chapter.

The main aim of the present studies was to examine the hydrodynamics of soluble rat and rabbit lung beta-adrenoceptors using gel exclusion chromatography. This technique was established as a first step towards a determination of the molecular size of these soluble beta-adrenoceptor...
preparations, and to test whether detergent-receptor complexes of beta-1- and beta-2-adrenoceptors had different hydrodynamic properties. Solubilised canine liver and heart beta-adrenoceptors had been reported to elute from sephadex G200 columns with apparent Stokes radii of 5.1 and 4.2 nm respectively (Fraser et al., 1978). The studies to be described will examine the hydrodynamic properties of detergent extracts of unoccupied beta-adrenoceptors. Previous work had established that these beta-adrenoceptor preparations were relatively stable at low temperature in the presence of NaCl (See Chapter 8). In contrast, the hydrodynamic properties of beta-adrenoceptors from S49 lymphoma cells (Haga et al., 1977) and canine heart and liver (Strauss et al., 1979) could only be performed on 125I-HYP pre-labelled receptors.

9:2 Methods
9:2:1 Gel Filtration

Pre-swollen sephacryl S200 or S300 superfine (Pharmacia Ltd.) was suspended in 100mM NaCl/50mM Tris HCl pH 7.8 and allowed to settle out under gravity. The 'fines' were removed by decanting the supernatant, a process which was repeated 3-4 times. Dissolved gases were removed from the suspension under reduced pressure, and the slurry was applied to a Pharmacia column KL6 having dimensions 1.6cm diam x 60cm. The gels were packed and equilibrated overnight at 2°C by pumping 'degassed' NaCl/Tris-HCl pH 7.6 through the columns at flow rates of 60ml/h (S200) and 45ml/h (S300). The gels were continuously eluted with buffer during the course of these experiments at flow rates of 15ml/h, which maintained constant elution properties of the gels.

Standard marker proteins or soluble receptor preparations were applied to the columns in 0.5-1ml volumes, which was <1% of the bed volume. Elution of proteins was performed using 100mM NaCl, 50mM Tris-HCl pH 7.6 (±1% digitonin) at 2°C, using a flow rate of 15ml/h. Protein peaks
were determined by continuously monitoring the optical density of the eluate at 280nm using a Cecil CE 272 spectrophotometer equipped with a flow cell. Eluates were collected using an LKB 2070 Ultrorac II fraction collector set to collect specified volumes by monitoring time intervals or drop number. The latter method was not used where the detergent concentration varied, since drop volume was dependent on detergent concentration. In general the elution volumes of each fraction varied by <5%.

Elution of proteins from these columns was determined by measuring the $K_{AV}$ (the fraction of the stationary gel volume which was available for diffusion of the solute), which was independent of the bed dimension and packing. This value was obtained from the expression $K_{AV} = (V_e-V_0)/(V_t-V_0)$. $V_e$ was the elution volume, which was the volume of eluate up to the protein peak maxima. $V_t$ was the volume of gel (120cm$^2$) and $V_0$ was the void volume, determined for each experiment using blue dextran 2000. Before gel chromatography was performed, soluble rat and rabbit lung preparations were concentrated by centrifugation at 800g for 1-5 min through Amicon Centriflow ultrafiltration cones, which excluded the passage of solutes having molecular weights ($M_r$) > 25,000. Experiments performed with soluble rat lung beta-adrenoceptors indicated that the recovery of $^3$H-DHA binding sites after filtration was >95%.

9:2:2 Affinity chromatography

The (-) alprenolol-sepharose 4B linked affinity chromatography gel used for these experiments was a generous gift of Dr S. Harding. Synthesis of the gel had been performed as described by Caron et al (1979). The alprenolol gel (0.5ml vol) was packed in a Pasteur pipette and washed overnight with 100mM NaCl 10mM Tris HCl pH 7.8 at 22°, using a flow rate of 16ml/h (400 column vols) followed by 0.2% digitonin/100mM NaCl) 10mM Tris HCl pH 7.8 (25 column vols) at a flow rate of 13ml/h. Concentrated soluble rat lung preparation (2ml, $\approx$ 4pmoles $^3$H-DHA binding sites) was applied to the column at a flow rate of 3ml/h at 22°. Digitonin, 0.2%
100mM NaCl/10mM Tris HCl pH 7.8 was used as the eluting solvent and 0.5mL
elution fractions were collected. Removal of adsorbed receptor was
performed by eluting the column with (+)salbutamol, 2×10^{-4}M, in digitonin-
Tris buffer at 40°C. The salbutamol extracts were combined, concentrated by
filtration through Amicon cones, diluted in 100mM NaCl/10mM Tris HCl pH 7.8
and re-centrifuged. This procedure was repeated 8-10 times in order to
remove the salbutamol, and the final concentrate was assayed for specific

\textsuperscript{3}H-DHA binding.

9:3 Results

9:3:1 Affinity chromatography of soluble rat lung beta-adrenoeceptors

Initial experiments aimed at adsorbing rat lung beta-adrenoeceptors
to alprenolol-sepharose gel were performed using a batch procedure.
Soluble rat lung beta-adrenoeceptors were added to washed gel, and
adsorption of receptors was monitored by assaying aliquots of supernatant.
Results indicated that \textsuperscript{3}H-DHA binding of the supernatant was decreased,
which suggested that adsorption of receptor to the affinity labelled gel
had occurred. However, further experiments soon established that the
decreased \textsuperscript{3}H-DHA binding related to the release of inhibitory substances
from the gel, probably alprenolol derivatives. Thus, after 90 min
exposure to the gel, the supernatant contained sufficient inhibitory
material to decrease \textsuperscript{3}H-DHA binding to a soluble beta-adrenoeceptor
preparation by 70%.

As a result of these findings, further experiments were conducted
using column chromatography conditions, such that extensive washing of the
gel could be performed. After application of soluble rat lung beta-
adrenoceptors to the column, the gel was washed and eluates collected and
assayed for \textsuperscript{3}H-DHA binding and for released alprenolol derivatives. Results
suggested that continuous release of alprenolol derivatives had occurred
but that the levels were less than those obtained previously using the
batch procedure. Following the washing phase, salbutamol was used to
elute potentially adsorbed material. This agent was chosen for its ability to more rapidly dissociate from the released receptor. Other workers have favoured the use of full agonists adrenaline (Wrenn and Haber, 1979); isoprenaline (Caron et al, 1979) or antagonists alprenolol (Vauquelin et al, 1977; Caron et al, 1979).

The elution fractions which contained the $^3$H-DHA binding activities were pooled, washed free of salbutamol and assayed. Results indicated that the soluble beta-adrenoceptor preparation had a slightly greater $^3$H-DHA binding specific activity ($B_{\text{max}} \approx 880 \text{ fmol/mg protein}$) than the original preparation ($B_{\text{max}} = 570 \text{ fmol/mg protein}$), but the degree of purification was negligible compared to values of 100-fold reported by Caron et al (1979). The estimated yield of $^3$H-DHA sites was 15% which was approximately half that obtained by these workers.

9:3:2 Gel filtration of soluble rat and rabbit lung beta-adrenoceptor preparations

Initial experiments on the hydrodynamic properties of soluble beta-adrenoceptors were performed using sephacryl S200 gel chromatography. This gel had the advantage of possessing high porosity and rigidity. It was suitable for globular proteins over the molecular weight range 5000-250,000, which seemed appropriate in view of reports that digitonin-solubilised frog erythrocyte beta-adrenoceptors eluted with an apparent molecular weight of 130-150,000 (Caron and Lefkowitz, 1976). Typical results from a gel filtration experiment performed with soluble rat lung beta-adrenoceptors are shown in Figure 9:1. The receptor protein was eluted in these experiments with NaCl-Tris buffers, and yields of specific $^3$H-DHA binding sites were 22($\pm3$)%, $n=4$. The major peak of specific $^3$H-DHA binding activity eluted after ferritin ($M_r=440,000$) and before human gamma globulin ($M_r=160,000$). In three out of four runs, a minor peak of specific $^3$H-DHA binding activity was observed, which had been retarded.
Figure 9:1

Sephacryl S200 chromatography of soluble rat lung beta-adrenoceptor. 0.35 ml of concentrated soluble rat lung preparation (∆800 fmoles ³H-DHA binding sites) were applied to a pre-equilibrated column (see 9:2), and eluted with 100 mM NaCl, 50 mM Tris-HCl, pH 7.6, at 2°C using a flow rate of 15 ml/h.

One ml fractions were collected and aliquots assayed for total and non-specific receptor binding. The protein elution profile was determined spectrophotometrically. Results show a representative experiment which was performed four times.
by the column and which eluted before ovalbumin ($M_r = 45,000$). This minor peak of activity accounted for $14(\pm 2)\%$ of the total specific binding recovered from the column.

The apparent molecular weights of these detergent extracts were calculated by reference to a calibration curve obtained with standard marker proteins, the elution profiles of which were determined under identical conditions. Figure 9:2 shows the positions of the major and minor peaks of soluble rat lung $^3$H-DHA binding activity, relative to proteins of known molecular weights. The minor and major peaks of $^3$H-DHA binding activity had mean apparent molecular weights of 51,700 ($\pm 8,000$), $n=3$, and 290,000 ($\pm 18,000$), $n=4$ respectively. An accurate assessment of the molecular weight of the major peak was hampered by the poor resolving power of the chromatography gel for $M_r > 250,000$. Therefore, experiments were repeated using sephacryl S300 superfine, which was reported to allow resolution of proteins over the molecular weight range $10^4$- $1.5 \times 10^6$. The results shown in Figure 9:2 confirmed the greater resolution of sephacryl S300 at high molecular weights, and all subsequent work was performed with this gel.

In the following series of experiments soluble beta-adrenoceptor preparations were eluted from sephacryl S300 columns using 100mM NaCl/50mM Tris HCl pH 7.6/1% digitonin. The addition of digitonin significantly increased the yield of soluble specific $^3$H-DHA binding sites from the columns (mean yields from five experiments were 69(\pm 7)\%). Its presence did not alter the elution profile of soluble beta-adrenoceptors, nor affect the calibration curve of standard proteins (data not shown). The major disadvantage in the use of digitonin-containing buffers was the precipitation of digitonin on prolonged standing in the cold, which necessitated the use of freshly prepared buffers. Other workers have overcome this problem by using digitonin which was obtained from freeze dried solutions (Caron, personal communication).
Figure 9:2
Calibration curves for sephacryl S200 and S300 gel filtration of standard proteins. The curves were constructed by plotting the known molecular weights of the protein markers against their relative elution volume (KAV), which was calculated as described in 9:2:1. Proteins were eluted at 15 ml/h with 100 mM NaCl/50 mM Tris-HCl, pH 7.6, at 2°C. R major and R minor were the peaks of soluble rat lung receptor observed in the experiment described in Fig. 9:1. The above data were the mean of 2-6 determinations for each protein.
Figure 9:3 shows the results of sephacryl S300 chromatography of a soluble rat lung beta-adrenoceptor preparation. Protein was eluted from the column in a broad band after the blue dextran peak. Soluble specific $^3$H-DHA binding sites also eluted as a rather broad band and with a peak activity which was located between ferritin and human gamma globulin. No discrete peaks of minor binding activity were apparent in the two experiments performed with the soluble rat lung preparation, which suggested that the previous observations may have related to the absence of digitonin in the eluting buffers. The apparent molecular weight of the soluble rat lung beta-adrenoceptor was 320,000 (n=2), which was similar to the previous value obtained using sephacryl S200 gel.

The hydrodynamic properties of soluble preparations of rabbit lung were investigated (Figure 9:4). The preparations used for these experiments contained a mixture of beta$_1$ and beta$_2$-adrenoceptors in the proportions of approximately 40% beta$_1$: 60% beta$_2$. The soluble preparation was retarded by the sephacryl S300 column, and it eluted as a rather broad band. The peak of specific $^3$H-DHA binding activity occurred, like the rat lung beta-adrenoceptor, between ferritin and human gamma globulin. The mean apparent molecular weight of the preparation, which was determined from three column runs, was 370,000 ($\pm$19,000).

Column eluates from the preceding experiments were concentrated, and their pharmacological properties were analysed, in a single experiment, by measuring atenolol displacement of $^3$H-DHA binding. The data obtained for soluble rat lung preparation suggested that the binding characteristics before and after gel filtration were similar, and indicative of a homogeneous beta$_2$-adrenoceptor population. After gel filtration, the soluble rabbit lung preparations had diminished proportions of beta$_1$-adrenoceptors, which probably reflected the lability of these receptors (See Chapter 8). In most cases, however, heterogeneous binding sites for atenolol were still apparent, which suggested that both beta$_1$ and beta$_2$-adrenoceptors were present.
Figure 9:3
Sephacryl S300 chromatography of soluble rat lung beta-adrenoceptor.
Concentrated soluble rat lung preparation (0.5 ml, ~ 4.5 mg protein) was applied to the column and eluted with 100 mM NaCl/50 mM Tris-HCl, pH 7.6/1% digitonin at 2\(^{\circ}\)C, using a flow rate of 15 ml/h. 0.75 ml fractions were collected and aliquots assayed for specific \(^{3}H\)-DHA binding. The optical density of the eluting fluid was monitored continuously (unbroken line) at 280 nm. Results shown were from a representative experiment performed twice. Inset: The molecular weight of standard proteins (see Fig. 9:2) have been plotted against their K\(_{AV}\). Soluble rat lung beta-adrenoceptors eluted with an apparent molecular weight of 320,000.
Figure 9:4
Sephacryl S300 chromatography of soluble rabbit lung beta-adrenoceptors.
Concentrated soluble rabbit lung preparation (0.35 ml, ≥ 8 mg protein) was applied to the column and eluted using an identical protocol to that described in Fig. 9:3. The data shown were a representative experiment, which was repeated three times. Inset: Calibration curve of standard proteins shown in Fig. 9:2. In this experiment the soluble rabbit lung beta-adrenoceptor had an apparent molecular weight of 400,000.
9:4 Discussion

The purification of receptors from solubilised membrane preparations required the application of protein separation techniques, of which the most successful and most extensively used was affinity chromatography. Affinity gels usually had a ligand linked via a long spacer arm to a solid agarose or glass matrix, and the most effective affinity adsorbents fulfilled the following criteria:-

(i) the binding of non-receptor proteins to the immobilised ligand and spacer arm was minimal.

(ii) the immobilised ligand retained specificity and high affinity for the receptor.

(iii) the affinity gel was stable.

The design of the spacer arm and its position of attachment to the ligand has been shown to be critical for the production of high affinity immobilised ligands. Thus, for a series of spacer arms linked to DHA, the affinity of the gel for beta-adrenoceptors was diminished 800-fold by decreasing the spacer arm chain length from 13-4 atoms (Pitha et al., 1980). The spacer arms have also been reported to bind solubilised proteins presumably by hydrophobic interactions since the introduction of an aromatic moiety into the spacer arm increased this non-specific binding (Vauquelin et al., 1978). A number of affinity gels have been described which linked beta-adrenoceptor agonists to supports via the ethanolamine side chain (Lefkowitz et al., 1972) or via the aromatic ring (Venter et al., 1972). These affinity gels have been reported to be less satisfactory than alprenolol linked to spacer arms via the allyl side chain (Vauquelin et al., 1978), although substantial purification of canine myocardial beta-adrenoceptors has been claimed using the former affinity gel (Wrenn and Haber, 1979).
Caron et al (1979) have also described an affinity gel which linked alprenolol to agarose via the allyl side chain, and the experiments described in this chapter were performed with this gel. The experience gained in this study suggested that the gel which was used had a number of problems. Clearly, there was significant leaching of ligand and/or spacer arm-ligand from the affinity gel, as shown by the fact that the buffer washings inhibited $^3$H-DHA binding to soluble beta-adrenoceptors. This apparent instability contradicted the claims that the gel had the advantage over cyanogen bromide activated sepharose gels of possessing enhanced stability (Caron et al, 1979). In addition, the former study reported the effective removal of non receptor protein, and an overall purification of 100-200 fold, whereas the present work failed to obtain significant purification of soluble rat lung beta-adrenoceptors. Clearly the synthesis of the affinity gel must be examined in order to determine the causes of the stability problems and the lack of effective receptor purification.

The present study has also examined the hydrodynamic properties of digitonin-solublised preparations of rat and rabbit lung beta-adrenoceptors using gel exclusion chromatography. The apparent molecular weights of these preparations were 370,000 (rabbit lung) and 300,000 (rat lung) which were equivalent to Stokes radii of 5.8nm and 5.5nm respectively. These findings did not indicate marked differences between the hydrodynamic properties of soluble beta$_2$ or beta$_1$-adrenoceptor preparations, which contrasted with the preliminary results of Fraser et al, 1978. These authors reported an apparent molecular weight disparity of soluble canine liver beta$_2$ and heart beta$_1$-adrenoceptors, although surprisingly the pharmacological specificies of membrane bound beta$_1$-adrenoceptors were not apparent. However, these apparent differences in the molecular weights of detergent-associated soluble receptors should be viewed with
caution. These results may not have related to true differences in receptor protein size, but merely reflected different amounts of detergent which were associated with hydrophobic regions of the receptors. Alternatively, there could have been differences in the proportion of receptors which were solubilised in association with other membrane components e.g. Nprotein (Limbird and Lefkowitz, 1978).

The molecular weights observed in this study were comparable to values obtained for digitonin-solubilised beta-adrenoceptor preparations of frog erythrocytes (Limbird and Lefkowitz, 1978) and rat reticulocytes (Limbird et al 1980, b) using similar gel filtration techniques. In general, detergent solubilised preparations of receptors have been shown to elute from gel filtration columns with high apparent molecular weights. Thus, the Stokes radii of detergent-extracts of pituitary TRH receptor (Hinkle and Lewis, 1978); rat brain etorphine receptor (Puget et al, 1980); rat liver alpha-adrenoceptor (Guellaen et al, 1979); platelet serotonin transporter (Talvenheimo and Rudnick 1980); rat brain muscarinic receptor (Haga 1980) and insulin receptor (Kahn, 1976) were reported to be: 4.6, 6.7, 5.7, 7.5, 6.8 and 7.0nm respectively. The presence of considerable amounts of detergent in these preparations undoubtedly accounted for the large apparent molecular sizes. Indeed, it was possible that receptors solubilised in the presence of digitonin bound the digitonin micelle, which has been reported to exhibit a molecular weight of approximately 75,000 (Hubbard R. 1954).

In spite of the problems of detergent interference with these measurements, gel filtration techniques have been successfully used by Limbird and colleagues to provide evidence that agonist occupation of beta-adrenoceptors resulted in a coupling of receptor and N protein (Limbird et al, 1980b). Thus, digitonin-solubilised rat reticulocyte beta-adrenoceptors were estimated to have an apparent \( M_r \approx 250,000 \), whereas agonist induced association of receptor with N protein resulted in a complex which eluted with an apparent \( M_r \approx 380,000 \). Similar observations
have also been made for the platelet alpha_2-adrenoceptor, where agonist occupation resulted in a complex, which after digitonin solubilisation, exhibited a greater sedimentation coefficient upon sucrose density centrifugation (Michel et al., 1981). These observations were interpreted as indicating that agonist occupation of receptors, which were either positively or negatively coupled to adenylate cyclase, resulted in the formation of receptor-Nprotein complexes. The precise stoichiometry of these receptor-Nprotein complexes could not be established however, since the contribution of lipids and detergent to the overall molecular size were not determined.

The molecular weight of Nprotein from turkey erythrocyte membranes has been determined using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and results implicated a subunit of M_r=42,000 (Pfeuffer, 1977) which was catalytically ADP-ribosylated by cholera toxin (Cassel and Pfeuffer, 1978). More recently this Nprotein has been reported to be composed of two subunits of M_r=45,000 and 35,000 (Sternweis et al., 1981), whereas rabbit liver membranes contained Nprotein complexes which contained 3 subunits of M_r=52,000, 45,000, and 35,000 (Northup et al., 1980). Hydrodynamic studies of detergent solubilised Nprotein have suggested that it bound less detergent than receptor protein (Howlett and Gilman, 1980) which has led to the suggestion that it contained a relatively small hydrophobic surface area. This finding implied that the Nprotein may not penetrate deeply into the membrane (Limbird, 1981).

Haga et al. (1977) have estimated the molecular weight of the beta-adrenoceptors from S49 lymphoma cells using gel exclusion chromatography and sucrose density centrifugation in H_2O and D_2O. The latter protocol enabled the contribution of detergent to the overall molecular weight to be determined, and a value for the M_r of the protein portion of the detergent-protein complex was 75,000. This molecular weight was similar to the values obtained for liver and kidney angiotensin
receptors, $M_r = 80,000$ (Guillon et al., 1980); hepatic alpha-adrenoceptors, $M_r = 96,000$ (Guellaen et al., 1979) and rat brain muscarinic receptors, $M_r = 86,000$ (Haga 1980). The $M_r$ value obtained for the rat brain muscarinic receptor using hydrodynamic techniques correlated well with its reported value of 83,000 using the denaturing conditions of SDS-PAGE (Birdsall et al., 1979a) which suggested that this receptor existed as a monomer in membranes (Haga 1980). In contrast, the Torpedo californica nicotinic acetylcholine receptor has been shown to be composed of several subunits, which may be organised as dimers (Raftery et al., 1976). Recent studies have provided evidence that beta-adrenoceptors of avian erythrocytes may also be composed of more than one subunit. Thus, two subunits have been reported for turkey (Atlas and Levitzki, 1978a; Charlton et al., 1980) and duck (Rashidbaigi and Ruoho, 1981) erythrocyte beta-adrenoceptor using SDS-PAGE of affinity labelled receptors. In contrast, frog erythrocyte (Shorr et al., 1981) and mammalian lung (Soiefer and Venter, 1980) beta-adrenoceptors were shown to be composed of single beta-adrenoceptor binding subunits of $M_r = 58,000$ and 38,000 respectively.

The results of the present study clearly represented the initial steps towards identifying the subunits of mammalian beta₁ and beta₂-adrenoceptors. In future, sucrose density centrifugation techniques will need to be applied to correct the $M_r$ values for the contribution of receptor-bound digitonin. In addition, purified, affinity labelled, or radioiodinated receptors will need to be subjected to SDS-PAGE. Data from these techniques should provide information on the molecular organisation of the receptors in the membrane. The exclusive use of the latter technique should be viewed with caution, since SDS-treatment has been known to cause dissociation of oligomeric proteins into subunits (See Helenius and Simons, 1975).

In recent years, the purification and characterisation of beta-
adrenoceptor subunits has progressed with the development of affinity labels. Bromoacetylated derivatives of propranolol (Atlas and Levitzki, 1978b) and alprenolol (Caron et al. 1980) have been described and used to label avian and amphibian erythrocyte beta-adrenoceptors. Recently, photoaffinity labels for the beta-adrenoceptors have been introduced (Dorfler and Marinetti, 1977; Wrenn and Homcy, 1980; Rashidbaigi and Ruoho, 1981). These agents had the advantage that they did not require specific orientation of reactive groups at the binding site, since U.V. activation allowed chemical insertion of ligand into C-H bonds of the receptor protein. Their main disadvantage was the low yield of covalent photoincorporation, although the use of a high affinity, high specific activity, radioiodinated, ligand has circumvented this problem (Rashidbaigi and Ruoho, 1981). A radioiodinated photoaffinity label has recently been described based on hydroxybenzylpindolol which will be very useful as a probe for beta-adrenoceptors. In future, however, photoaffinity labels based on iodocyanopindolol may prove more advantageous in view of the ability of \( ^{125}\text{I}-\text{HYP} \) to bind to indole recognition sites (Engel et al., 1981).
CHAPTER 10

Concluding Discussion
The present study has examined the pharmacological characteristics of in vitro tissue preparations of beta-adrenoceptors using the radioligand $^3$H-DHA. Results of these studies demonstrated that membranes of rat myocardium, lung, cerebral cortex and rabbit lung possessed specific $^3$H-DHA binding sites which had the characteristics of beta$_1$ and beta$_2$-adrenoceptors. These findings supported the suggestion of Carlsson et al. (1972) that beta-adrenoceptor subtypes may coexist in a tissue. The pharmacological characteristics of these sites were conserved between tissue and species, and they were independent of the relative proportion of beta-adrenoceptor subtype in the preparation. Moreover, the properties of these in vitro preparations of beta-adrenoceptor binding sites were similar to the pharmacological characteristics of intact tissue responses. These data supported the contention that there were only two subtypes of beta-adrenoceptor in mammalian tissues which were equivalent to those proposed by Lands et al. (1967a).

These radioligand binding studies did not, however, provide information on the functional activity of these specific binding sites, nor their cellular location. Thus, the coexistence of both subtypes in a tissue homogenate could have reflected the cellular heterogeneity of the tissue. Indeed, the studies conducted with rat erythrocytes and reticulocytes, which consisted of homogeneous cell types, indicated the presence of a single population of beta$_2$-adrenoceptors. Recently, however, the presence of both beta$_1$ and beta$_2$-adrenoceptors on C6 glioma cells has been reported (Homburger et al., 1981). Moreover, studies of the ontogeny of beta-adrenoceptor subtypes in rat lung suggested that both beta$_1$ and beta$_2$-adrenoceptors were on a single cell type (Ashton et al., 1981; Whitsett et al., 1981). The nature of this cell type is presently unknown although bronchiolar smooth muscle was a prime candidate in view of reports that beta$_1$ and beta$_2$-adrenoceptors could mediate relaxant responses in guinea pig lung (Furchgott...
and Wakade, 1975). Alternatively, Type II pneumocytes might contain the beta-adrenoceptors since the proportion of this cell type in each alveoli did not alter markedly with age (Balis and Conen, 1964), which correlated with the unaltered proportion of beta-adrenoceptor subtypes during development (Ashton et al, 1981).

The important question of the location of beta-adrenoceptor subtypes in tissues has received relatively little attention in recent years. A number of workers have suggested a neuronal location for striatal and cortical beta\textsubscript{1}-adrenoceptors following radioligand binding studies of brain preparations from chemically, or surgically lesioned animals (Nahorski et al, 1979b; Minneman et al, 1979d; U'Pritchard et al, 1980). Cortical beta\textsubscript{2}-adrenoceptors have been suggested to be located on glial cells or cerebral vasculature (Minneman et al, 1979d). An alternative approach has been to localise beta-adrenoceptors in tissues by fluorescent probes (Atlas and Levitzki, 1978b) drug-avidin-ferritin conjugates (Atlas et al, 1978) or autoradiographic techniques coupled with radiolabelled ligand binding (Palacios and Kuhar, 1980). In future, the availability of high affinity, radiiodinated, photoaffinity ligands coupled with the use of selective beta-adrenoceptor agents should allow the location of beta-adrenoceptor subtypes in tissues.

An attractive theory for the physiological significance of beta\textsubscript{1} and beta\textsubscript{2}-adrenoceptors was provided by Ariens and Simonis (1976), who suggested that beta\textsubscript{1}-adrenoceptors were primarily responsive to neuronally released noradrenaline, whereas beta\textsubscript{2}-adrenoceptors were thought of as responsive to circulating adrenaline. Thus, the relative proportions of beta\textsubscript{1}/beta\textsubscript{2}-adrenoceptors have been proposed to be dependent upon the density of the adrenergic innervation (Carlsson and Hedberg, 1976). This concept has been extended by Bryan et al (1981) to suggest that extraneuronal uptake might be associated with beta\textsubscript{2}-adrenoceptors. In view of reports that the chronotropic responses in rat atria were mediated exclusively through beta\textsubscript{1}-
adrenoceptors (Bryan et al, 1981) it could be speculated that the high proportion of beta₂-adrenoceptors found in rat heart may have related to sites which were not coupled to physiological responses but to extra-neuronal uptake. Indeed, Wrenn et al (1979) have shown that the beta-adrenoceptor agent soterenol stimulated membrane-bound COMT activity in canine cardiac microsomes by a beta-adrenoceptor mediated mechanism. Moreover, Baker et al (1980) have reported that the distribution of beta-adrenoceptor binding sites in canine heart was related to that of blood flow and the retention of circulating catecholamines. The relationship of beta₂-adrenoceptors to extraneuronal uptake systems in non-cardiac tissue is presently uncertain, although it was of interest that rat reticulocytes contained approximately four times the number of beta₂-adrenoceptors as rat erythrocytes and five times greater membrane-bound COMT activity (Quiring et al, 1978).

An alternative source of the beta₂-adrenoceptors in the above tissue preparation may have been from pre-synaptic locations. Pre-synaptic beta₂-adrenoceptors have been shown to augment the release of noradrenaline in rat portal vein (Dahlof et al, 1979) and guinea pig vas deferens (Stjarne et al, 1979). The quantities of such receptors are likely to be very small, however, since chemical sympathectomy of rat spleen failed to reveal any significant proportion of pre-synaptic beta-adrenoceptors (Nahorski et al, 1979a).

The concept of beta₁-adrenoceptors as the 'innervated' and beta₂-adrenoceptors as the 'hormonal' receptor cannot be applied to certain non-mammalian species. Thus, the frog sympathetic neurotransmitter has been shown to be adrenaline, and the cardiac beta-adrenoceptors controlling inotropic and chronotropic responses had the characteristics of beta₂-adrenoceptors as classified by Lands et al (1969). Moreover, the neuronal uptake system was selective for the natural neurotransmitter adrenaline, whereas extra-
neuronal uptake was selective for noradrenaline (Stene-Larsen, 1981). These findings have led Stene-Larsen to propose a new terminology for beta-adrenoceptors as adrenaline and noradrenaline receptors.

The beta-adrenoceptor binding sites on erythrocytes of the non-mammalian species aves and amphibia have been examined in these studies. The results indicated that certain beta-adrenoceptor agents had affinities for frog and chick erythrocyte beta-adrenoceptors which differed significantly from those for mammalian beta-adrenoceptors. These differences were particularly evident for selective beta-adrenoceptor antagonists but the rank order of potencies of catecholamines was indicative of beta$_1$-classifications for chick erythrocytes and beta$_2$-classification for frog erythrocytes. In view of this result it is debatable whether these non-mammalian beta-adrenoceptors represented further subclasses of beta-adrenoceptors, although these receptors clearly possessed accessory binding sites which differed from those of mammalian beta-adrenoceptors.

These results raised the question of the relative merits of using agonist potencies to characterise receptors. Indeed, Arnold has suggested that adrenoceptor classification should be undertaken primarily with catecholamines and that non-catecholamines or antagonists should be used only in a corroborative role (Arnold, 1972). However, the use of agonist potency ratios to classify receptors in intact tissue preparations was hazardous in view of the potential differences in tissue stimulus-response relationships (see Jenkinson, 1973). In vitro binding studies also had the complication that agonist displacement curves obtained in the absence of GTP consisted of agonist-induced high and low affinity states of the receptor (Kent et al, 1980; DeLean et al, 1980). In view of these problems it was surprising that the relative potency of catecholamine agonists for beta-adrenoceptors on intact tissue and membrane (and soluble) preparations correlated with those values obtained originally by Lands et al (1967a), (see Daly and Levy, 1979). It was perhaps doubly surprising when one reflects that Lands and
co-workers failed to take into consideration differences in neuronal uptake of the catecholamines or the contribution of reflex mechanisms in their in vivo experiments.

Methods for the solubilisation and assay of mammalian lung beta-adrenoceptors have been developed in order to determine whether the pharmacological properties of the receptor subtypes were conserved upon solubilisation. The results indicated that the major characteristics of beta₁ and beta₂ adrenoceptors were similar in solution and in membranes. These findings argued against the concept that the properties of the beta-adrenoceptor subtypes were provided by the lipid environment (Ahlquist, 1977). The differential stabilities of soluble beta₁ and beta₂-adrenoceptors provided support for the concept that they were separate molecular entities. Direct proof of this suggestion will undoubtedly be provided in the near future by the application of purification techniques. The recent development of photoaffinity labels should provide the tools with which to define the binding site subunits of beta₁ and beta₂-adrenoceptors.

The methods which were developed for the determination of radiolabelled ligand binding to soluble beta-adrenoceptor preparations have found application for the measurement of plasma levels of beta-adrenoceptor antagonists. Previous radioreceptor assays for beta-adrenoceptor agents have used membrane preparations of brain, lung and erythrocytes and problems of plasma interference have been noted (Innis et al, 1978; Bilezikian et al, 1979). The replacement of membrane preparations by soluble beta-adrenoceptor protein resulted in considerably less plasma interference in the subsequent assay (Barnett et al, 1981). The use of solubilised receptor protein coupled with charcoal separation technique has improved the radioreceptor assay, which now provides sensitivity without prior extraction of plasma samples and does not require specialised vacuum filtration apparatus.
These studies have examined the interactions of agonists with beta-adrenoceptors and the influence of guanine nucleotides. Most of the results could be fitted by the receptor-effector coupling model of Lefkowitz and co-workers (Kent et al., 1980). Thus, agonists, but not antagonists, were thought to induce (or stabilise) the formation of a high affinity GTP-sensitive, state of the receptor, which might be a ternary complex of agonist-receptor-$N_{\text{protein}}$ (DeLean et al., 1980), and which is probably an essential intermediate in the activation of adenylate cyclase (Stadel et al., 1980). Guanine nucleotides have been suggested to destabilise this complex by a dissociation reaction which results in the release of agonist-bound receptor. The major finding of the present study was that the dissociation reaction, which was studied in a number of beta$_1$ and beta$_2$-adrenoceptor systems, had differential ion and temperature requirements. Thus, agonist binding to the beta$_2$-adrenoceptor preparations of frog erythrocyte and rat lung induced a high affinity state of the receptor which was interconverted to a uniform low affinity state by GTP under standard assay conditions. In contrast, guanine nucleotide modulation of agonist binding to rabbit lung, chick erythrocyte and rat reticulocyte required elevated temperatures and/or the presence of Mg$^{2+}$/EDTA.

The reasons for these differences are presently speculative, but they may involve differences in the affinities of Mg$^{2+}$ and/or GDP for the $N_{\text{protein}}$. Recently, the rates of agonist-induced release of $^3$H-GDP from frog and turkey erythrocytes was shown to differ substantially (Pike and Lefkowitz, 1981). Moreover, this release reaction was temperature dependent which suggested that tightly bound GDP may have contributed to the insensitivity of certain systems to guanine nucleotides. An attractive, alternative, hypothesis is that these differences related to different subunit compositions of the $N_{\text{protein}}$. The 52,000 Mr subunit has recently been postulated as responsible for binding GTP and facilitating GTP/GDP exchange (Limbird, 1981). Therefore, differences in the stoichiometry of the $N_{\text{protein}}$ subunits may
have contributed to the differential regulatory properties of certain preparations.

Clearly further work will be required to resolve which of these alternatives is correct. Initial experiments should include determination of the relative contributions of Mg\textsuperscript{2+} and EDTA to the above results. In order to study the effects of differentially liganded $N_{\text{protein}}$ upon adenylate cyclase activation and agonist binding characteristics, cation-free and GDP-free membranes will need to be prepared. Work here would be aimed at investigating the postulate of Maguire and co-workers that agonist-receptor-$N_{\text{protein}}$ complex, which has $N_{\text{protein}}$ containing Mg\textsuperscript{2+} but not guanine nucleotides, is the required intermediate for adenylate cyclase activation (Cech et al., 1980). The tightness of GDP binding to the receptor preparations also needs to be elucidated, by measuring the ability of Gpp(NH)p to activate adenylate cyclase in the absence and presence of agonists (Lad et al., 1980a) or by measuring the agonist induced release of GDP directly (Cassel and Selinger, 1977). Studies aimed at investigating the involvement of the $N_{\text{protein}}$ structure could include complementation experiments (Lad et al., 1980b) or direct analysis of the subunit structures (Northup et al., 1980).

The results of this study suggested that agonist binding to beta\textsubscript{1}-adrenoceptors was more sensitive to alterations in temperature and certain cations. In support of this conclusion, the affinities of agonists for atrial beta\textsubscript{1}-adrenoceptors, but not tracheal beta\textsubscript{2}-adrenoceptors, were decreased with increasing temperature (Reinhardt et al., 1972, Wagner et al., 1972). These studies also suggested that, under certain rather non-physiological conditions, beta\textsubscript{1} and beta\textsubscript{2}-adrenoceptors could be differentially coupled to the effector system. These findings raised the possibility that appropriate interventions could change the relative contribution of the beta-adrenoceptor subtypes which mediated certain physiological
responses. Studies by Zaagsma et al (1979) have not supported this postulate, however, since the ratios of beta₁/beta₂-adrenoceptors coupled to relaxant responses in guinea pig trachea were not significantly altered by lowering the temperature from 37°C to 25°C.

In future, it will be important to establish, in tissues where both subtypes coexist, whether perturbations in the coupling of one beta-adrenoceptor subtype affects the functioning of the other. Apparent desensitisation of beta₂-adrenoceptors of guinea pig trachea by salbutamol has been suggested to result in hypersensitisation of the beta₁-adrenoceptors (Omini et al, 1979). Further work will be needed to resolve the questions of whether beta₁ and beta₂-adrenoceptors share common pools of N_protein; whether they are similarly affected by alpha₂-adrenoceptor occupation (Woodcock and Johnston, 1980); and whether they desensitise differentially.

In general, the studies with partial agonists confirmed the reports that intrinsic activity was related to the degree of GTP-sensitive binding (Lefkowitz et al, 1976) and to the proportion of agonist-induced high affinity state of the receptor (Kent et al, 1980). Salbutamol, which exhibited beta₂-adrenoceptor selectivity in vivo was shown not to have selective affinity for beta₂-adrenoceptors but to possess selective efficacy. This drug was capable of coupling receptor occupation to adenylate cyclase activation in beta₂ but not beta₁-adrenoceptor systems. This result could have accounted for the in vivo selectivity of the drug, although another important consideration was the stimulus-response relationships of the tissues involved. Recently prenalterol, a partial agonist which expressed beta₁-adrenoceptor selectivity in vivo (Carlsson et al, 1977) was shown to possess neither selective affinity nor efficacy for beta₁-adrenoceptors. Its functional organ selectivity was related to the differential stimulus-response relationships of the tissues studied (Kenakin and Beek, 1980).
Hybridisation experiments have indicated that the intrinsic activities of beta-adrenoceptor agents were expressed through membrane components distal to the receptor (Pike et al., 1979). Moreover, the intrinsic activities correlated with the drugs' ability to stimulate a GTPase (Pike and Lefkowitz, 1980), which related to the rate at which GDP was released from the N<sub>protein</sub> (Pike and Lefkowitz, 1981). These results implied that the N<sub>protein</sub> defined the magnitude of the response to agonist occupation. In the light of these findings and recent work by Larner and Ross (1981) it can be postulated that the inability of salbutamol occupation of beta<sub>1</sub>-adrenoceptors to result in ternary complex formation and activation of adenylate cyclase was due to differences in the N<sub>protein</sub> associated with beta<sub>1</sub> and beta<sub>2</sub>-adrenoceptors.

The recent advances in the methodological approaches to the study of beta-adrenoceptors has enabled these systems to be examined under different hormonal influences. The number of beta-adrenoceptors in lung tissue has been shown to be regulated by glucocorticoids (Mano et al., 1979). Moreover, the proportion of beta<sub>1</sub> and beta<sub>2</sub>-adrenoceptors in uterus appeared to be controlled by the steroid balance of the tissue. Thus, the progesterone primed uterus had beta<sub>2</sub>-adrenoceptors which dominated, whereas, the oestrogen primed uterus had both beta<sub>1</sub> and beta<sub>2</sub>-adrenoceptors (Richardson, 1979; Johansson et al., 1980). The coupling efficiency of the beta-adrenoceptor effector system also appeared to be under hormonal control. Thus, the ability of cardiac beta-adrenoceptors to form the high affinity coupled state has been shown to be influenced by adrenal steroid hormones (Davies et al., 1981). Similarly, the ability of guanine nucleotides to modulate agonist affinities for fat cell beta-adrenoceptors was regulated by the thyroid status of the animal (Malbon, 1980). These results suggested that the beta-adrenoceptor-effector system in tissues is regulated by a number of hormones, and the characterisation of this system in disease states will be a fruitful area of future research.
Appendix 1: Chemicals, radiochemicals, enzymes and drugs

Chemicals

All chemicals were of analytical reagent grade and were purchased from the indicated companies: Cyclic AMP, Gpp(NH)p.Li, 5'GTP, Na3, ATP Na2 (Boehringer Mannheim); 5'GMP Na, phenylhydrazine HCl, cis vaccenic acid, NEM, polyethylene glycol 6000, digitonin, n-octylglucoside, Triton-X-100, Deoxycholate (Sigma); Blue dextran 2000 (Pharmacia Limited); digitonin (Fischer Limited); Norit GSX charcoal (Hopkins & Williams); dithiothreitol (BDH Biochemicals).

Radiochemicals

(-) propyl-2-3-3H -dihydroalprenolol, 50-102 Ci/m mole (New England Nuclear); 5'-8-3H -cyclic AMP, 40-60 Ci/m mole (Radiochemical Centre, Amersham).

Drugs

(-)-Isoprenaline bitartrate, (-)-adrenaline bitartrate, (-)-noradrenaline bitartrate, 3-isobutyl-1-methyl xanthine, phentolamine (Sigma); (+)-isoetharine (Riker Laboratories); (+)-salbutamol (Glaxo-Allenburys); (-)-timolol (Leo Laboratories); (-)-alprenolol, H35/25 (Haessle); (+)-procaterol (Otsuka); (+)-metoprolol (Astra); (+)-atenolol, (+)-practolol, (+)-propranolol, (-)-propranolol and ICI 118.551 erythro-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol (ICI Pharmaceuticals); betaxolol (Synthelabo).

Proteins/Enzymes

Bovine serum albumin, ferritin, human-γ-globulin, chymotrypsinogen A (bovine pancreas), ribonuclease A (bovine pancreas), ovalbumin (Sigma).
APPENDIX 2  Scintillation fluid

(i) 4g 2,5-diphenyloxazole
0.1g 1,4- bis \([2(4\text{-methyl-5-phenyl-oxazoyl})]\) benzene
500ml Triton X100 or Fisons Emulsifier mix
1 l. Toluene

(ii) Fisofluor scintillation cocktail (Fisons Ltd.)

APPENDIX 3  Reagents for protein assay

A 2% Na$_2$CO$_3$ in 0.1N NaOH
B 1% CuSO$_4$ 5H$_2$O
C 2% Potassium sodium tartrate
D Freshly prepared 100ml solution A
  1ml solution B
  1ml solution C
E Folin-Ciocalteau reagent diluted 1:3
APPENDIX 4 (i) The relationship of the inhibition constant (Ki) and the concentration of displacing drug causing 50% displacement in a competition binding assay
From Cheng and Prusoff (1973)

The binding of radioligand can be described by:

\[ B_0 = \frac{B_{\text{max}} \cdot S}{K_D + S} \]

where \( B \) = bound radioligand in the absence of competing drug;
\( B_{\text{max}} \) = maximum binding capacity; \( K_D \) = equilibrium dissociation constant; \( S \) = radioligand concentration.

When a competitive inhibitor is present:

\[ B_i = \frac{B_{\text{max}} \cdot S}{K_D \left(1 + \frac{I}{K_i}\right) + S} \]

When \( I = IC_{50} \), \( B_0 = 2B_i \) and:

\[ \frac{B_{\text{max}} \cdot S}{K_D + S} = \frac{2 \cdot B_{\text{max}} \cdot S}{K_D \left(1 + \frac{IC_{50}}{K_i}\right) + S} \]

By rearrangement \( Ki = \frac{IC_{50}}{\frac{T + S}{K_D}} \)

This equation is valid if the binding of labelled ligand follows the principles of mass action behaviour and if the inhibitor binds competitively at the same binding site. It can be applied only when bound ligand is a small % of the total ligand and when \( K_D > \) the [Receptor site]. The applicability of
this correction was verified for (-) alprenolol displacement of 
$^3$H-DHA binding to rat erythrocyte membranes over the range 0.8-6nM (data not shown).

APPENDIX 4 (ii) Association rate constant ($k_+\)\)

For the reaction: \[ R + L \xrightarrow{k_+} RL \xrightarrow{k_-} R + L \]

The association rate equation can be described by:
\[ \frac{d[R]}{dt} = k_+ [L] [R] - k_- [RL] \]  

If the experiment is performed such that at equilibrium <10% of radioligand is bound, $[L]$ remains constant and the reaction can be regarded as pseudo first order.

\[ k'_1 = k_+ [L] \]

Substituting 2 into 1.
\[ \frac{d[R]}{dt} = k'_1 [R] - k_- [RL] \]  

At time $t$.
\[ [R] = [R]_0 - [RL]_t \]

At equilibrium.
\[ \frac{d[R]}{dt} = 0 \]

Substituting 4 into 3.
\[ k'_1 ([R]_0 - [RL]_{eq}) = k_- [RL]_{eq} \]

\[ k_- = \frac{k'_1 [R]_0}{[RL]_{eq}} - k'_1 \]
Substituting 4 and 6 into 3 after rearrangement and integration between \(t_1\) and \(t_2\):

\[
\ln \frac{[RL]_{eq} - [RL]_{t_1}}{[RL]_{eq} - [RL]_{t_2}} = \frac{k'_1 [R]_0 (t_2 - t_1)}{[RL]_{eq}}
\]

When \(t_1 = 0\):

\[
\ln \frac{[RL]_{eq}}{[RL]_{eq} - [RL]_t} = \frac{k'_1 [R]_0}{[RL]_{eq}} t_2
\]

Substituting 2 and 6 into 8:

\[
\ln \frac{[RL]_{eq}}{[RL]_{eq} - [RL]_t} = \left(k_{-1} + k_{+1} [L]\right) t
\]

The slope of the plot of \(\ln \frac{[RL]_{eq} - [RL]_t}{[RL]_{eq}}\) vs \(t\) = \(K_{OBS}\) (the observed pseudo first order rate constant), and the second order rate constant \(k_{+1} = \frac{K_{OBS} - k_{-1}}{[L]}\)

This equation is valid when <10% of the initial free ligand is bound at equilibrium

**APPENDIX 4 (iii) Dissociation rate constant \(k_{-1}\)**

For the reaction: \(RL \xrightleftharpoons[k_{-1}]{k_{+1}} R + L\), elimination of the reverse reaction by adding an excess of unlabelled inhibitor, allows the dissociation reaction to be described by the first order equation:

\[
\frac{d [RL]}{dt} = -k_{-1} [RL]
\]

After integration and rearrangement:

\[
\ln \left(\frac{[RL]}{[RL]_0}\right) = -k_{-1} t
\]
For experiments conducted after the attainment of equilibrium, the negative slope of the plot of $\ln\left(\frac{B_t}{B_{eq}}\right)$ vs $t = \text{dissociation rate constant } (k_\text{d}).$

**APPENDIX 4 (iv) Multiple site analysis (From Richardson, 1979)**

Inhibition curves of drug displacement of $^3$H-DHA binding were fitted using an iterative curve fitting computer program (NAG library, E04FBF, NAG Ltd., Oxford 1977), arriving at the best fit parameters by minimisation of the sum of squares.

The mathematical model of binding for a two site, non-interacting, system was:

$$B = \frac{B_{max_1}}{[I] + IC_{50_1}} + \frac{B_{max_2}}{[I] + IC_{50_2}}$$

where $B = \%$ of binding displaced, $[I] = \text{concentration of inhibitor, } IC_{50_1}, IC_{50_2} = \text{concentration of ligand causing 50\% inhibition of binding to each site, } B_{max_1}, B_{max_2} = \text{proportion of each site contributing to the total. 'Goodness of fit' was estimated as described by Richardson (1979). This procedure involved the use of the error sum of squares which was defined by:}$

$$\text{Error s.o.s.} = \frac{\text{Residual s.o.s.}}{(M - N)}$$

where $M = \text{number of observations, and } N = \text{number of parameters,}$ and the stability of the best fit data over increased values of accuracy (the limits to which the sum of squares is required).
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