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BIOCHEMICAL STUDIES ON MUSCARINIC CHOLINERGIC RECEPTORS

Susan Carson

Lincoln College, Oxford

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A thesis submitted for the degree of Doctor of Philosophy at the University of Oxford.

Michaelmas Term, 1982

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"La méthode expérimentale considérée en elle-même, n'est rien autre chose qu'un "raisonnement" à l'aide duquel nous soumettons méthodiquement nos idées à l'expérience des "faits"....Tout le problème expérimentale se réduit à ceci: "Prévoir et diriger les phénomènes"."

Claude Bernard(1865)

Introduction à l'Etude de La Medicine Expérimentale

"They sought it with thimbles, they sought it with care;
They pursued it with forks and hope;
They threatened its life with a railway-share;
They charmed it with smiles and soap."

Lewis Carroll

"The Hunting of the Snark"

ABSTRACT

BIOCHEMICAL STUDIES ON MUSCARINIC CHOLINERGIC RECEPTORS

S. CARSON, LINCOLN COLLEGE

D. Phil.

Michaelmas Term, 1982

A novel solubilising agent(0.1% sodium cholate-1M NaCl) has been developed which will solubilise 10-30% of muscarinic cholinergic receptors from bovine caudate nucleus. Using the muscarinic antagonist quinuclidinyl benzilate(QNB), a single saturable binding component was found with an equilibrium constant of 200pM, approximately 10-fold higher than the membrane receptor and 4-fold higher than the ratio k_{-1}/k_{1} determined kinetically in the soluble material. This latter difference may indicate that the binding of QNB to the solubilised receptor is not a simple second-order process. Inhibition constants for a variety of muscarinic agonists and antagonists were 10 to 20-fold higher than in the membrane state and non-muscarinic ligands were without effect. The decrease in affinity was shown to be due to the presence of high salt.

Evidence was presented that the apparent increase in Hill coefficient for muscarinic agonist binding to soluble material was not due to a differential solubilisation of muscarinic receptors or to a conformational change of high to low affinity agonist sites during the solubilisation. Instead the Hill coefficient of the soluble material decreased as the percentage of total binding sites solubilised increased. The stability of receptor binding at different temperatures was shown to be dependent on the protein: cholate(w/w) ratio. Results from gel filtration , affinity chromotography and immunization studies are also reported. The results of this thesis are discussed in the light of the possible importance of phospholipids for receptor activity.

ACKNOWLEDGEMENTS

The work described in this thesis was carried out over a period of three years in the University Department of Pharmacology, Oxford under a grant from the Wellcome Trust. I would like to thank Professor W.D. Paton for allowing me to do research in his department and all the members of the department who have helped to make my stay here a pleasant one.

I would especially like to thank my supervisor Dr. A. Claudio Cuello for his patience and understanding and who, although allowing me a total freedom in my research, has influenced me and this thesis greatly.

I would also like to thank Drs. Lorenza Eder-Colli and John Powell for critical comments and encouragement during the course of this work.

To the many people from whom I have borrowed equipment over the past three years my sincere thanks: Derek Hope's Group(in particular Ruth Baker and Sylvia Phipps for their unfailing generosity), David Smith's Group and finally Dr. Eric Sidebottom(Department of Pathology, Oxford) for his generous lending of his tissue culture facilities.

I would particularly like to thank Dr. Edward Gill for his synthesis of the affinity compounds described in

this thesis and much helpful advice and Dr. David Wing for his phospholipid determinations.

Finally, I would like to thank the many people who have been associated with "Cuello's Crowd" and who have made my stay so memorable ,in particular: Adriana Consolazione, Rejean Couture, Erica Jaffe, Sara Patel, John Priestley and Steven Bramwell.

PREFACE

Part of the work presented in Chapter 3 of this thesis has already been published:

Carson, S. (1982) Cholate-salt solubilisation of bovine brain muscarinic receptors.
Biochemical Pharmacology 31, 1806-1809.

TABLE OF CONTENTS

		Page
CHAPTER 1	GENERAL INTRODUCTION	1
CHAPTER 2	MEMBRANE-BOUND MUSCARINIC RECEPTORS	26
CHAPTER 3	SOLUBILISATION OF BOVINE BRAIN MUSCARINIC RECEPTORS	33
CHAPTER 4	CHARACTERISATION OF CHOLATE-SALT SOLUBLE MUSCARINIC RECEPTORS	52
CHAPTER 5	AFFINITY COMPOUNDS	69
CHAPTER 6	MUSCARINIC ANTIGENS	80
CHAPTER 7	GENERAL CONCLUSION	87
CHAPTER 8	REFERENCES	93
APPENDIX I	SYNTHESIS OF AFFINITY COMPOUNDS	

CHAPTER 1

GENERAL INTRODUCTION

Historical Background

The study of the interaction between neurotransmitters and their receptors is important for the understanding of cellular function. In 1905, Langley wrote: "...there is evidence that the majority of substances which are ordinarily supposed to act upon nerve-endings (as nicotine, curari(sic), atropine, pilocarpine, strychnine) act upon the "receptive substances" of the cells...So we may suppose that in all cells two constitutents at least are to be distinguished, a chief substance, which is concerned with the chief function of the cell as contraction and secretion, and receptive substances which are acted upon by chemical bodies and in certain cases by nervous stimuli. The receptive substance affects or is capable of affecting the metabolism of the chief substance." Thus, a receptor can be said to combine the dual function of "recognition" (or binding) of the ligand or neurotransmitter and "interaction" with the membrane and cytoplasm which then leads to the physiological response.

Acetylcholine is a neurotransmitter in the peripheral and central nervous systems which mediates two types of

responses first classified by Dale in 1914: "In the action of choline, and, with varying degrees of intensification, in the action of certain ethers and esters of choline, two distinct types of action can be detected: a "muscarine" action, paralysed by atropine and a "nicotine" action paralysed by excess of nicotine." Muscarinic effects were produced by: "Stimulatory nerves belonging to the cranial and sacral division of the involuntary(autonomic) system...the constrictor effect of the third cranial nerve on the pupil; the inhibition of the heart, constriction of the bronchioloes(sic) and contraction of the muscular walls of the alimentary canal produced by the vagus, the motor action of the pelvic nerve on the large intestine and urinary bladder...all these are reproduced faithfully by muscarine." Evidence for the role of acetylcholine as endogenous mediator for nicotinic and muscarinic effects(in parallel to work on adrenaline) accumulated throughout the twenties and thirties until Dale wrote in 1933 that: "...we seem to need words which will briefly indicate action by two kinds of chemical transmission...I suggest the words "adrenergic" and "cholinergic" respectively, for use in this sense. We can then say that postganglionic parasympathetic fibres are predominantly, and perhaps, entirely, "cholinergic", and that postganglionic sympathetic fibres are predominantly, though not entirely, "adrenergic", while some and probably all, of the preganglionic fibres of the whole autonomic system are "cholinergic"." Thus, by the 1930's several principles of molecular pharmacology existed in embryonic form: 1) the action of a drug on a given receptor required binding to take place; 2) the action of

neurotransmitters could be imitated(agonists) or blocked(antagonists) by certain drugs; 3) the presence of the physiological response indicated the existence of receptors in these tissues; and 4) receptor subtypes(such as nicotinic and muscarinic) were defined by different chemical specificities and different tissue localisation.

Several good review articles on receptor mechanisms and binding in general(Cuatrecasas and Hollenberg, 1976; Colquhoun, 1978; Hollenberg and Cuatrecasas, 1978; Burgen, 1979; Orrego, 1979), acetylcholine receptors(Rang, 1974; Krnjevic, 1975) and muscarinic receptors in particular(Snyder et al., 1975; Birdsall and Hulme, 1976; Massoulie et al., 1977; Heilbronn and Bartfai, 1978; Bolton,1979; Richelson and El-Fakahany, 1981) exist and this introduction will limit itself to a discussion of the literature on muscarinic cholinergic receptors necessary for a proper understanding of the work presented in this thesis.

The Muscarinic Cholinergic System

The acetylcholine receptor recognises the endogenous neurotransmitter acetylcholine and mediates two different biological responses through different macromolecular complexes. Table 1 summarises some of the differences between nicotinic and muscarinic receptors. In general, the nicotinic response such as the contraction of skeletal muscle is fast which reflects well the close association of the agonist-regulated cation channel with the receptor monomer(for reviews see: Changeux, 1975; Eldefrawi and

Table 1

ACETYLCHOLINE RECEPTOR

NICOTINIC	MUSCARINIC
striatal muscle	smooth muscle
electric organ	major AChR in brain
fast response	slow response
less than 1 msec latency	latency 100 msec
duration less than 100 msec	duration greater than 0.5 sec
agonists: nicotine, ACh	muscarine, ACh, oxotremorine
antagonists: curare	atropine, scopolamine, QNB
purification:	purification:
electric organ	thousand fold less in brain (2 nmoles/g protein)
easily solubilised	difficult to solubilise (non-ionic detergents)
affinity compounds (a-Bungarotoxin)	none to date
pathological states: Myasthenia gravis	Huntington's Chorea Parkinsonism (DA-ACh?) Tardive dyskinesia

Eldefrawi, 1977; for reconstitution see: Anholt et al., 1981). The muscarinic response is more complex, slower and longer acting than the nicotinic response. The cholinergic neuron shown in Figure 1(from Heilbronn and Bartfai, 1978) represents cholinergic synapses in the central nervous system(brain) where muscarinic receptors are the major cholinergic receptor type(Krnjevic, 1975). There is some evidence that they exist presynaptically(Giorguieff et al., 1977; de Belleroche and Bradford, 1978; Bowen and Marek, 1982; de Belleroche et al., 1982; Nordstrom et al., 1982) as well as post-synaptically. The bias of this introduction will be towards central post-synaptic receptors. The recognition function of the receptor will be discussed in detail later, therefore we will at present focus on the "interaction" of the receptor with other systems which eventually lead to the physiological response.

It has been shown that acetylcholine increases the cellular concentration of cyclic GMP by activating a guanyl cyclase which in turn induces the cellular response perhaps by phosphorylating key proteins(Greengard, 1976). Muscarinic agonist-induced increases in cGMP have been found in isolated bovine adrenal medullary cells(Yanagihara et al., 1979) and in the mouse neuroblastoma cell line NIE-115(Richelson et al., 1978) while a decrease is found in cAMP levels in the neuroblastoma x glioma clone Ng108-15(Lichtshtein et al., 1979). This occurs at the same time as a slowly reversible decrease in adenylate cyclase activity(Nathanson et al., 1978) while a more stable decrease is seen in the rat parotid gland(Oron et al., 1978) after muscarinic agonist stimulation.

FIGURE 1

Figure 1 CNS Cholinergic Synapse

The components of the CNS cholinergic synapse are: 1)pre-synaptic muscarinic receptor(autoreceptor); 2)post-synaptic muscarinic receptor; 3)Ca^{2+}-channel; 4)synaptic vesicle; 5)high-affinity choline uptake system; 6)choline acetyltransferase; 7)mitochondrion; 8)receptors for other neurotransmitters which influence ACh release; 9)acetylcholinesterase; 10)Ca^{2+}-dependent protein kinase; 11)membrane bound guanylate cyclase; 12)soluble guanylate cyclase; 13)cGMP-dependent protein kinase; 14) endogenous membrane protein which might be involved in regulation of membrane potential(a substrate for the protein kinase); 15)ion channels; 16)protein phosphatase; 17)3',5'-cyclic nucleotide phosphodiesterase. Most of the data on generation of cGMP through muscarinic stimulus refer to a post-synaptic site; however, the existence of pre-synaptic guanylate cyclases is also likely. Phosphatidylinositol turnover has similarly been described mostly as a post-synaptic event. An adenylate cyclase should also be included in the scheme.

From: Heilbronn and Bartfai (1978)

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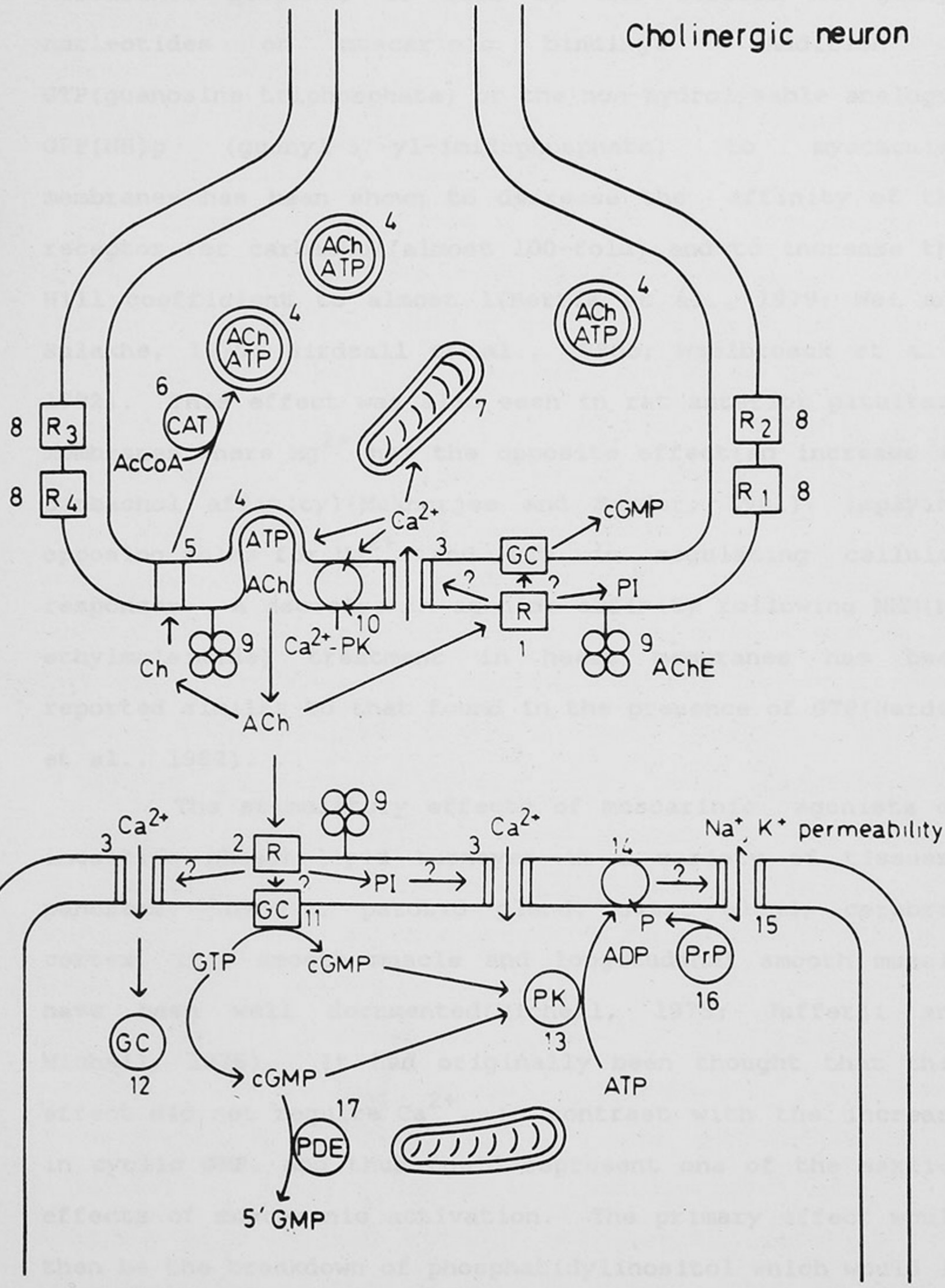


Additional evidence for the role of adenylate cyclase and possible GTP regulatory protein(Rodbell, 1980) in the muscarinic response is seen by the effects of guanyl nucleotides on muscarinic binding. Addition of GTP(guanosine triphosphate) or the non-hydrolysable analogue GPP(NH)p (guanyl-5'-yl-imidophosphate) to myocardial membranes has been shown to decrease the affinity of the receptor for carbachol(almost 100-fold) and to increase the Hill coefficient to almost 1(Berrie et al., 1979; Wei and Sulakhe, 1979; Birdsall et al., 1980b; Waelbroeck et al., 1982). This effect was also seen in rat anterior pituitary membranes where Mg^{2+} had the opposite effect(an increase in carbachol affinity)(Mukherjee and Snyder, 1981), implying opposing roles for Mg^{2+} and GTP in regulating cellular responses. A decrease in agonist affinity following NEM(N-ethylmaleimide) treatment in heart membranes has been reported similar to that found in the presence of GTP(Harden et al., 1982).

The stimulatory effects of muscarinic agonists on inositide phospholipid turnover in a variety of tissues: pancreas, thyroid, parotid gland, sweat gland, cerebral cortex, iris smooth muscle and longitudinal smooth muscle have been well documented(Michell, 1975; Jafferji and Michell, 1976). It had originally been thought that this effect did not require Ca^{2+}, in contrast with the increase in cyclic GMP, and thus could represent one of the earlier effects of muscarinic activation. The primary effect would then be the breakdown of phosphatidylinositol which would in turn increase the Ca^{2+} permeability of the membrane, thereby activating the guanylate cyclase and thus inducing indirectly the physiological response. Evidence for the

contrary, at least in the case of guinea-pig cerebral cortex synaptosomes, has appeared in the recent work of Fisher and Agranoff(1982). They demonstrated the presence of a Ca^{2+}-dependent increase in the level of ACh-stimulated phosphatidylinositol labeling which could be abolished by the addition of calcium-chelating agents EGTA or EDTA. They also showed that the stimulated labeling of phosphatidylinositol (and phosphatidate) appeared to result from an increased availability of diglyceride brought about by the breakdown of polyphosphoinositides rather than of phosphatidylinositol(Fisher and Agranoff, 1981). The addition of the the calcium ionophore A23187 also increased phosphatidylinositol labeling which was atropine insensitive, and the simultaneous addition of A23187 and acetylcholine(ACh) resulted in a synergistic increase in labeling which appeared to suggest a common site of action. Jones and Michell(1975,1978), however, did not find an effect of the calcium ionophore on phosphatidylinositol metabolism in the parotid gland where the changes in phospholipid labeling are not accompanied by calcium mobilisation. This might represent a fundamental difference between the two systems.

Recent evidence has appeared on the importance of calcium in muscarinic receptor regulation. Short-term desensitisation of muscarinic receptors in NIE-115 cells brought about after a 30 minute incubation with muscarinic agonists brings about a rapid and specific loss of cGMP responses with no loss of binding sites. This short-term desensitisation is thought to result from inactivation of calcium channels(El-Fakahany and Richelson, 1980).

Lanthanides(terbium) which are non-competitive blockers with Ca^{2+} for the calcium channel have been shown to both decrease the maximal cGMP response to carbachol stimulation and to decrease (^{3}H)-QNB (quinuclidinyl benzilate) binding in neuroblastoma cells and in rat brain homogenates (El-Fakahany and Richelson, 1981).

Long-term densitisation or "down-regulation" of muscarinic receptors(loss of binding sites) occurs after incubation for more than one hour with agonists and has been documented in neuroblastoma cell lines(Review: Klein, 1980b; Shifrin and Klein, 1980; Siman and Klein, 1981; Higuchi et al., 1982; Taylor et al., 1982) and in vivo(Ben-Barak et al., 1981; McKinney and Coyle, 1982). Burgoyne(1980) has suggested that in rat brain this down-regulation could be mediated by a protein phosphorylating mechanism requiring the presence of cAMP. This receptor loss under phosphorylating conditions could be abolished by addition of calcium chelators and was subsequently shown to depend on the the presence of calmodulin indicating a role for both a cAMP and a calmodulin-dependent protein kinase(Burgoyne, 1981). After exposure of rat cerebellar cultures to carbachol, 3 polypeptides of MW 75,000, 67,000 and 62,000 were phosphorylated which followed a time course of phosphorylation similar to that of carbachol-induced loss of receptor binding(Burgoyne and Pearse, 1982). A tentative suggesetion was made that muscarinic receptor regulation by phosphorylation of the receptor itself occurred since the 75,000MW polypeptide was similar to the molecular weight obtained by irreversibly labeling the muscarinic receptor with the antagonist propylbenzilylcholine mustard(Birdsall et al., 1979a) followed by SDS gel electrophoresis.

In summary, the muscarinic response can be considered to consist of the following sequence of events: 1) acetylcholine binds to the receptor which in turn has an effect via an, as yet unknown, protein resulting in an increase of calcium necessary for the subsequent effects seen on the increase in cGMP levels and on phosphatidylinositol metabolism. Adenylate cyclase and a GTP regulatory protein are also implicated as shown in the studies on heart muscarinic receptor. These effects then lead via a probable phosphorylating mechanism to the physiological effect. 2) The activation of the receptor by agonists leads to the phenomenon of desensitisation. A cAMP- and a calmodulin-dependent protein kinase has been shown to be implicated in the phosphorylation of membrane proteins which follow the same time course as desensitisation of the receptor. It is unclear as to whether the membrane mechanisms responsable for (1) and (2) are the same or represent different regulatory systems. Although the exact order of each of these events has not yet been determined, all three systems: 1) adenylate and guanylate cyclase, 2) phosphatidylinositol metabolism and 3) protein phosphorylation are involved in the muscarinic response. A complete understanding of the system will depend on the purification of the muscarinic receptor which can then be inserted into artificial membrane systems to see which of the systems mentioned and in what sequence are necessary for the muscarinic response. One of the difficulies in the study of the muscarinic receptor has been the difficulty in obtaining the receptor in a stable soluble form. The rest of this chapter will concern itself with

studies on the recognition or binding site of the receptor.

Muscarinic Ligands

The binding properties of muscarinic receptors have been studied by taking advantage of their pharmacological specificity. The use of radiolabeled antagonists and agonists have allowed the recognition function to be dissociated from the physiological response of the receptor. This has been of much use when following the receptor through purification steps; however, the caveat is necessary that all studies are therefore performed on the "binding site" of the receptor which may not be synonymous with the whole receptor protein.

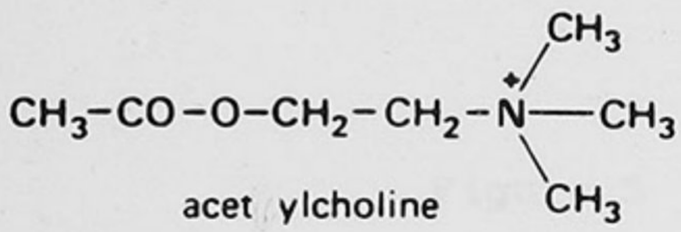
To establish that binding of a radioactive drug to whole tissue or to subcellular fractions of it represents specific binding to a specific receptor, rather than non-specific binding, requires a careful quantitative study of the binding properties of the preparation and a comparison with the pharmacological properties of the whole tissue. In general, three basic criteria must be fulfilled for receptors in general and for muscarinic receptors in particular(Birdsall and Hulme, 1976): 1) Saturability: the component of binding which is being studied should saturate with increasing concentrations of the labeled drug. By definition, non-specific binding increases linearly with increasing concentrations of the ligand. 2) Specificity: muscarinic drugs should inhibit specific binding to the receptors at pharmacologically active concentrations while non-muscarinic and non-cholinergic drugs should have no

effect on specific binding. 3) Localisation: specific binding should be localised to tissues and specific regions of tissues known from pharmacological studies to contain the receptor.

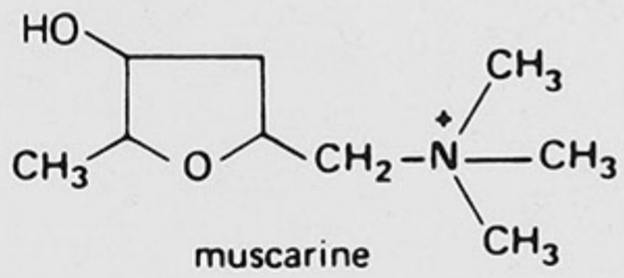
Some examples of muscarinic agonists and antagonists are shown in Figures 2 and 3. As can be seen from the variety of compounds shown, muscarinic ligands generally possess a cationic head, an ester group and a cyclic component which is usually aromatic. Hydrophobic binding is also important as shown by the studies of Baumgold et al. (1977) on the potency of psychotomimetic glycolates. Enantiomers also have been of great use in binding studies in different receptor systems since the inactive form is then able to be used to measure the non-specific binding of the drug. Dexetimide((+)-benzetimide) binds to the receptor with an affinity 100 times greater than its inactive (-) form(Soudijn et al., 1973). The L-enantiomer of quinuclidinyl benzilate has 15 times the affinity for the receptor than the D-form(Baumgold, 1977). Recently, an agonist aceclidine(3-acetoxy quinuclidine, a tertiary amine originally characterised by Barlow and Casy(1975)) known for its central(tremor and analgesia) as well as peripheral effects has been studied(Ringdahl et al., 1982). This compound combined the possibility of comparing stereoselectivity in binding studies with the central and peripheral effects observed <u>in vivo</u>. A correlation was found between the high affinity agonist binding site and the effects observed <u>in vivo</u>. The ratio (+)-aceclidine / (-)-aceclidine was approximately 14 with the (+)- form showing the expected 2 agonist binding sites while the (-)-form

Figure 2

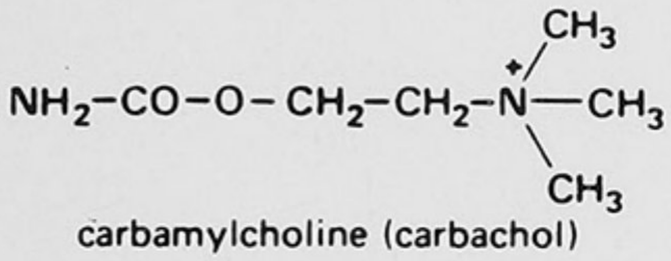
Muscarinic agonists



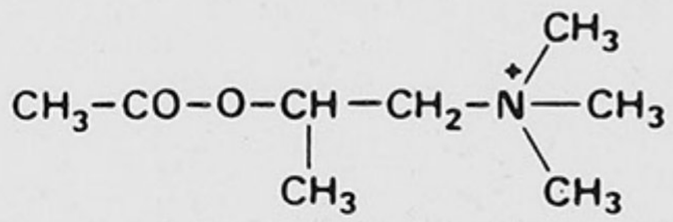
acet ylcholine



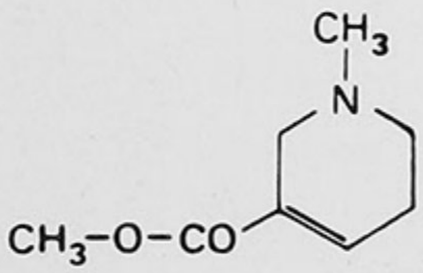
muscarine



carbamylcholine (carbachol)



acetyl · β · methylcholine



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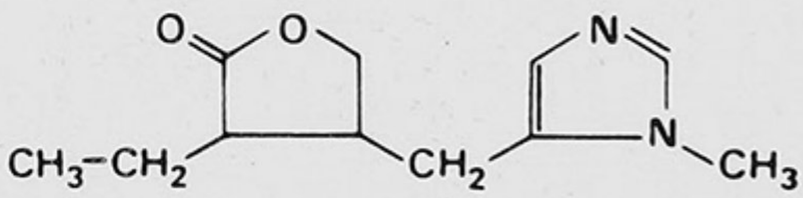
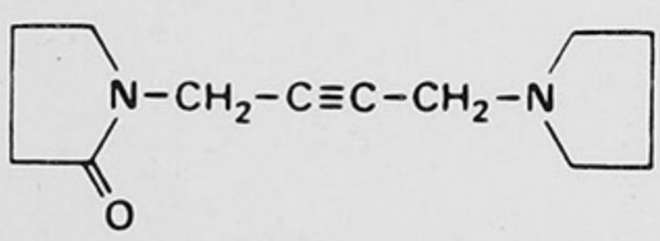
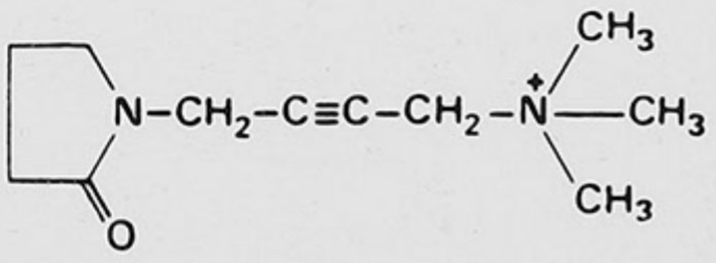


pilocarpine



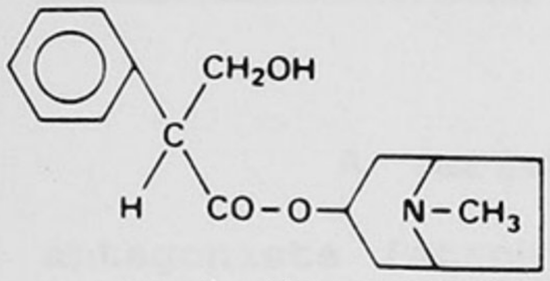
oxotremorine



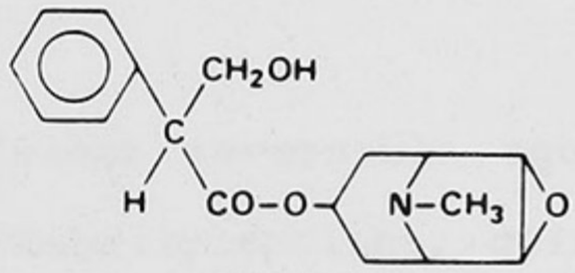
oxotremorine – M

Figure 3

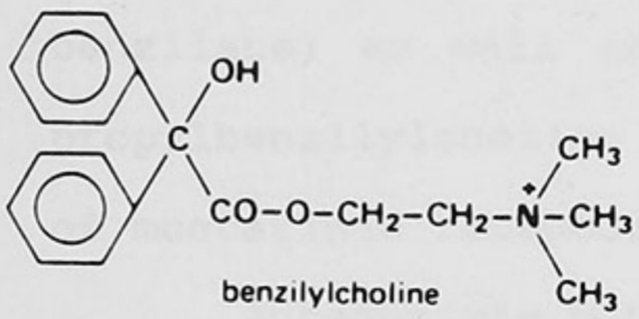
Muscarinic antagonists



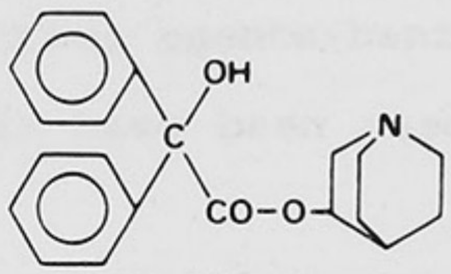
atropine (± hyoscyamine)



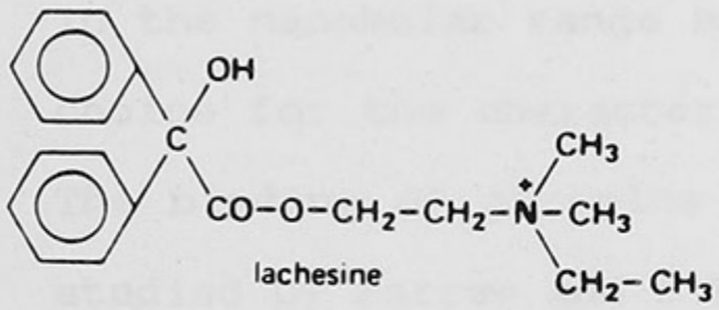
scopolamine



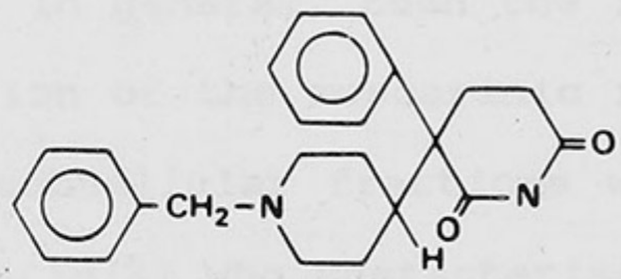
benzilylcholine



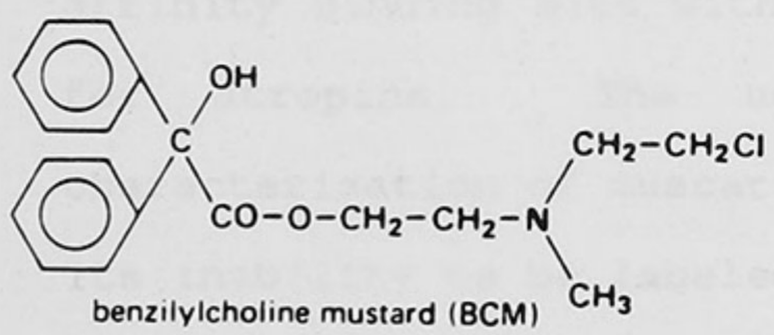
quinuclidinyl benzilate (ONB)



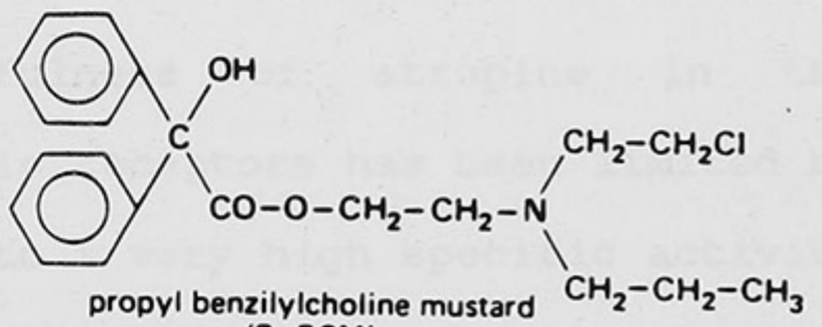
lachesine



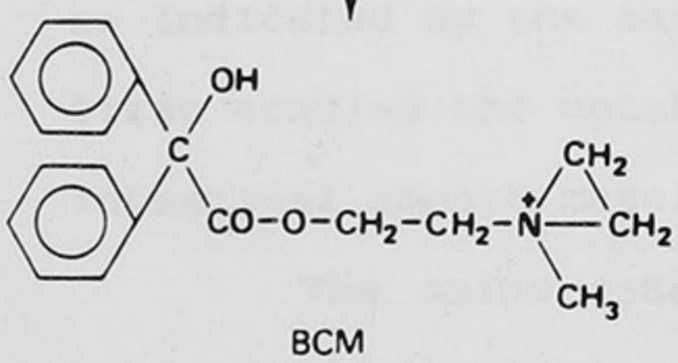
benzetimide



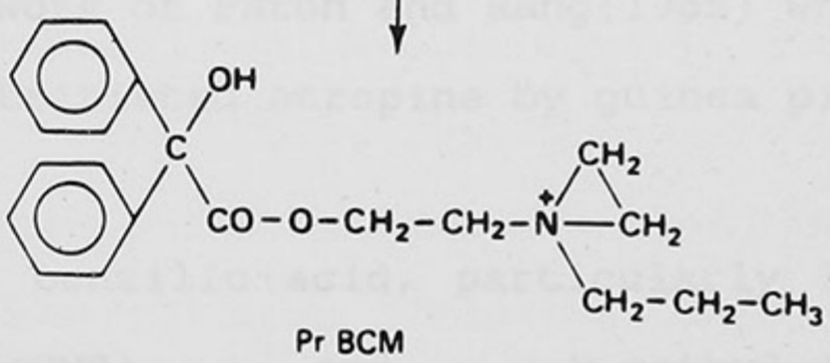
benzilylcholine mustard (BCM)



propyl benzilylcholine mustard (Pr BCM)



BCM



Pr BCM

ethyleniminium ions (alkylating agents)

showed only a single site.

Antagonist Binding

A variety of different reversible agonists and antagonists (atropine, propylbenzilylcholine, quinuclidinyl benzilate, dexetimide, oxotremorine-M, N-M-piperidyl benzilate) as well as alkylating agents(benzilylcholine or propylbenzilylcholine mustard) have been used in the study of muscarinic receptors.

Antagonists with their high affinity for the receptor in the nanomolar range have, in general, been the ligand of choice for the characterisation of the muscarinic receptor. The binding of atropine to subcellular fractions was first studied by Farrow and O'Brien(1973) who characterised a high affinity binding site with a dissociation constant of 0.6nM for atropine. The usefulness of atropine in the characterisation of muscarinic receptors has been limited by its inability to be labeled to a very high specific activity and by the presence of as much as 50% non-specific binding as indicated by the earlier work of Paton and Rang(1965) who first studied the uptake of tritiated atropine by guinea pig intestinal smooth muscle.

The amino esters of benzilic acid, particularly 3-quinuclidinyl benzilate(QNB) and N-methyl-4-piperidylbenzilate(NMPB), have been widely used in the study of muscarinic receptors. QNB is one of the most widely used ligands as it can be highly labeled(up to 60 Ci/mmole) and has a very high affinity for the receptor. Yamamura and Snyder(1974a,b) originally showed that this ligand bound to

rat brain or guinea pig ileum homogenates exhibiting a saturable component sensitive to muscarinic agents with a dissociation constant of less than 0.1nM. Using this compound the muscarinic receptor has been shown to be specifically localised in the following tissues: neuroblastoma cell lines(Burgermeister et al., 1978; Strange et al., 1978); mammalian myocardium(Wei and Sulakhe, 1978; Mukherjee et al., 1979); cerebral blood vessels(Estrada and Krause, 1982); Torpedo electric organ(Strange et al., 1980); cat esophageal sphincter(Rimele et al., 1979); rat colonic epithelial cells(Rimele and Gaginella, 1982); human erythrocyte membranes(Aronstam et al., 1977b); bovine adrenal medulla(Kayaalp and Neff, 1979); goldfish(Moreno-Yanes and Mahler, 1979) and mammalian retina(Hruska et al., 1978). QNB binding has also been extensively studied in different areas of the brain which will be discussed in a separate section.

However, there are some instances where a very high affinity ligand exhibiting a single binding site and having the same affinity in all tissues is not appropriate. If the K_d of the ligand is very low such as for QNB, the concentration of receptors must be less than or equal to the K_d of the drug, otherwise a significant depletion of the ligand will occur at equilibrium which can lead to anomolous binding curves and the masking of high affinity sites if left uncorrected(Wells et al., 1980). Propylbenzilylcholine (K_d=1.2x10^{-8}M) and N-methylscopolamine (K_d=1nM) have been introduced as muscarinic antagonists binding with slightly lower affinity to a single population of sites(Hulme et al., 1978).

The group of Sokolovsky have extensively studied the antagonist N-methyl-4-piperidyl benzilate (NMPB) ($K_d=4x10^{-10}M$) and have used it to study receptors in the mouse brain(Kloog and Sokolovsky, 1977; 1978a,b), in cat iris(Sachs et al., 1979) and in Torpedo electric organ(Kloog et al., 1978). Their data indicated that the binding of NMPB to the receptor could be explained theoretically as a single population of sites during association and two populations of sites appearing during dissociation experiments. They explained this by assuming an isomerization step to occur after binding of the ligand to receptor($RL---R^*L$) which was much slower than the association and dissociation reactions of both RL and R^*. Similar results have been described for the neuroblastoma x glioma cell line using (^3H)-QNB(Klein, 1980a) and for smooth muscle using the antagonists NMPB and QNB(Jarv et al., 1979). Using NMPB, Kloog et al.(1979a,b) were able to show a heterogeneity in the dissociation constants between the different regions of the mouse brain.

Regional heterogeneity in binding constants has also been studied using another antagonist pirenzepine(Hammer et al., 1980). The <u>in vitro</u> effects found with pirenzepine in binding studies resemble the pharmacological specificity of the drug; in so far as the affinity of the drug for sublingual, parotid and lacrimal glands is greater than that found for the heart. Unfortunately, the parietal cells of the gastric mucosa(the <u>in vivo</u> site of action of the drug) could not be assayed due to the low number of binding sites, and the binding found to different brain regions has little physiological significance since <u>in vivo</u> very little of the drug crosses the blood-brain barrier. Pirenzepine is,

however, of great theoretical interest since it exhibits heterogeneous binding curves in the hippocampus, cortex, medulla-pons and salivary glands while the heart, parotid and lacrimal glands appear to contain a single population of sites. The differences seen with pirenzepine could be due to the enviromental constraints on muscarinic receptors or due to intrinsic differences between receptors detected by the antagonist.

The alkylating mustards have also been useful in muscarinic studies particularly in characterisation of solubilised prelabeled sites(to be discussed later). Benzilylcholine(Gill and Rang, 1966) and propylbenzilylcholine mustard(Young et al., 1972) have been used to study receptors in rat brain and small intestine(Burgen et al., 1974a,b).

In summary, tritiated reversible antagonists binding to a single high affinity site(10^{-11}-10^{-8}M K_d) or to a heterogeneity of sites(pirenzepine and NMPB) and alkylating agents(mustards) are all available for the study of muscarinic cholinergic receptors.

Agonists(such as methylfurmethide, carbachol and acetylcholine) generally have 100-1000-fold lower affinity for the receptor than the most potent antagonists and therefore are not widely used for receptor localisation but mainly in competition studies with radiolabeled antagonists for indirect studies on agonist binding sites. A distinct heterogeneity appears to exist in strong agonist binding to the muscarinic receptor which have been divided into superhigh, high and low affinity sites(Birdsall et al.,

1978). A heterogeneity in the distribution of the three types of agonist sites also appears to occur in the rat brain: the cerebellum appears to have the highest concentration of super-high sites while the hippocampus appears to consist of mainly low affinity sites(Birdsall et al., 1980c). Regional heterogeneity has also been found in agonist binding in mouse brain(Aronstam et al., 1979). In an effort to further study the interaction of receptor sites in the membrane different protein modifying agents have been used.

Protein-Modifying Reagents

The heterogeneity seen classically with agonists and more recently with antagonists could mean that muscarinic receptors exist in distinct states, either because of intrinsic molecular differences, or because of their association with different effector systems(such as phosphatidylinositol metabolism, guanylate or adenylate cyclase, etc.). The work of Aronstam's group on rat brain(1977c, 1978a) indicated a role for sulfhydryl groups in receptor-ligand interactions. The results demonstrated that reaction of sulfhydryl groups with p-chloromercuribenzoate(pCMB) inhibited both agonist and antagonist binding while reductive alkylation of -SH groups with N-ethyl-maleimide(NEM) converted the low affinity agonist site into its high affinity state while not affecting antagonist binding. The heterogeneity for agonist binding, which had previously been described seemed therefore, to reflect the existence of two allosteric type

states of the receptor which are controlled by sulfhydryl group alkylation.

However, whereas this group found inhibition of binding by pCMB, Birdsall et al. (1980a) found only a 20% decrease of antagonist binding accompanied by a large decrease in the affinity of carbachol for sites in pCMB treated membranes(reduced to a single affinity of $4x10^{-3}$M). Other groups have found varying results in the effects of sulfydryl reagents on muscarinic binding. Variations were shown to be due in one case to the presence of Ca^{2+} which, when added, revealed an inhibitory effect of the reducing agent dithiothreitol and a reduction, not an increase, in agonist affinity sites as a result of NEM treatment(Hedlund and Bartfai, 1979). A possible species difference and temperature dependence of the NEM effect on agonist binding was reported where an increase in agonist binding affinity was seen only in rat(not bovine) membranes at 22^{o}C but not at 37^{o}C(Carson, 1980). Differences in disulfide reducing agents were also reported(Aronstam and Eldefrawi, 1979b). A recent report (Ikeda et al., 1980) indicated that while NEM always increased agonist affinity, the extent of this increase was not uniform throughout the different areas of the brain and suggested that these differences could not be explained by a simple interconversion between high and low affinity agonist populations.

In general, the muscarinic receptor has been shown to be insensitive to the more common ions such as Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, although a decrease in affinity is found at higher concentrations of monovalent ions(0.5M) for both agonists and antagonists(Birdsall et al., 1979b). Studies

have also been carried out on heavy metal inhibition of muscarinic binding(Aronstam and Eldefrawi, 1979a) and cadmium was shown to preferentially inhibit muscarinic receptors in the striatum(Hedlund et al., 1979).

The discrepencies between results obtained by the different groups indicate the need for a systematic study of protein modifying agents as a function of protein and ion concentration and temperature using different ligands before any final conclusions are drawn as to the precise role of different sulfhydral groups in receptor binding. It might be tentatively suggested, however, that the differences seen could result from variation in the phospholipid composition of the of the membranes used which could cause sensitive disulfide bonds or sulfhydral groups to be hidden within the receptor and thus be differentially available for modification depending on temperature, ion composition or ligand used. It is also possible that the various treatments by different authors are removing different membrane constraints(phospholipids, -SH, S-S) which will eventually lead to some "ground state" of the receptor(Wong, 1981).

Interaction with Other Systems

Muscarinic receptors have recently been shown to interact with other receptor systems, notably the dopaminergic system and more recently have been shown to interact with the polypeptide VIP(vasoactive intestinal peptide). A thorough discussion of these systems is out of the scope of this introduction and for further reading the

following reviews are suggested(Westfall, 1977; Roth and Bunney, 1976; Burnstock, 1976;1978).

VIP has been shown to increase muscarinic binding in cat submandibular salivary gland. Carbachol was shown to have a 1000-fold higher affinity for the receptor in the prresence of VIP(10nM) than in its absence(Lundberg et al., 1982). Carbachol was also shown to potentiate the cyclic AMP response to VIP in the submandibulary gland which was atropine-sensitive(Enyedi et al., 1982). Although the physiological interaction of ACh and VIP has been well documented(Lundberg et al., 1979,1980), the binding results have not been so far reproduced by other groups. Since the binding was performed under non-equilibrium conditions further work from other groups will have to be awaited before a final conclusion is drawn.

Localisation of mAChR

The distribution of mAChR in brain has been studied using the binding of reversible(Yamamura et al., 1974; Kobayashi et al., 1978; Rea and Simon, 1981; Takano et al., 1981) or irreversible(Hiley and Burgen, 1974) ligands to homogenates from different brain areas. Initially, autoradiographic stuides were performed using (^{3}H)-QNB (Kuhar and Yamamura, 1975;1976). Recently, an elegant autoradiographic study using the irreversible ligand(^{3}H)-propylbenzilylcholine mustard has been published(Rotter et al., 1979a-d). Further work localising mAChR to synapses at the EM level has been published (Kuhar et al., 1981) as well

as autoradiography of high and low affinity agonist binding sites using (^{3}H)-N-methylscopolamine(Wamsley et al., 1980). The results are in general agreement from all methods: with highest binding(or concentration of receptors) occurring in the caudate-putamen, high binding in the nucleus accumbens, cerebral cortex, hippocampus, pontine nuclei and hypoglossal nucleus and low binding in the globus pallidus, substantia nigra and the cerebellum.

The lack of an adequate cholinergic marker for the central nervous system(recent work using antibodies to choline acetyltransferase has only just begun(Kimura et al., 1981; Sofroniew et al., 1982)) has made localisation of acetylcholine to specific cell types and the mapping of cholinergic pathways difficult. Since the muscarinic receptor makes up the majority of cholinergic receptors in the CNS, an antibody to the muscarinic receptor or conjugates of muscarinic ligands to markers such as horseradish peroxidase(for a-bungarotoxin:Vogel et al., 1979; Zucher and Yazulla, 1982) or biotin(Bayer and Wilchek, 1980) would be of great use.

To better understand the muscarinic response, the interaction of the muscarinic receptor with other systems and its role in such diseases as Huntington's Chorea(Wastek et al., 1976; Wastek and Yamamura, 1978), Parkinsonism(Javoy-Agid et al., 1981; Ruberg et al., 1982) and Tardive dyskinesia(Ansell, 1981) as well as its fine neuroanatomical localisation, a purified muscarinic receptor is necessary.

Receptor Purification

The studies on the binding of muscarinic agents by smooth muscle strips or tissue homogenates and subcellular particulate preparation, reveal a heterogeneity of binding sites. It appeared possible that such a heterogeneity is at least in part due to environmental constraints on the receptor protein and in order to understand the precise functioning of muscarinic transmission it is necessary to study the receptor in an isolated state. There are two approaches which can be used in protein purification: biochemical and immunological as shown in Table 2. We will first discuss attempts at biochemical purification and its necessary first step: solubilisation.

Unlike nicotinic receptors which do not lose their binding properties upon solubilisation with non-ionic detergents(Changeux, 1975), muscarinic receptors seem to be totally inhibited by these compounds(Beld and Ariens, 1974, among others) and solubilised proteins fail to show any muscarinic binding(Fewtrell and Rang, 1973).

One method of avoiding the problem of inhibition upon solubilisation is to first covalently label the membrane bound receptor and then to do a series of studies on the solubilised labelled receptor. Fewtrell and Rang(1971, 1973) using tritiated benzilylcholine mustard(BCM) have characterised two polypeptide bands in SDS polyacrylamide gel electrophoresis, with molecular weights of 23,000 and 50,000 for smooth muscle preparations. Birdsall and Hulme(1976) using the same procedure with tritiated propylbenzilylcholine mustard(PrBCM) for rat cortex membranes obtained a single band of higher molecular weight 87,000. Possibly some proteolysis took place in the small

Table 2

PROTEIN PURIFICATION

BIOCHEMICAL

IMMUNOLOGICAL

SOLUBILISATION

AFFINITY CHROMATOGRAPHY

OTHER:

ION-EXCHANGE CHROMATOGRAPHY

GEL FILTRATION

SUCROSE GRADIENTS

CsCl GRADIENTS

SDS PAGE

intestine preparation which could account for the difference in molecular weights. For accurate characterisation of the receptor however, a solubilised preparation which can be assayed directly by such techniques as equilibrium dialysis or gel filtration is necessary.

Solubilisation of proteins with muscarinic binding characteristics using high salt(2M NaCl) has been described by several groups(Bartfai et al., 1974; Alberts and Bartfai, 1976; Carson et al., 1977). However, these results were unable to be reproduced by other groups(Filbert et al., 1976; Rueβ and Lieflander, 1979; Repke and Matthies, 1980; Gorissen et al., 1981). Possible explanations for the discrepency has been the presence of small membrane fragments in the "soluble" material. Solubilisation by mild detergents such as Lubrol PX(Haga, 1980a) has more recently been reported. Haga characterised the soluble material as having a Stokes radius of 6.9 and a sedimentation coefficient of 3S corresponding to a molecular weight of 86,000 which is quite similar to that obtained from (^{3}H)-PrBCM prelabeled material on SDS gels. Lysolecithin has also been shown to be successful as a solubilising agent(Haga, 1980b) although the prohibitive cost of this detergent makes it less useful for preparative work. The most popular detergentused so far is digitonin, first introduced by Beld and Ariens(1974). Recently, several laboratories(Hurko, 1978; Aronstam et al., 1978b); Gorissen et al., 1978) have characterised the digitonin solubilised receptor.

Hurko's group using a variety of techniques(dialysis, precipitation by ammonium sulfate, filtration on DEAE filters) reported the solubilisation of about 50% of total

receptor activity from bovine brain. The soluble material had a dissociation constant for (^{3}H)-QNB of the order of 370pM; antagonist affinity remained unchanged while strong agonist affinity was reduced to the low affinity state with a Hill coefficient of close to 1 indicating the presence of a homogeneous population of sites. Similarly, Aronstam's group also reported the solubilisation of 50% of total receptor sites in a bovine caudate nucleus preparation. They reported a K_d of 190pm for (^{3}H)-QNB and no noticeable change in affinity for agonists or antagonists, although complete binding curves were not shown in this study. Finally, Gorissen's group studied digitonin-solubilised receptors using (^{3}H)-dexetimide, and performed binding by the method of gel filtration. Approximately 5-12% of total receptor sites were solubilised, with no change in antagonist affinity being reported(K_d for (^{3}H)-dexetimide was 500pM; however, in this case, only the high affinity binding component for agonists appeared to have been solubilised. The sedimentation coefficient for the digitonin-solubilised material was determined to be 9S(Hurko, 1978; Gorissen et al., 1978). The difference in sedimentation coefficient between lubrol and digitonin solubilised material could be due to the different amounts of lipids and detergent associated with each receptor in each detergent.

Certain problems are associated with the use of digitonin as a solubilising detergent. Aronstam found that the solubilising ability of digitonin varied from batch to batch, which was later shown to be due to the proportion of gitonin present in the digitonin preparations(Repke and

Matthies, 1980). Hydrodynamic studies are difficult to do in the presence of digitonin and, as can be seen from above, binding characteristics of the digitonin solubilised material differed from group to group. At the present time, however, digitonin seems to represent the most efficient solubilising agent available for quantitative isolation of the muscarinic receptor(reviewed: Laduron and Ilien, 1982), although the definitive solubilisation and purification of the muscarinic receptor has not yet been reported.

Another approach to protein purification can be that described as immunological. Antibodies made to proteins of interest can be linked to a Sepharose column to form an immunoabsorbant column. The problem of impure antigens(such as the muscarinic receptor) can be circumvented by the use of monoclonal antibody technology. Immune serum from an animal contains many different antibodies to different antigenic determinants(polyclonal serum), the more impure the preparation is, the more non-specific(non-receptor) antibodies the serum will contain. In some instances, the non-specific components of the preparation will be more immunogenic than the desired antigen and the sera will thus contain a large proportion of one or several antibodies to the exclusion perhaps of all others, including the antibody of interest. The monoclonal antibody technique can help to isolate an antibody that is already present in the polyclonal serum(Kohler and Milstein, 1975;1976; Milstein and Kohler, 1977). Briefly, this technique allows for the the immortalisation of a single antibody secreting cell(or clone) which can be dissected out from even a very high background of non-specific antibodies. This approach ahs been used in the purification of interferon. Secher and

Burke(1980) were able to make an immunoabsorbant column from a monoclonal antibody to interferon which gave a 5000-fold purification of their initial impure interferon preparation(only 0.1-1% of total protein).

Such an approach is theoretically possible for the muscarinic receptor. However, the muscarinic receptor in even its most purified membrane state represents only 0.005% of total membrane protein, almost 100 times less pure than the interferon preparation used for immunisation in the work of Secher and Burke. Difficulties arise when using such impure preparations since, even if one antibody was made to each of the molecules present(of which only 5 out of 100,000 would be directed towards the muscarinic receptor), a minimum of 100,000 clones would have to be screened. The possibility must also be considered that no antibodies at all would be directed against the receptor(as in the case of antigenic competition). The monoclonal technique is a powerful tool but a minimum amount of purification is almost certainly necessary.

Aims of Thesis

The muscarinic cholinergic receptor has been extensively characterised in its membrane state. Using neural cell lines for regulation and desensitisation studies and tissue homogenates for classical binding studies, the interaction of muscarinic receptors with differnt membrane and cytoplasmic systems has been explored. The exact sequence of events leading from interaction between receptor

and ligand through to physiological response is not yet known. A major stumbling block has been the lack of a purified receptor due in part to the absence of any rich source of the receptor(as the electric organ for the nicotinic receptor) and a stable soluble preparation.

The original goal of this thesis was to obtain an enriched preparation of the receptor based on biochemical methods which would be suitable for use as an antigen. Specific antibodies would be obtained by using the monoclonal technique for an eventual use in the immunohistochemical localisation of central cholinergic systems. Difficulties encountered in the preparation of antibodies to impure antigens made it obvious that a purer receptor preparation and a better biochemical understanding of the receptor would be necessary before any immunological work could be undertaken. This thesis has therefore focused its attention on a more biochemical approach to the study of muscarinic receptors. A novel method of receptor solubilisation is described(Chapters 2 and 3) and the stability of the soluble material examined(Chapter 4). Chapter 5 describes our efforts at finding an affinity compound while our attempts to obtain a specific muscarinic antibody are summarized in Chapter 6. Chapter 7 is a general discussion of the results in this thesis.

CHAPTER 2

MEMBRANE-BOUND MUSCARINIC RECEPTORS

Introduction

Muscarinic receptors have been extensively characterised in the membrane state in rat brain and smooth muscle(for references see Introduction). Although it is expected that the muscarinic receptor will show similar characteristics in central and peripheral tissues in other mammalian species, a thorough understanding of the native state of the receptor is necessary before any purification can be attempted. Three criteria must be fulfilled when using radioligand binding assays to define receptors in tissue homogenates: 1) Saturability of binding must be seen which 2) is specifically displaced only by muscarinic drugs and 3) which is localised to those areas known to contain muscarinic receptors from physiological studies.

An optimal membrane preparation(on regional and subcellular levels) containing the highest concentration of receptors possible is also necessary for undertaking solubilisation studies which are a prequisite for the study of membrane proteins. An alternative approach would be to use cell lines enriched in muscarinic receptors as a possible source of receptors. To date, only the mouse neuroblastoma cell line N1E-115 has been used extensively in muscarinic studies and the concentration of receptors in this cell line is rather low(<50 pmoles/ g protein). This

chapter describes a new human neuroblastoma cell line(TR14) which may be of future use in receptor studies(Rupniak et al., 1982, submitted) and studies the conditions necessary for optimal membrane preparations used in subsequent solubilisation studies.

Methods

Preparation of Membranes

Ox brains were obtained fresh from the slaughterhouse within four hours of death. The appropriate areas(usually the caudate nucleus) were immediately dissected out and used at once or frozen at -20°C until use. No loss in receptor sites was noticed up to 2 months after freezing whole brain regions.

The subcellular fractionation scheme of de Blas and Mahler(1978) was used as described in Figure 1. Homogenates could be frozen for up to one month without loss of binding activity; however, they were usually used within two weeks after freezing.

Binding Assay

The binding assay was a modification of the method described by Yamamura and Snyder(1974a): Membranes (usually microsomal at a concentration of 0.02-0.03 mg protein/ml) were incubated with the appropriate concentrations of (^{3}H)-quinuclidinyl benzilate(QNB) for 60 minutes at 37°C in a total volume of 2 mls in 25mM Na-phosphate buffer, pH 7.4 ±

1mM EGTA(as noted). When unlabeled ligand was included in the assay it was incubated for 15 minutes at 37°C before addition of (^{3}H)-QNB.(Unlabeled ligand could also be added at the same time as labeled ligand without any difference being seen in the binding curves). The suspension was then rapidly filtered through Whatman glass-fibre filters(GF/B) and washed with 10 mls ice cold 10mM phosphate buffer, pH 7.4; all points were performed in duplicate or triplicate as indicated. The filtration apparatus was commercially supplied by Holzel(Technik, Munchen). The filters were oven dried and 6 mls Packard Toluene Scintillation Fluid (0.5%(w/v) 2,5-diphenyloxazole(PPO), 0.01%(w/v) 1,4 bis-(2,(4-methyl-5-phenyloxazolyl)benzene(dimethyl POPOP) in toluene) was added. The vials were counted in a Packard Tri-Carb 2660 Liquid Scintillation Counter at an efficiency of 40%. Specific binding was defined as the difference between total binding and binding in an excess of atropine(1-10μM as noted). Saturation curves were always performed at 20pM of sites and inhibition curves were performed at 50pM (^{3}H)-QNB(32 Ci/mmole). Protein was determined by the method of Lowry et al. (1951).

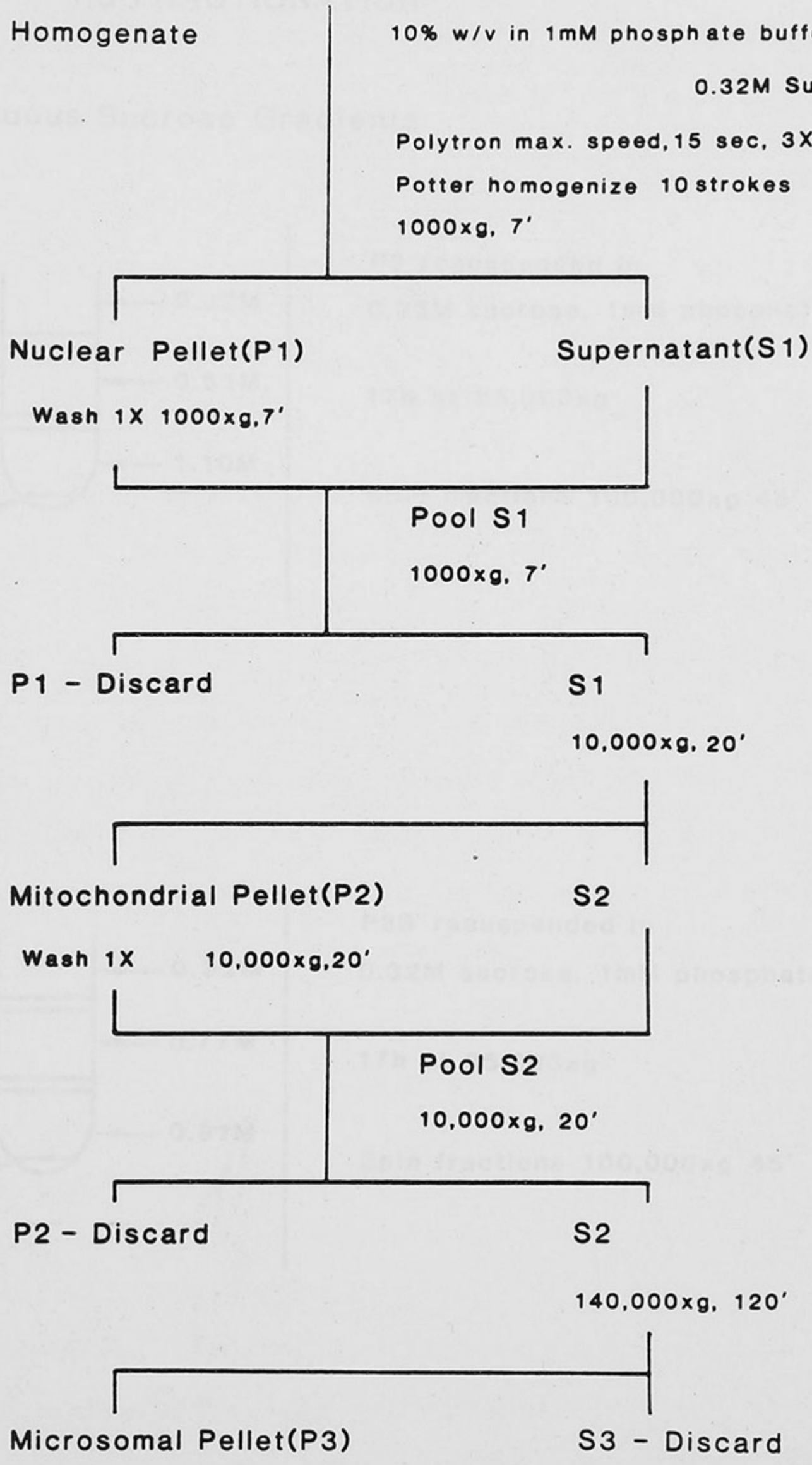
(±)-(^{3}H)-QNB(32 Ci/mmole) was obtained from the Radiochemical Centre(Amersham, U.K.). Benzhexol, propylbenzilylcholine and benzilylcholine were gifts of Dr. J.M. Young(Department of Pharmacology, Cambridge). All other ligands and supplies were from Sigma Chemical Co.(London, U.K.).

TR14 Cells

TR14 cells were a gift from Dr. T. Rupniak(Imperial

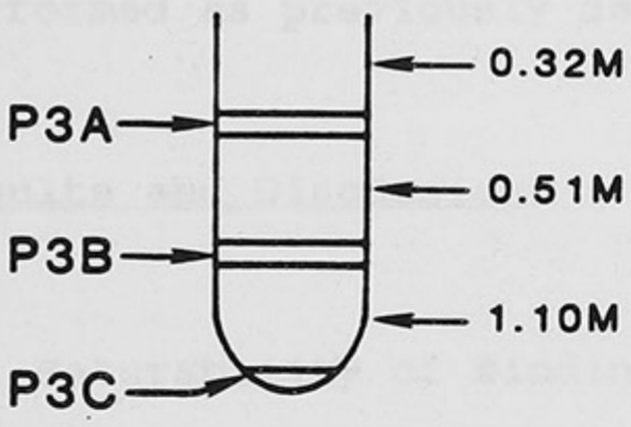
FIGURE 1

BOVINE CAUDATE NUCLEUS MEMBRANE FRACTIONATION



P3 FRACTIONATION

Discontinuous Sucrose Gradients

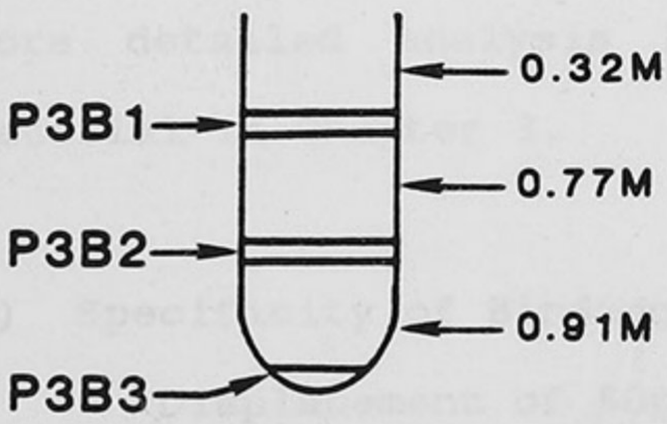


P3 resuspended in

0.32M sucrose, 1mM phosphate buffer

17h at 95,000xg

Spin fractions 100,000xg 45'



P3B resuspended in

0.32M sucrose, 1mM phosphate buffer

17h at 95,000xg

Spin fractions 100,000xg 45'

Cancer Research Fund, London). The cell line was established from biopsy material from a primary adrenal tumour. Primary cell cultures from this biopsy could be serially propagated and thus gave rise to the neuroblastoma cell line TR14(as described in Rupniak et al., 1982, submitted). Cells were homogenised in phosphate buffer, pH 7.4 at a protein concentration of 200-600 µg/ml and binding performed as previously described for bovine brain material.

Results and Discussion

1) Saturability of Binding

Figure 2 shows binding of the reversible antagonist (^{3}H)-QNB to a microsomal preparation from bovine caudate nucleus. The apparent K_d of 20pM is similar to that found by other groups for brain and binding is seen to be to a single site. Since binding characteristics have been extensively described in the membrane state by many other groups only a minimum of data will be presented here with a more detailed analysis being undertaken on the soluble material in Chapter 3.

2) Specificity of Binding

Displacement of 50pM (^{3}H)-QNB by a variety of muscarinic agonists and antagonists is shown in Figure 3. As reported previously(Birdsall et al., 1978; Hulme et al., 1978), antagonists show binding to a single site while strong agonists(carbachol and methylfurmethide) have a Hill coefficient less than one indicating the presence of more than one binidng site. The affinity of antagonists for the

receptor was approximately 100-1000-fold higher than that of agonists. Table 1 summarises the inhibition constants and compares them to rat values. In general, there is good agreement between values except in the case of scopolamine which has a 10-fold lower affinity in the bovine brain than in rat. The higher affinity seen for QNB is probably due to the more optimal conditions of binding(lower protein concentration)used in the bovine brain study than initially used in the rat brain.

3) Localisation of Binding

Figure 4 shows binding of 500pM (^{3}H)-QNB to different regions of rat and bovine brain. As seen in earlier work(Yamamura and Snyder, 1974a), the highest concentration of receptor is found in caudate nucleus with lowest binding being seen in the cerebellum and brain stem. Rat brain shows a higher concentration of sites in the cortex than in bovine brain(1200 vs 600 pmoles/ g protein) but the general distribution of receptor binding is the same. The bovine caudate nucleus was chosen as the optimal preparation to use due to the quantity of tissue available(10 grams per brain) and high density of receptors(average of 1500 pmoles (^{3}H)-QNB binding sites/ g protein).

4) Subcellular Localisation of Receptor Binding

In an attempt to further purify the receptor, the subcellular fractionation procedure of de Blas and Mahler(1978) was used as described in Methods(Figure 1). The highest concentration of receptor sites was found in the microsomal pellet(P3) with little purification being seen in the mitochondrial pellet(Figure 5). Further purification of

FIGURE 2

FIGURE 2

Specific and Non-specific Binding of (^{3}H)-QNB(quinuclidinyl benzilate) to a Microsomal Preparation from Bovine Caudate Nucleus.

The concentration of receptor sites was 20pM. Binding as in methods.

Upper figure: Binding of (^{3}H)-QNB in the presence (▲ - ▲) and absence (● - ●) of 10 µM atropine.

Lower figure: Subtraction of the two curves gives specific binding.

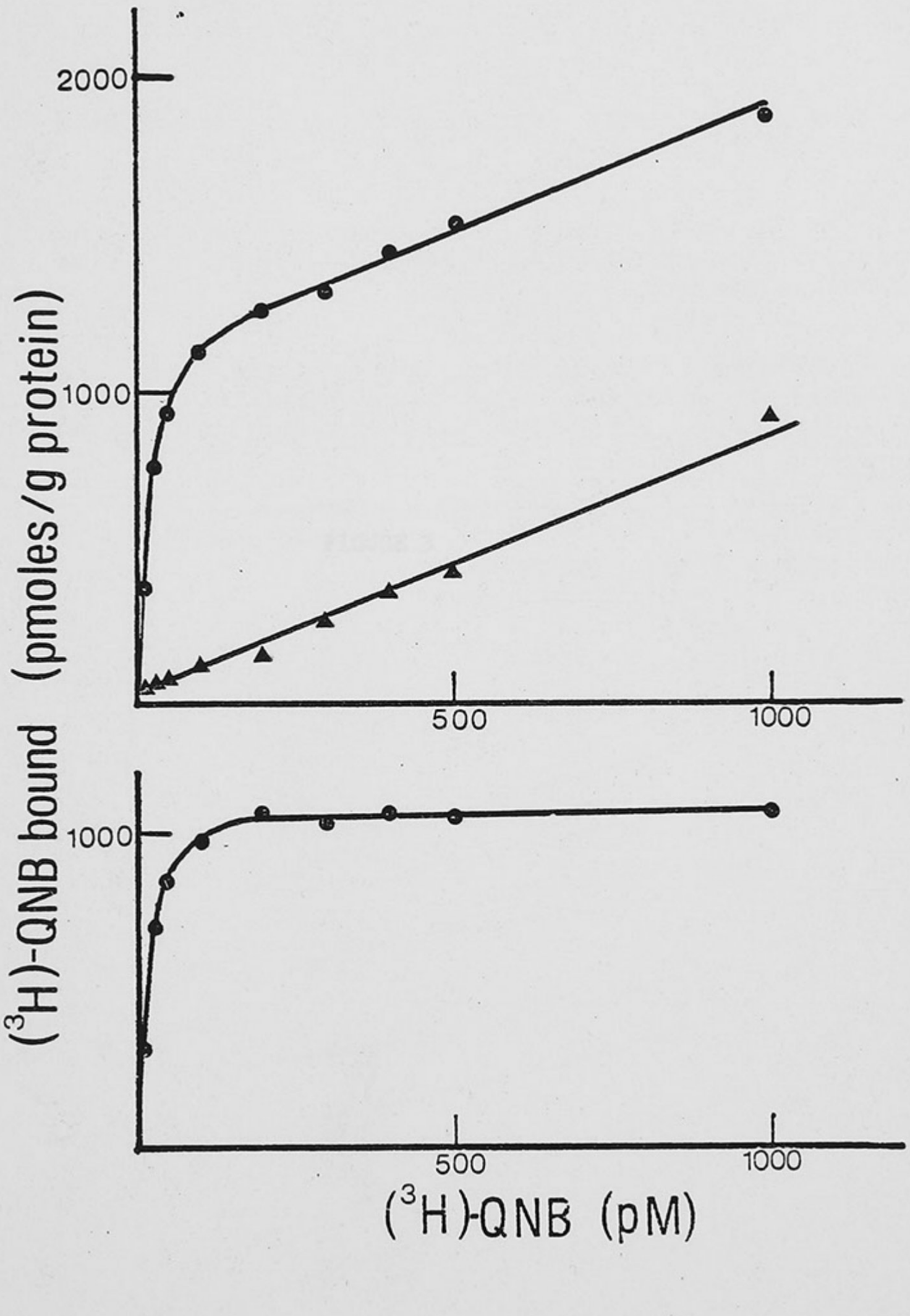

2000
1000
(pmoles/g protein)
500
1000
1000
500
1000
(3H)-QNB bound
(3H)-QNB (pM)

FIGURE 3

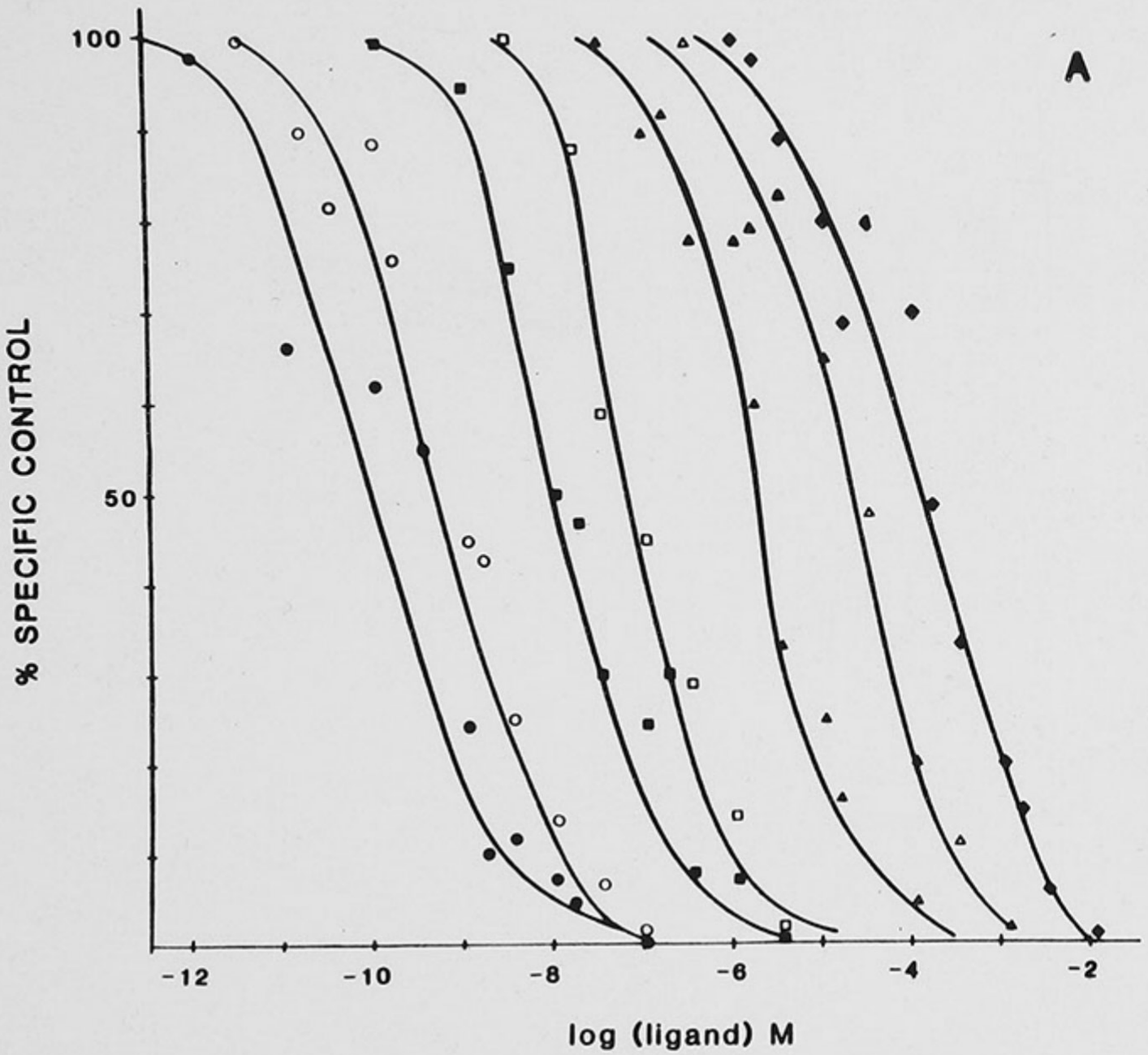
FIGURE 3

Inhibition of (^{3}H)-QNB Binding by Muscarinic Agonists and Antagonists.

A. 50pM (^{3}H)-QNB was incubated with the appropriate concentration of ligand at 37^{o}C as indicated in Methods.

B. The corresponding Hill plots for the inhibition curves shown in A. log %C/%I is plotted as a function of the log of the unlabeled ligand(M).

QNB(●); atropine(○); benzilylcholine(■);
chlorpromazine(□); oxotremorine(▲);
methylfurmethide(△); carbachol(◆).


A
100
50
% SPECIFIC CONTROL
-12
-10
-8
-6
-4
-2
log (ligand) M

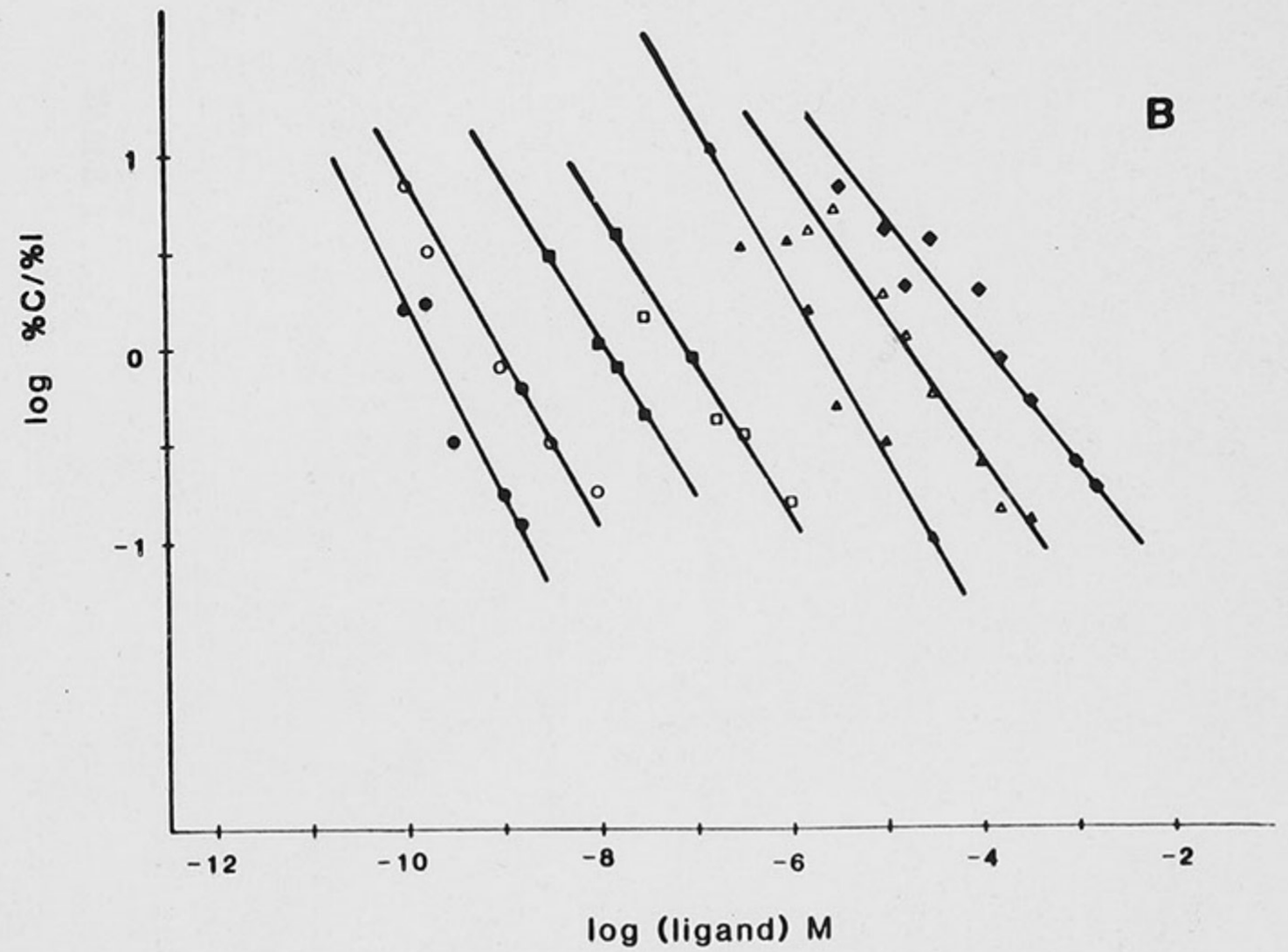

B
1
0
-1
log %C/%I
-12
-10
-8
-6
-4
-2
log (ligand) M

TABLE 1

TABLE 1

Inhibition constants were calculated from the equation:

$$K_i = \frac{ID_{50}}{1 + C/K_d}$$

where C is the concentration of the radioactive ligand(50pM) and K_d its equilibrium constant(20pM). Binding assays were performed as in Methods. Each value represents the average of at least 2 experiments agreeing within 10%. ID_{50} values were determined graphically. Non-muscarinic drugs(hexamethonium, decamethonium, d-tubocurare, nicotine and neostigmine were without effect up to 10μM).

Values for Rat brain were determined from the following references except for reference iii which was determined in guinea pig ileum.

i: Yamamura and Snyder(1974)
ii: Hulme et al.(1978)
iii: Reasbeck and Young(1973)
iv: Yamamura et al.(1976)
v: Birdsall et al.(1978)

TABLE 1

Inhibition Constants for Muscarinic Agonists and Antagonists in Rat and Bovine Membrane Material.

Ligand	Rat Brain		Ox Brain	
	K_i(M)	Ref	K_i(M)	n_H
QNB	$2x10^{-10}$	i	$2.5x10^{-11}$	1.0
atropine	$7x10^{-10}$	i	$3.6x10^{-10}$	0.95
scopolamine	$4x10^{-10}$	i	$2.1x10^{-9}$	1.0
benzhexol	$7.6x10^{-9}$	ii	$1x10^{-9}$	1.0
benzilylcholine	$1.8x10^{-9}$	iii	$2.8x10^{-9}$	0.9
propyl-benzilylcholine	$1.3x10^{-8}$	ii	$2x10^{-8}$	0.9
chlorpromazine	$5x10^{-8}$	iv	$2.5x10^{-8}$	0.9
oxotremorine	$3x10^{-7}$	i	$5x10^{-7}$	0.85
pilocarpine	$3x10^{-6}$	i	$2.1x10^{-6}$	1.0
methylfurmethide	$4x10^{-6}$	v	$8.5x10^{-6}$	0.65
carbamylcholine	$1x10^{-5}$	i	$2x10^{-5}$	0.55

FIGURE 4

FIGURE 4

Regional Localisation of (^{3}H)-QNB Binding Sites in Bovine and Rat Brain.

Microsomal preparations were prepared as described in Methods and binding assayed at saturation(500pM (^{3}H)-QNB) ± 10 µM atropine. Results are from a representative experiment. SA is specific activity in pmoles of (^{3}H)-QNB binding sites / g protein.

Abbreviations: cere: cerebellum; bs: brain stem; hipp: hippocampus; ctx: cortex; cn: caudate nucleus.

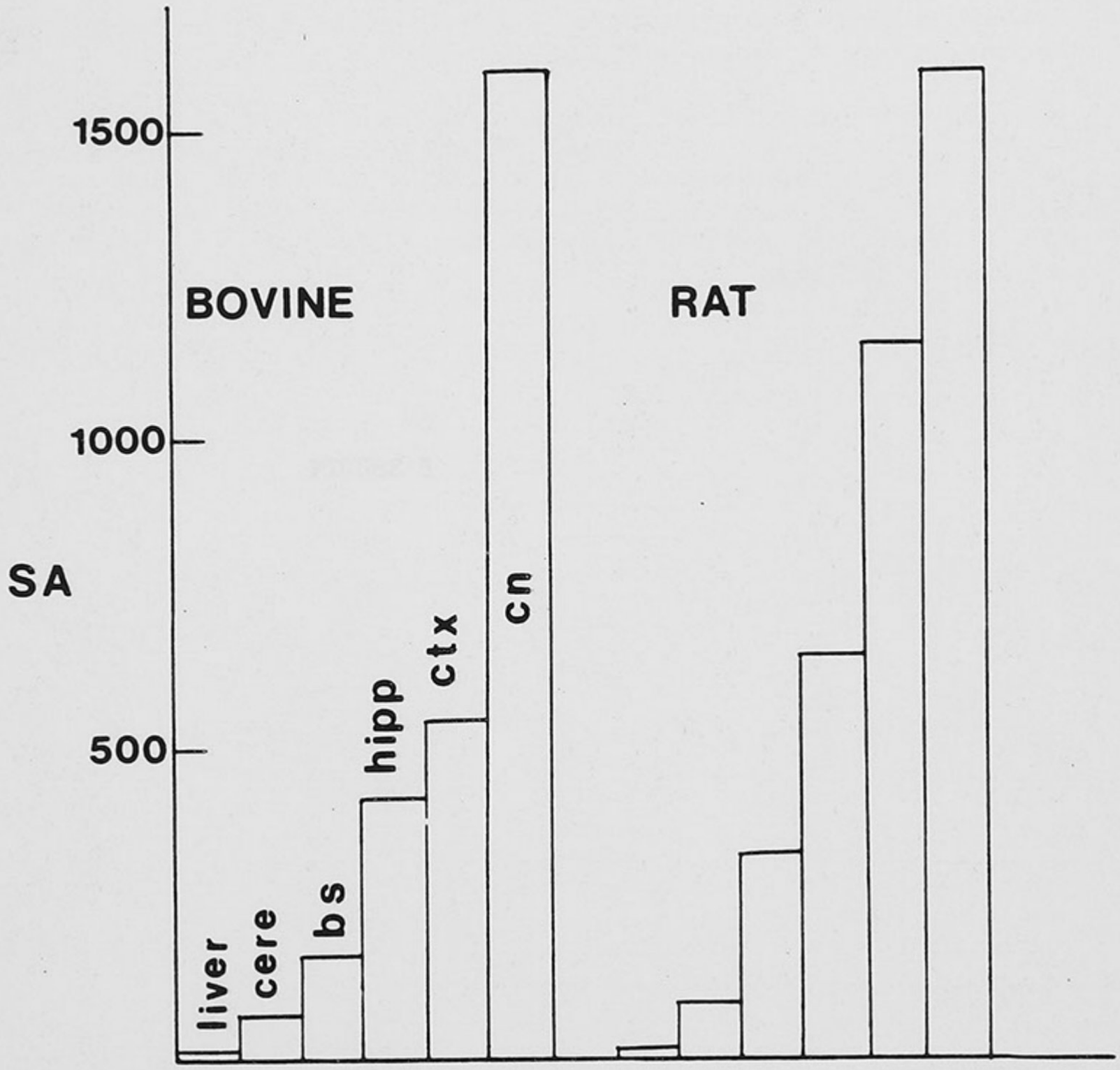
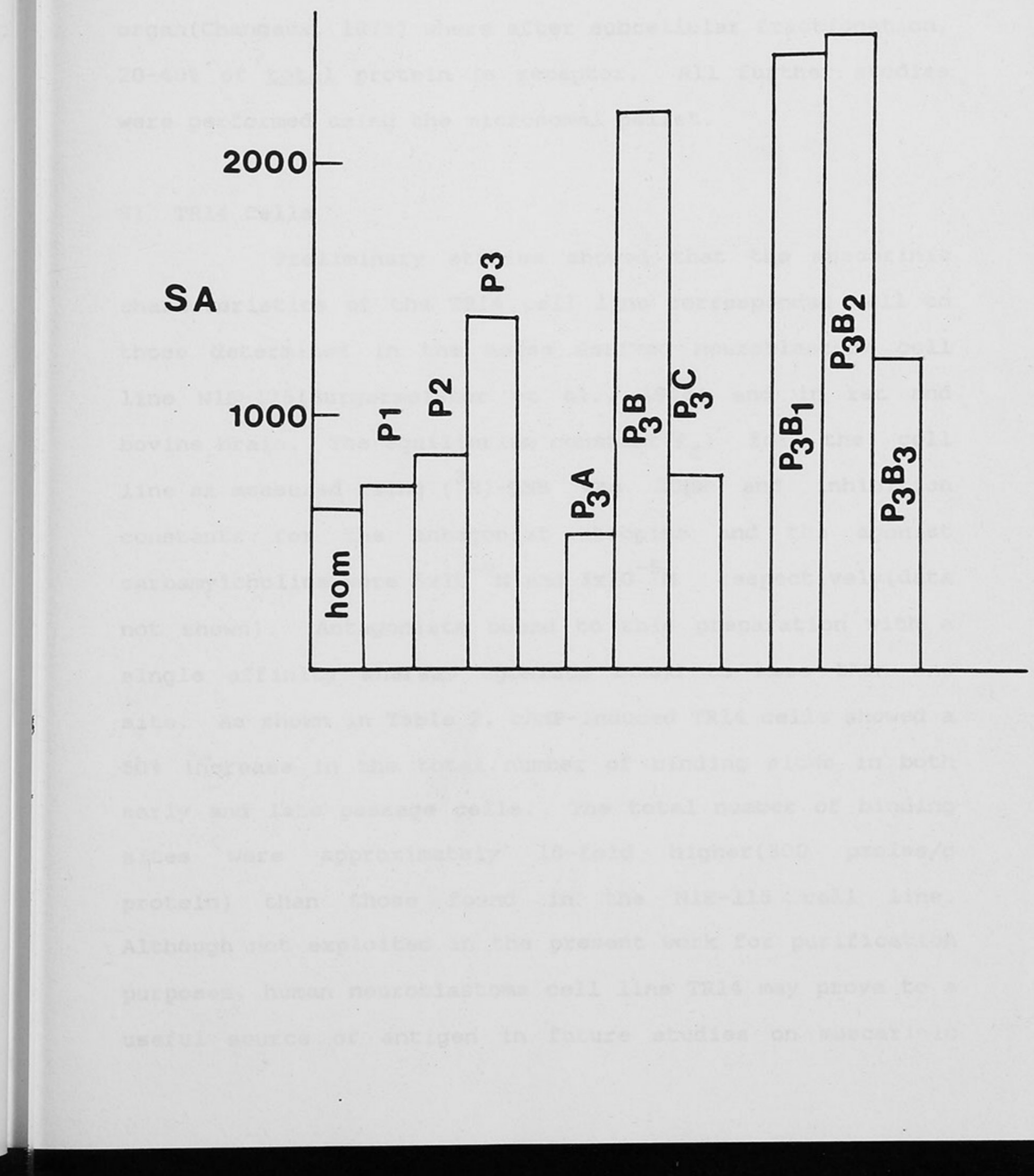

1500
1000
500
SA
BOVINE
RAT
liver
cere
bs
hipp
ctx
cn

FIGURE 5

FIGURE 5

Subcellular Fractionation of Muscarinic Receptors from Bovine Caudate Nucleus.

Fractionation method is described in Figure 1.
Binding of the individual fractions was assayed at a saturating concentration of (^{3}H)-QNB(500pM)$\pm$10uM atropine. Results are from a representative experiment.
SA is specific activity in pmoles (^{3}H)-QNB binding sites/ g protein.


SA
2000
1000
hom
P1
P2
P3
P3A
P3B
P3C
P3B1
P3B2
P3B3

the P3 pellet by discontinuous sucrose gradients led to an approximate 2- to 3-fold increase in binding sites. These fractions were used as antigens as will be described in Chapter 6. In general, little purification over the homogenate concentration of receptors was seen upon subcellular fractionation(maximum 5-8 fold) unlike that found for the nicotinic receptor of the electric organ(Changeux, 1975) where after subcellular fractionation, 20-40% of total protein is receptor. All further studies were performed using the microsomal pellet.

5) TR14 Cells

Preliminary studies showed that the muscarinic characteristics of the TR14 cell line corresponded well to those determined in the mouse derived neuroblastoma cell line N1E-115(Burgermeister et al., 1978) and in rat and bovine brain. The equilibrium constant(K_d) for the cell line as measured using (^{3}H)-QNB was 50pM and inhibition constants for the antagonist atropine and the agonist carbamylcholine were $5x10^{-9}$M and $3x10^{-5}$M respectively(data not shown). Antagonists bound to this preparation with a single affinity whereas agonists bound to more than one site. As shown in Table 2, cAMP-induced TR14 cells showed a 60% increase in the total number of binding sites in both early and late passage cells. The total number of binding sites were approximately 10-fold higher(300 pmoles/g protein) than those found in the N1E-115 cell line. Although not exploited in the present work for purification purposes, human neuroblastoma cell line TR14 may prove to a useful source of antigen in future studies on muscarinic

TABLE 2

TABLE 2

Muscarinic Binding to TR14 Cells

Receptor binding measured at 500pM (^{3}H)-QNB ±5uM atropine. Values represent specific binding and are the mean and standard deviation of three independent experiments. Cells were induced using cAMP.

TABLE 2

Muscarinic Receptor Binding

(pmoles/g protein)

Early Passage	211±78
induced	377±88
Late Passage	156±20
induced	256±13

receptors.

Conclusion

This chapter presented a pharmacological analysis of bovine brain membrane muscarinic receptors. The agonist and antagonists affinities and localisation of receptors were similar to those described extensively by other groups(see General Introduction). The microsomal pellet from bovine caudate nucleus was shown to be the optimal membrane preparation under the experimental conditions described in this chapter.

The first step in the purification of any membrane protein is its solubilisation. The screening for an optimal detergent and subsequent pharmacological characterisation of the soluble material is described in Chapter 3.

CHAPTER 3

SOLUBILISATION OF BOVINE BRAIN MUSCARINIC RECEPTORS

Introduction

Previous work on muscarinic cholinergic receptors has shown that they are membrane-bound proteins(Carson, 1979). Membrane proteins can generally be divided into two classes: peripheral(or extrinsic) and integral(or intrinsic) proteins. Peripheral proteins can classically be solubilised by chelating agents or by lowering or increasing the ionic strenght or pH without dissociating the lipid matrix of the membrane. These proteins(such as rhodopsin) are presumably bound by predominantly polar interactions to proteins or to lipids in the membrane. Integral proteins are tightly bound to the membrane and can only be solubilised by disrupting the membrane with organic solvents or detergents. These proteins have been suggested to have an amphiphilic structure with a polar portion in contact with the extracellular fluid and a lipophilic portion which is inserted into the membrane(Helenius and Simons, 1975). Lipids generally contain an apolar group of aliphatic or aromatic nature(called tails) and a charged(phosphate, amino, sulphate, carboxyl) or uncharged(hydroxyl, carbonyl) polar group(called heads). Polar lipids can be divided into insoluble and soluble amphiphiles. Insoluble amphiphiles

constitute the major lipid groups(triacylglycerols, cholesterol, phospholipids, glycolipids) while soluble amphiphiles include the detergents.

There are two types of soluble amphiphiles which can be distinguished by their methods of solubilising membrane proteins: Type A(Figure 1) include most anionic, cationic and non-ionic detergents, lysolecithin and gangliosides. These form micelles, thermodynamically stable colloidal aggregates formed spontaneously above a narrow concentration range(the CMC or critical micellar concentration), as indicated in Figure 2. Since the driving force for micellar formation is hydrophobic, the hydrophilic head groups tend to oppose micellar formation(as in ionic detergents) but a high counter ion concentration can reduce the CMC by reducing the electrostatic repulsion between head groups allowing association to occur at a lower monomer concentration. The CMC for non-ionic detergents(such as the Triton or Tween series) is generally 100-1000 times lower than the ionic detergents. Type B detergents(the bile salts and digitonin, Figure 3) do not form micelles(per se) such as the Type A detergents do at higher concentrations, due to their bulky and complicated cyclic or aromatic hydrophobic moieties. Bile salts do not contain a defined polar head group but instead contain a polar and an apolar face which tend to form small aggregates(2-8 molecules) above the CMC. At higher counter ion concentrations, larger aggregates may form secondary micelles(Figure 4) which are probably made up of primary micelles bound to each other by polar interactions and forming approximately globular clusters. Both detergent Types A and B have been used in the study of muscarinic receptors.

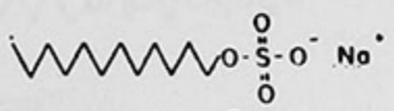
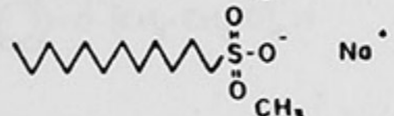
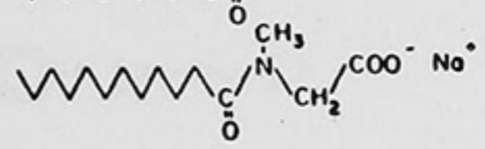
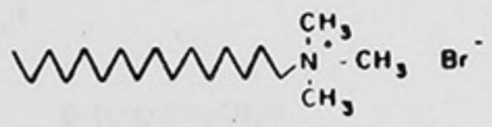
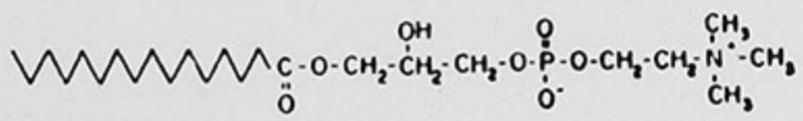
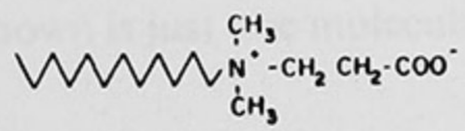
FIGURE 1

FIGURE 1

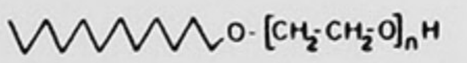
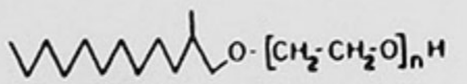
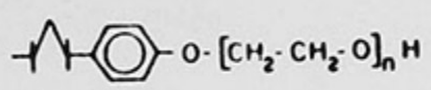
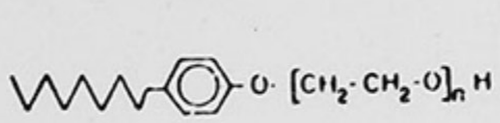
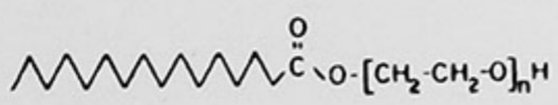
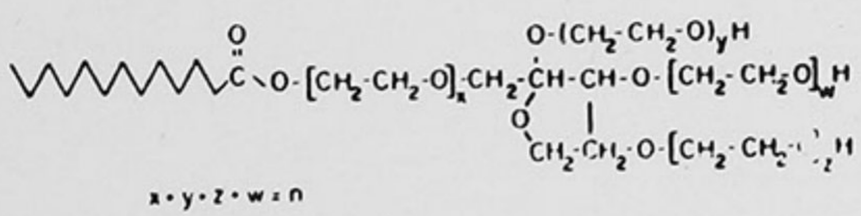
Type A Soluble Amphiphiles
(from Helenius and Simons (1975))

TABLE II

SOME SURFACTANTS OF TYPE A

Structural formula	Chemical name	Examples of trade names
	Anionic detergents	
	Sodium dodecylsulphate	
	Sodium dodecylsulphonate	
	Sodium dodecyl-*N*-sarcosinate	
	Cationic detergents	
	Cetyltrimethylammonium bromide	
	Tetradecylammonium bromide	
	Dodecylpyrimidinium chloride	
	Ampholytic detergents	
	Palmitoyllysolecithin	
	Dodecyl-*N*-betaine	

Non-ionic surfactants

	Polyoxyethylene alcohol	Brij series, Lubrol W, AL series
	Polyoxyethylene isoalcohol	Sterox AJ, AP series Emulphogen BC series Renex 30 series
	Polyoxyethylene p-t-octyl phenol	Triton X series Igepal CA series Nonidet P 40
	Polyoxyethylene nonylphenol	Triton N series Igepal CO series Surfonic N series
	Polyoxyethylene esters of fatty acids	Sterox CO séries Myrj series
	Polyoxyethylene sorbitol esters*	Span series Tween series Emasol series

* The formula shown is just one molecular type in a complex mixture of different possible structures. n = average number of ethylene oxide units per molecule.

FIGURE 2

FIGURE 2

Equilibrium of Type A Detergents
(from Helenius and Simons (1975))

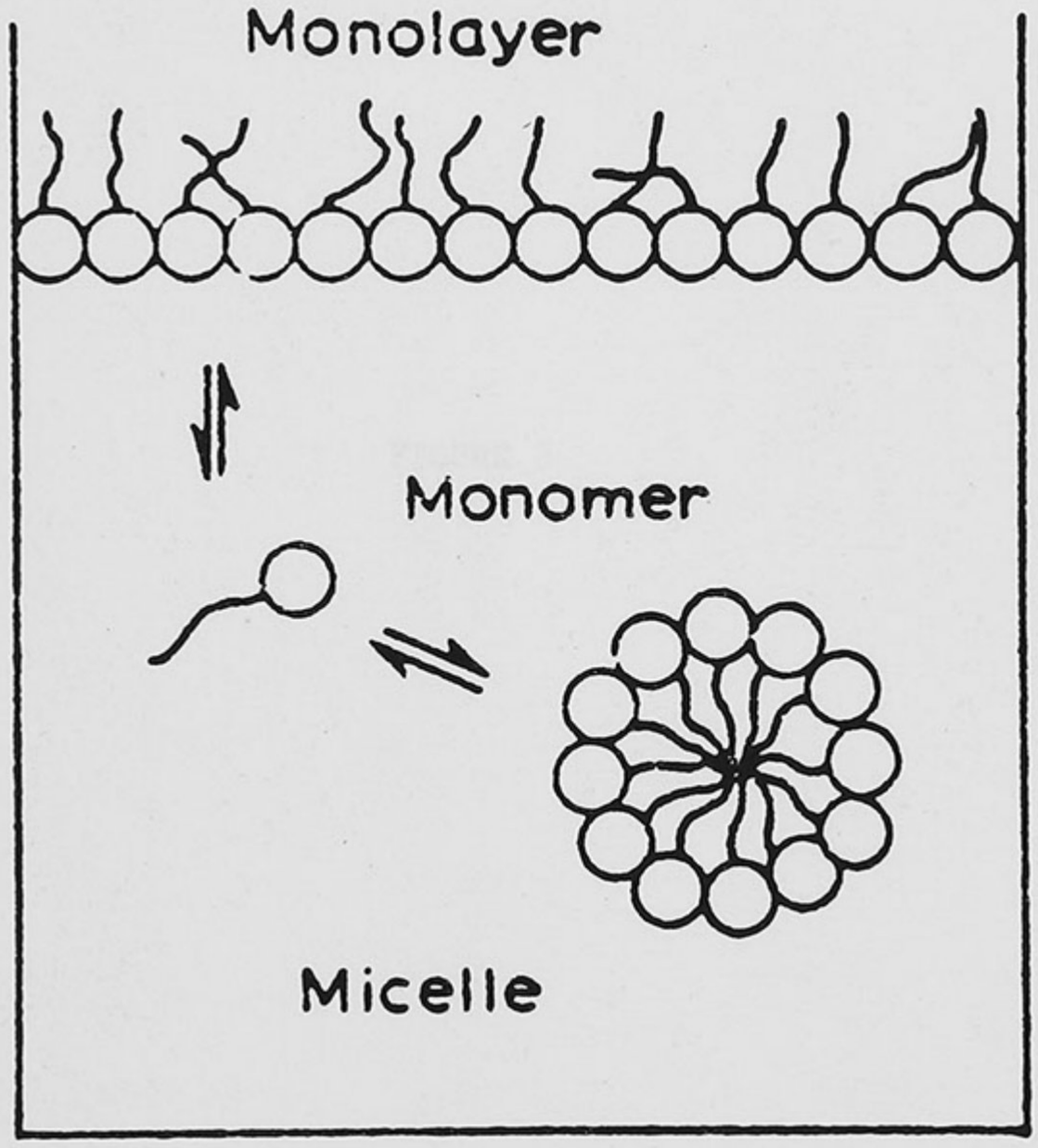

Monolayer
Monomer
Micelle

FIGURE 3

FIGURE 3

Type B Soluble Amphiphiles
(from Helenius and Simons (1975))

SOME SURFACTANTS OF TYPE B

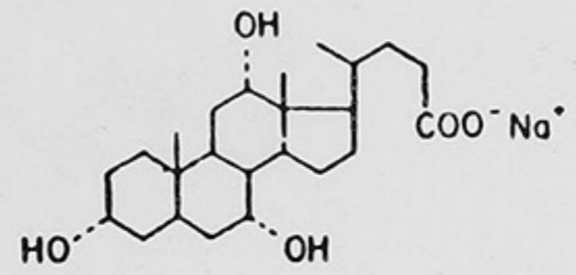
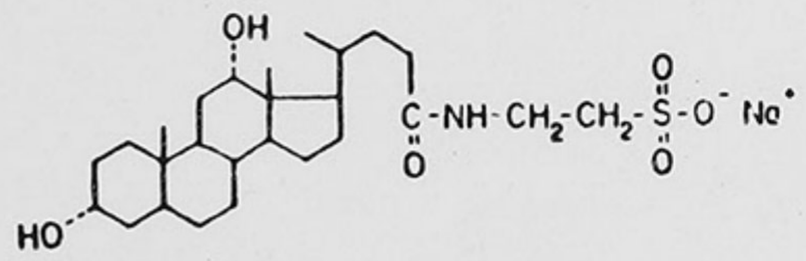
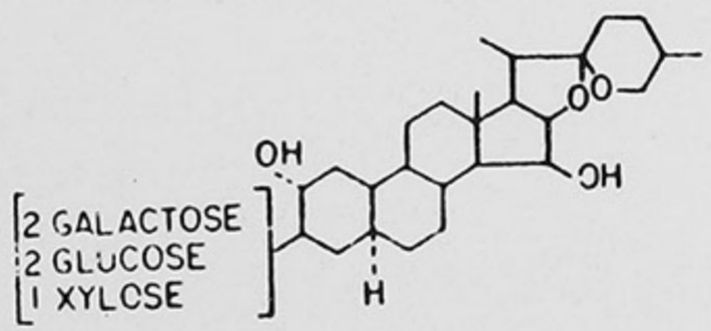
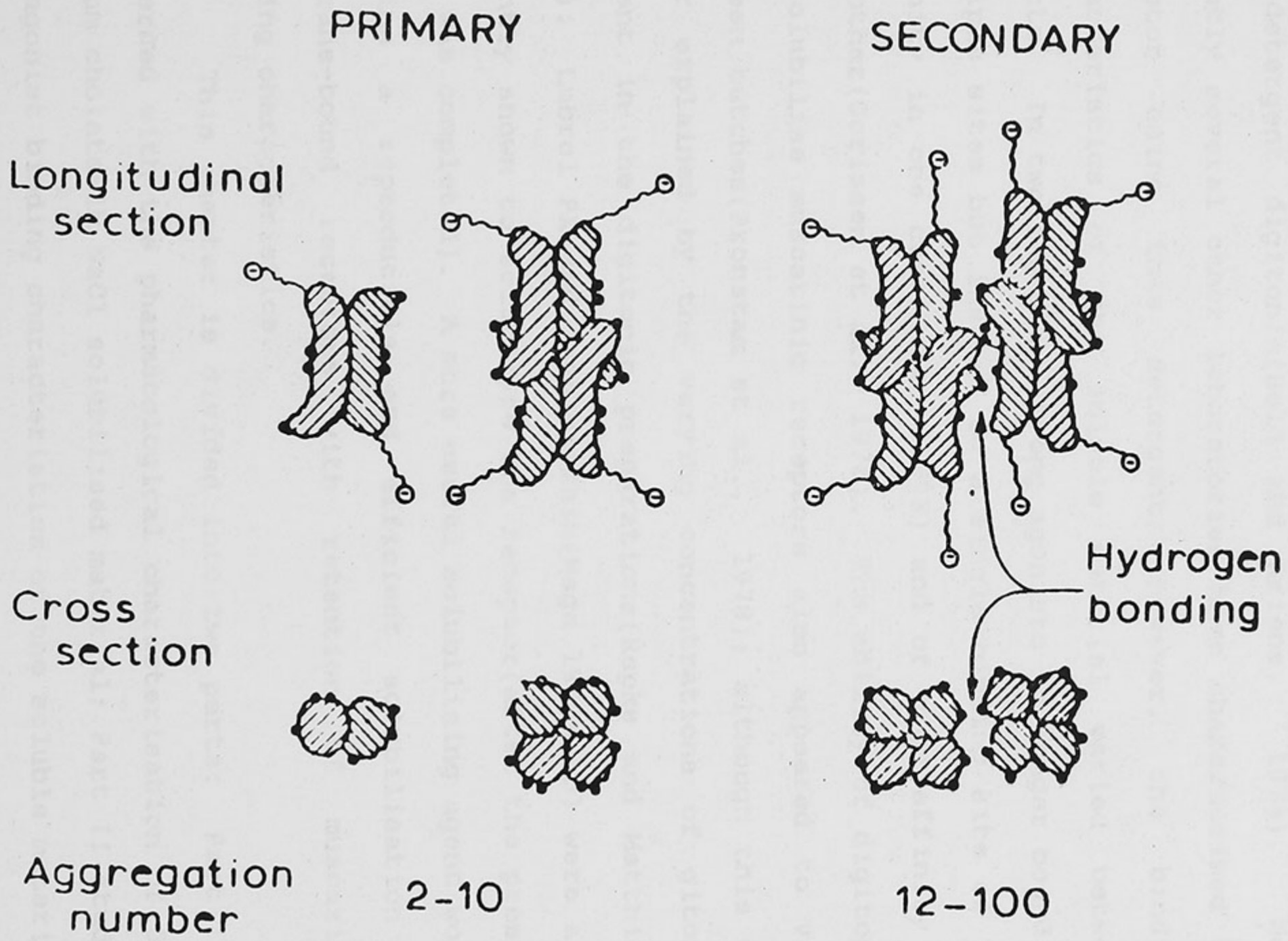
Structural formula	Name
	Sodium cholate (trihydroxy bile salt)
	Sodium taurodeoxycholate (dihydroxy bile salt)
	Digitonin

FIGURE 4

FIGURE 4

Proposed Structure of Bile Salt Micelles
(from Helenius and Simons (1975))



Proposed structures for the "primary" and "secondary" bile salt micelles. Taken from Carey and Small [25].

In order to understand the precise functioning of the muscarinic receptor, it is desirable to study the receptor in an isolated state; however, purification attempts have been hampered by the lack of an efficient solubilising agent. As mentioned in the General Introduction, earlier attempts to solubilise the muscarinic receptor made use of high salt(Alberts and Bartfai, 1976; Carson et al., 1977) or the detergent digitonin(Beld and Ariens, 1974). More recently several other laboratories have characterised the receptor using this detergent; however, the binding characteristics of the soluble material varied between reports. In two reports, strong agonists no longer bound to multiple sites but instead to a single binding site of low affinity in one case(Hurko, 1978) and of high affinity in the other(Gorissen et al., 1978). The ability of digitonin to solubilise muscarinic receptors also appeared to vary between batches(Aronstam et al., 1978); although this was later explained by the varying concentrations of gitonin present in the digitonin preparations(Repke and Matthies, 1980). Lubrol PX and lysolecithin(Haga 1980a, b) were also recently shown to solubilise the receptor(after the present work was completed). A more useful solubilising agent would combine a reproducible and efficient solubilisation of membrane-bound receptors with retention of muscarinic binding characteristics.

This chapter is divided into two parts: Part I is concerned with the pharmacological characterisation of 0.1% sodium cholate-1M NaCl solubilised material; Part II studies the agonist binding characteristics of the soluble material.

Methods

Ox brains were obtained fresh from the slaughterhouse within 4 hours of death. The caudate nucleus was dissected out and a microsomal pellet(1500-2500 pmoles (^{3}H)-QNB binding sites/ g protein) was prepared as described in Chapter 2. Protein was assayed by the method of Lowry et al.(1951).

Prelabeling of Microsomal Pellets

(^{3}H)-PrBCM(20Ci/ mmole) was a gift from Dr. N.J.M. Birdsall(National Institute for Medical Research, London). Cyclisation of the mustard to its reactive aziridinium ion was as described by Burgen et al.(1974a): the stock solution was diluted approximately 100-fold in 10mM phosphate buffer, pH 7.4(to a final concentration of 2µM) and left to cyclilize at room temperature for 1 hour. Membranes were incubated in 2nM (^{3}H)-PrBCM at a final concentration of 200 µg/ml for 30 minutes at 30^{o}C. The reaction was stopped by the addition of 10mM Na thiosulphate. Free mustard was removed by washing and centrifuging the membranes twice at 187,000xg for 30 minutes(48,000rpm in a Beckman 50.2Ti rotor).

Some membrane preparations were incubated with (^{3}H)-QNB at a final concentration of 1nM for 60 minutes at 37^{o}C, membranes were then washed and centrifuged as above.

Addition of an excess of atropine to the above preparations allowed for determination of non-specific binding. Both pre-labeled membrane preparations were stable for months at -20^{o}C,with less than 10% of the total original

bound cpms dissociating after storage for 3 months.

Solubilisation of Prelabeled Material

Prelabeled membranes were diluted to a final protein concentration of 1-2mg / ml and incubated with the appropriate concentration of detergent for one hour at 4°C. The membranes were then centrifuged for one hour at 100,000xg and the supernatent counted for activity which was taken to represent the amount of receptor that had been solubilised.

Solubilisation and Assay of Cholate-Salt Soluble Material

The final optimal solubilisation procedure and assay procedure are summarised in Table 1. Several other methods for separating free from bound radioactive ligand(PEG, charcoal, gel filtration) were tried without success.

Microsomal pellets(2mg protein/ml) were homogenised in 0.1% Na cholate, 1M NaCl, 1mM EGTA, 25mM Na-phosphate buffer(pH7.4) for 10 strokes and shaken at room temperature(or 4°C) for one hour. The mixture was diluted 1:1 in phosphate-EGTA buffer(final concentration now 0.05% cholate-0.5M NaCl) and centrifuged for 1 hour at 187,000xg in a Beckman 50.2Ti rotor at 4°C. The resulting supernatant was stored at 4°C(stable up to one week) and is referred to as soluble material. The remaining pellet could be reextracted yielding a preparation of increased specific activity(3-5 fold).

Binding studies on soluble material(150-300 µg

TABLE 1

SOLUBILISATION PROCEDURE

-P3(1:10) 2 mg protein/ml

-0.1% sodium cholate-1M NaCl

-1 hour at RT(or $4^{o}C$) shaking

-dilute 1:2 in phosphate-EGTA buffer

-Centrifuge at 187,000xg for 60 minutes

ASSAY

-Incubation with (^{3}H)-QNB for 60 min at $37^{o}C$ as previously described.

-1 ml soluble material + 1 ml saturated $(NH_4)_2SO_4$,pH7.0

-2 minutes on ice

-Filter GF/B

-Wash with 50% $(NH_4)_2SO_4$

protein/ml) were performed by a protein precipitation method using saturated ammonium sulphate(pH 7) essentially as described by Gavish et al.(1979): after incubation with the appropriate ligand as described in Chapter 2(60 minutes ± a preincubation of 15 minutes with unlabeled ligand), 1 ml of soluble material was precipitated with 1 ml of saturated ammonium sulphate(pH7) and left on ice for 2 minutes. The mixture was then filtered through Whaman GF/B glass fibre filters. The filters were dried and radioactivity determined by scintillation counting using Packard Toluene Scintillator and counted in a Packard Tri-Carb 2660 Scintillation Counter at an efficiency of 40%.

(±)-(^{3}H)-QNB(32 Ci/mmole) was obtained from the Radiochemical Centre(Amersham, U.K.). Benxhexol, propylbenzilylcholine and benzilylcholine were gifts from Dr. J.M. Young(Department of Pharmacology, Cambridge). All other ligands and supplies were from Sigma.

Analysis of Binding Data

The analysis of the binding data from the kinetic studies was based on the work of Maelicke et al. (1977) as thoroughly described by Wastek and Yamamura(1978). The analysis was based on the assumption of a simple bimolecular interaction between R(receptor) and L(ligand):

$$R + L \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} RL$$

Determination of $t_{1/2}$ dissociation:

An excess of atropine is added at saturation(t=0) and

binding is calculated at different times t. A semi-log plot of total specific binding at time t divided by total binding at saturation vs time(t) yields at 50% the value for $t_{1/2}$ dissociation. Specific binding is calculated(total binding minus non-specific binding) since this may increase more slowly than the nearly instantaneous non-specific binding.

$$\log \frac{B_t}{B_{max}} \quad \text{vs.} \quad t$$

Determination of $t_{1/2}$ association:

(^{3}H)-QNB is added at t=0 and binding is calculated at subsequent times t. A semi-log plot of the following equation(B always refers to specific binding) gives at 50% $t_{1/2}$ association.

$$\log \frac{B_{max}-B_t}{B_{max}} \quad \text{vs.} \quad t$$

Determination of Rate Constant k_{-1} dissociation:

Taken from Cuatrecasas and Hollenberg(1976):

$$k_{-1} = \frac{0.693}{t_{1/2}(\text{dissociation})} \quad \text{in min}^{-1}$$

This is derived from the first order rate equation which when integrated yields:

$$k_{-1} = (1/t)2.303\log(x/x-a)$$

where x = amount of RL complex at t = 0(before atropine is added)

x-a = amount of complex remaining at time t

when half the concentration has dissociated the above equation reduced to:

$$k_{-1} = \frac{2.303 \log 2}{t_{1/2}} = \frac{0.693}{t_{1/2}}$$

Determination of rate constant for association(k_1):

Rate of R-L complex formation

$$R + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RL$$

is: (1) $v=dx/dt$ where v is the reaction rate

x is concentration of RL complex at time t

(2) $v=k_1RL - k_{-1}(RL)$

at equilibrium $v=dx/dt=0$ and

$$k_1RL = k_{-1}(RL)$$

$$k_{-1} = \frac{k_1R_{eq}L_{eq}}{(RL)_{eq}}$$

where R_{eq}, L_{eq}, $(RL)_{eq}$ are the concentrations of complexes at equilibrium

Substitution into (2) yields:

$$v = k_1RL - k_{-1}(RL)$$

$$v = k_1RL - k_1\frac{R_{eq}L_{eq}}{(RL)_{eq}}(RL)$$

$$(3) \quad = k_1\left(RL - (RL)\frac{R_{eq}L_{eq}}{(RL)_{eq}}\right)$$

The concentration of free receptor R and free ligand L at time t:

$$R = R_o-(RL)_t \qquad L = L_o-(RL)_t$$

R_o = initial receptor concentration

$(RL)_t$ = concentration of receptor-ligand complex at time t

L_o = initial ligand concentration

Substitution into equation (3) followed by integration:

$$\ln \frac{(RL)_{eq}\left[L_T - (RL)_t\left(\frac{(RL)_{eq}}{R_T}\right)\right]}{L_T\left[(RL)_{eq} - (RL)_t\right]} = B$$

$$B = k_1 t\left[L_T \frac{R_T}{(RL)_{eq}} - (RL)_{eq}\right]$$

$(RL)_{eq}$ = (RL) complex at equilibrium

$(RL)_t$ = (RL) complex at time t

R_T = total bound receptor at saturation

L_T = total ligand added

Plot (B) vs time t; if ln B= $k_1 t$ (multiplied by some number n) then the slope m = k_1 n

$k_1 = M^{-1}min^{-1}$

$K_d = k_{-1}/k_1$ and for a simple bimolecular reaction, the ratio $k_{-1}/k_1 = K_d$

Scatchard Plot:

B/L vs B where L = total ligand - bound ligand

B = bound receptor in pmoles/g protein

1/slope(m) = pM value for K_d

B_{max} = x intercept

Hill Plot:

$$\log(B_i / B_T - B_i) \text{ or } \log\left(\frac{\%\text{inhibited}}{\%\ \text{control}}\right)$$

vs the ligand concentration $\log[M]$

Inhibition Constant:

$$K_i = ID_{50} / 1 + C/K_d$$

C is the concentration of radioligand used and K_d is the equilibrium constant

where $K_d \neq k_{-1}/k_1$ only the ID_{50} cor can be calculated.

Results

1) Screening of Detergents

The receptor was known to be difficult to solubilise reproducibly. Therefore, a total of 25 detergents were screened for possible use as solubilising agents. The results are shown in Table 2. The results represent the percentage of prelabeled material solubilised and are thus an indication of the detergent's ability to solubilise receptors but not necessarily in an active form. In general,the detergents which were good solubilisers also inhibited binding. Preliminary experiments using the technique of equilibrium dialysis established that a combination of 0.1% Na cholate(but not 0.1% deoxycholate)

TABLE 2

TABLE 2

Screening of Detergents

Equal amounts of (^{3}H)-PrBCM-labeled membranes were incubated for 1 hour at 4^{o}C with the appropriate amount of detergent. Solutions were then centrifuged at 100,000xg for 1 hour. Resulting cpms in supernatant were taken as % of initially added counts solubilised.

Concentrations of detergents were 1% unless otherwise stated in 25mM Na-Phosphate buffer, pH 7.4.

DETERGE

Triton

Octylgl
Digiton
Cholate
Triton

Triton
Tween

Tween

Brij 3
Cholat

Trito

Trito

Trit
Brij

Lubr
SDS

DETERGENT	% SOLUBILISED
Triton N-57 X-15 X-35	20
Octylglucoside Digitonin Cholate 1% Triton X-45 X-305	30
Triton X-405 Tween 20 40 85	40
Tween 60 80 Brij 35 Cholate 2%	50
Triton X-165 X-155	60
Triton X-100 X-102 X-114	70
Triton N-101 Brij 56 96 Lubrol SDS	80

and 1M NaCl would solubilise specific (^{3}H)-QNB binding sites from bovine brain(Carson, 1979). A more rapid and efficient filtration assay was later developed as described in Methods. Cholate-salt routinely solubilised between 10-30% of total receptor sites, reextraction of the pellet remaining after the first solubilisation resulted in a 3-5 fold increase in specific activity of the soluble material and solubilised a further 2-4% of total membrane binding sites. 0.1% Na cholate or 1M NaCl alone solubilised less than 1% of total specific activity. The soluble material was relatively stable at 4^{o}C and lost approximately 30% initial binding activity per week; however, all activity was lost after 5 minutes at 100^{o}C. Efforts to reduce the salt concentration below 0.5M NaCl resulted in a loss of binding sites, therefore all present studies were carried out at a final cholate-salt concentration of 0.05% and 0.5M respectively.

2) Saturation Curve of Soluble Material

Figure 5 shows a typical saturation curve for the binding of the antagonist (^{3}H)-QNB to a soluble preparation from bovine caudate nucleus. Non-specific binding(binding in the presence of 5μM atropine) was 10-20%. Scatchard plot analysis of the data(inset) yields a B_{max} of 800 pmoles/ g protein and an apparent dissociation constant(K_d) of 200pM. The total number of binding sites varied from 150-900 pmoles/ g protein and K_d values varied from 150-300pM for soluble preparations.

3) Kinetic Studies on Soluble Material

FIGURE 5

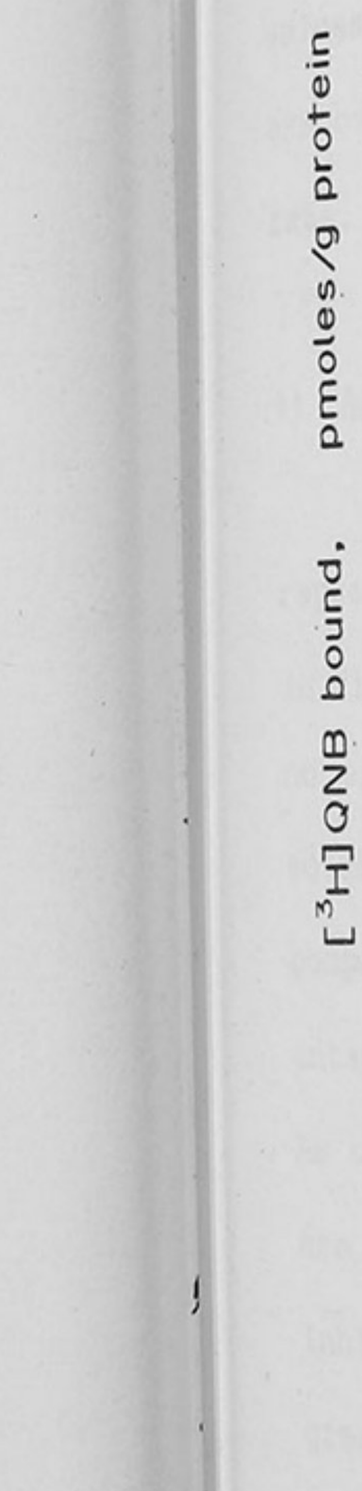
FIGURE 5

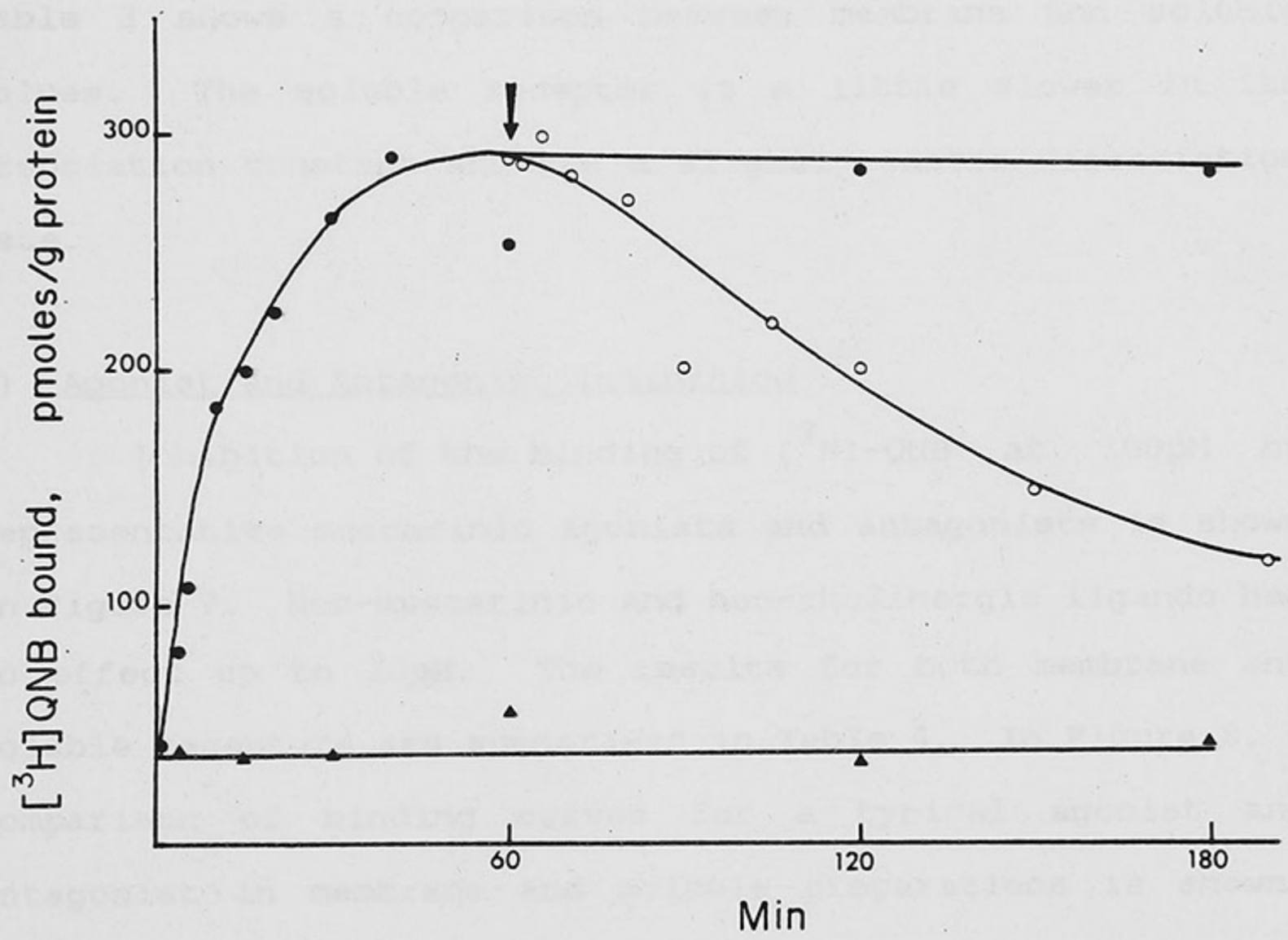
Specific and Non-specific Binding of (^{3}H)-QNB to a Soluble Preparation from Bovine Caudate Nucleus.

Binding was performed for 1 hour at 37^{o}C and bound ligand was determined by an ammonium sulphate precipitation assay.

Upper figure: Binding of (^{3}H)-QNB in the absence (● - ●) and presence(▲ - ▲) of 5 µM atropine.
Lower figure: Subtraction of the two curves gives specific binding.

NB: FIGURE is FIGURE 6




[3H]QNB bound, pmoles/g protein
300
200
100
60
120
180
Min

The association and dissociation of specific (^{3}H)-QNB binding is shown in Figure 6. The average half times of association and dissociation were respectively 10±2 minutes and 70±9 minutes(n=4). Analysis of the kinetic data gave rate constants of $1.85\pm0.6\times10^{8}M^{-1}min^{-1}$ for k_1 and $1.0\pm0.03\times10^{-2}min^{-1}$ for k_{-1}. Assuming a simple binding process the K_d calculated from the ratio k_{-1}/k_1 was 54pM as compared to 20pM for the membrane value(Carson, 1979). Table 3 shows a comparison between membrane and soluble values. The soluble receptor is a little slower in its association constant and has a slightly faster dissociation rate.

4) Agonist and Antagonist Inhibition

Inhibition of the binding of (^{3}H)-QNB at 100pM by representative muscarinic agonists and antagonists is shown in Figure 7. Non-muscarinic and non-cholinergic ligands had no effect up to 10μM. The results for both membrane and soluble receptors are summarised in Table 4. In Figure 8, a comparison of binding curves for a typical agonist and antagonist in membrane and soluble preparations is shown. As can be seen in Table 4, the membrane values in ox brain are comparable to those of other workers in the rat brain. Inhibition constants for soluble material were 10-20 fold greater than in the membrane state. Hill coefficients remained the same after solubilisation except for the agonists methylfurmethide and carbamylcholine where the Hill coefficient was increased to 1.0 from 0.65 and 0.55 respectively.

Discussion

FIGURE 6

FIGURE 6

Association and Dissociation of QNB Binding.

Assays were performed at 500pM (^{3}H)-QNB at 37^{o}C.

Total binding(● - ●); non-specific binding(▲ - ▲) in the presence of 5 μM atropine; total binding o-o after addition of 5 μM atropine at 60 minutes.

NB: FIGURE IS FIGURES

- ▲)
nding o-o

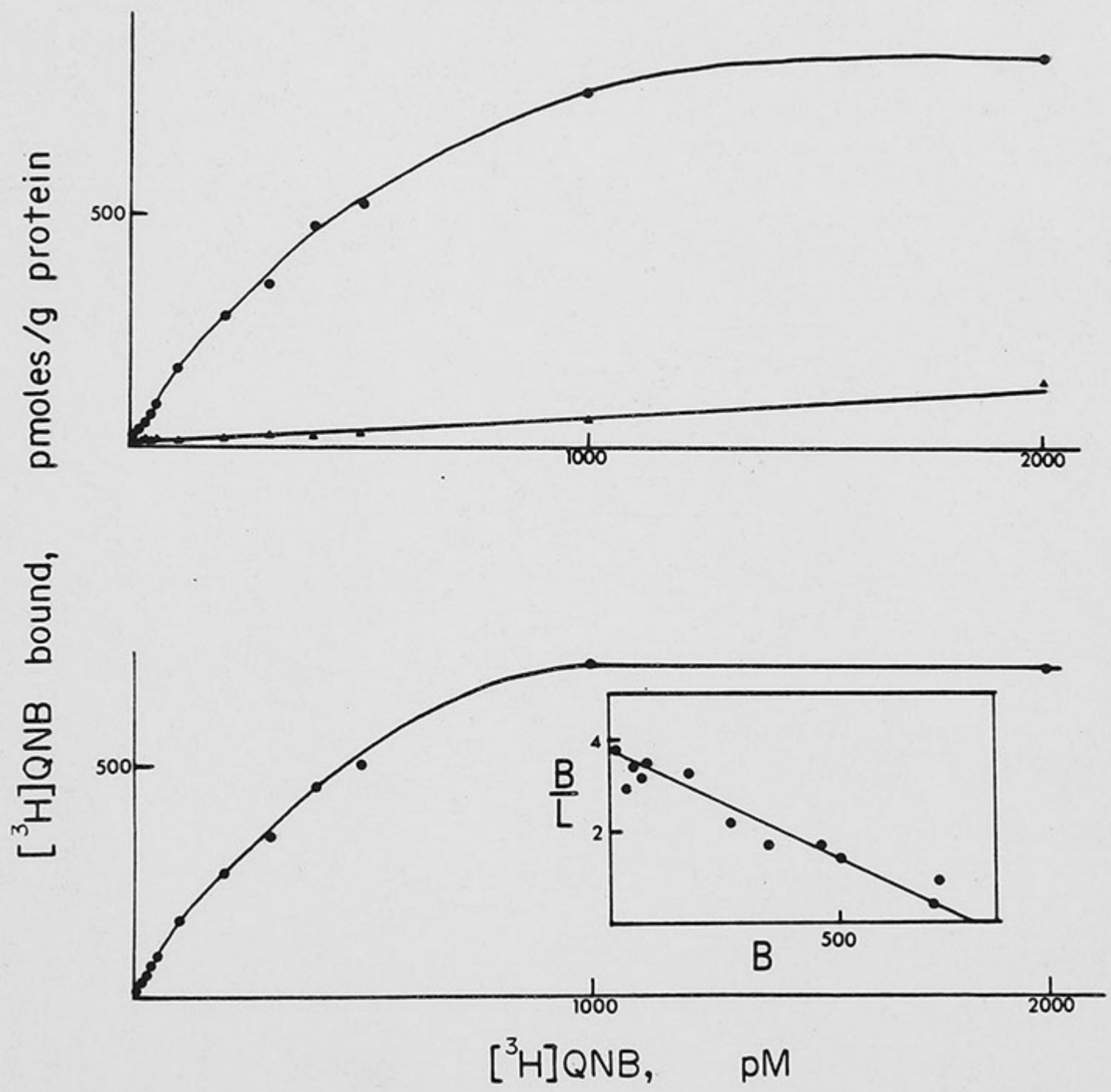

pmoles/g protein
500
1000
2000
[3H]QNB bound,
500
1000
2000
4
2
B
B
500
[3H]QNB, pM

TABLE 3

TABLE 3

Comparison of Kinetic Results: Membrane and Soluble Material

Membrane results were essentially determined as described in the text for soluble material but using bovine cortex(Carson, 1979).

K_d(app

k_{-1}/k_1

k_{-1}

k_1

$t_{1/2}$

$t_{1/2}$

	MEMBRANE	SOLUBLE
K_d(app)	15-30pM	150-300pM
k_{-1}/k_1	22 pM	54pM
k_{-1}	$6.0 \times 10^{-3} min^{-1}$	$1.0 \times 10^{-2} min^{-1}$
k_1	$2.8 \times 10^{8} M^{-1} min^{-1}$	$1.8 \times 10^{8} M^{-1} min^{-1}$
$t_{1/2}$ assoc.	6± 2 min	10± 2 min
$t_{1/2}$ dissoc.	100± 18 min	70± 9 min

FIGURE 7

FIGURE 7

Inhibition of (^{3}H)-QNB Binding to a Soluble Preparation by Muscarinic Agonists and Antagonists.

Curves were drawn to fit a simple mass action equation.

QNB(● - ●); atropine(○ - ○); benzilylcholine(▲ - ▲) chlorpromazine(△ - △); oxotremorine(◆ - ◆); methylfurmethide(◇ - ◇); carbachol(□ - □).

Soluble
and
action
)

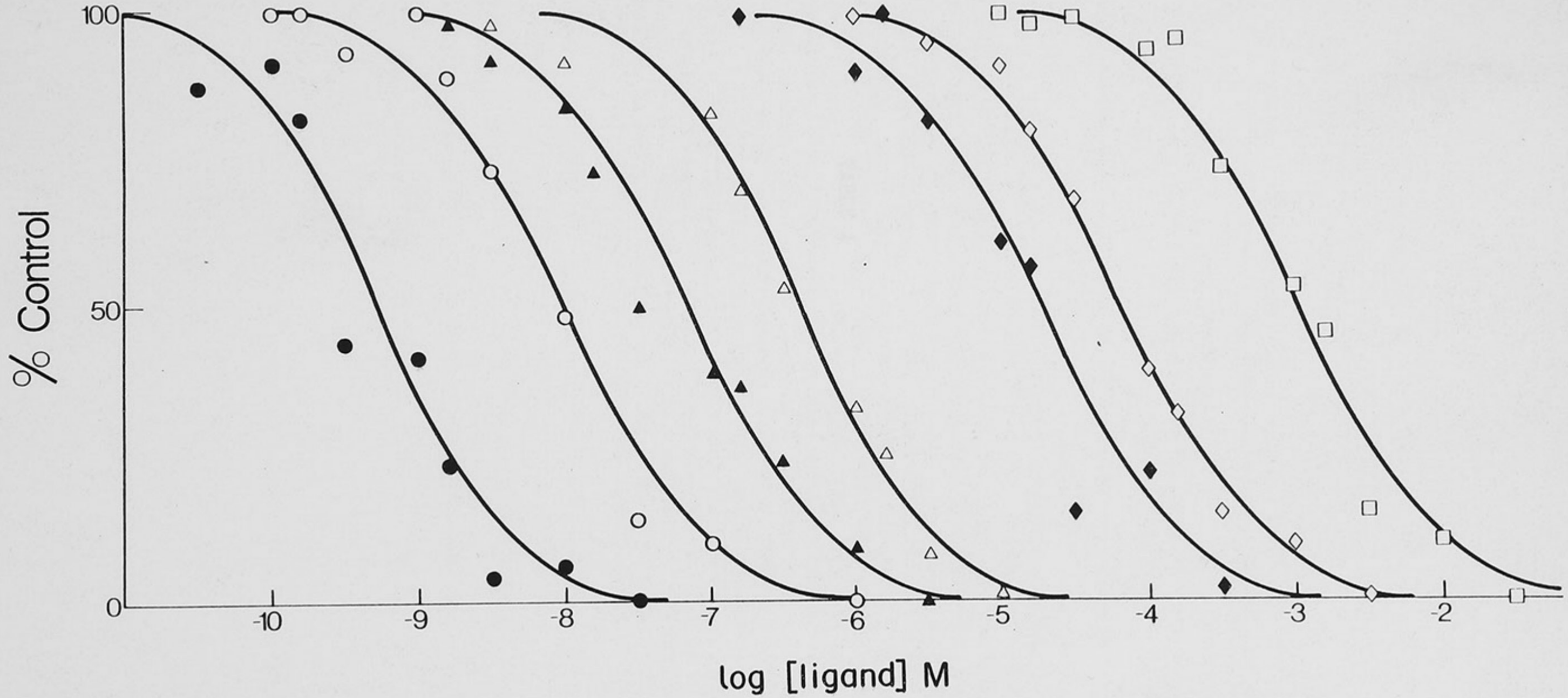


TABLE 4

TABLE 4

Inhibition Constants for Muscarinic Agonists and Antagonists in Membrane and Soluble Material

Inhibition constants were calculated from the equation:

$$K_i = \frac{ID_{50}}{1 + C/K_d}$$

where C is the concentration of the radioactive ligand(50 pM for membranes and 100 pM for soluble material) and K_d its equilibrium constant for (^{3}H)-QNB(20 pM and 200 pM for membrane and soluble material respectively). Due to the discrepency between the equilibrium constant(200 pM) and the ratio k_{-1}/k_1(54 pM) only the ID_{50} can be calculated from the above equation for the soluble material. Binding assays were performed as in Methods. Each value represents the average of at least 2 experiments agreeing within 10%. ID_{50} values were determined graphically. Non-muscarinic drugs(hexamethonium, decamethonium, d-tubocurare, nicotine and neostigmine) were without effect up to 10 µM. Membrane values already quoted in Chapter 2 were included for the sake of convenience.

TABLE 4

Inhibition Constants for Muscarinic Agonists and Antagonists in Membrane and Soluble Material

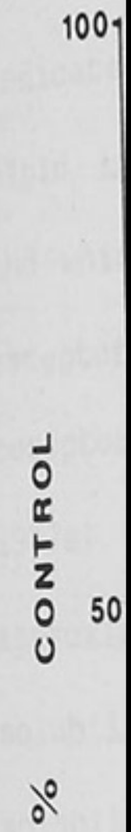
Ligand	Rat Brain Membrane			Ox Brain Soluble	
	K_i(M)	K_i(M)	n_H	ID_{50} cor	n_H
QNB	$2x10^{-10}$	$2.5x10^{-11}$	1.0	$4.6x10^{-10}$	1.0
atropine	$7x10^{-10}$	$3.6x10^{-10}$	0.95	$6.6x10^{-9}$	1.0
scopolamine	$4x10^{-10}$	$2.1x10^{-9}$	1.0	$6x10^{-9}$	0.9
benzhexol	$7.6x10^{-9}$	$1x10^{-9}$	1.0	$1.3x10^{-8}$	0.9
benzilylcholine	$1.8x10^{-9}$	$2.8x10^{-9}$	0.9	$4.6x10^{-8}$	0,85
propyl-benzilylcholine	$1.3x10^{-8}$	$2x10^{-8}$	0.9	$2x10^{-7}$	0,85
chlorpromazine	$5x10^{-8}$	$2.5x10^{-8}$	0.9	$4x10^{-7}$	0,85
oxotremorine	$3x10^{-7}$	$5x10^{-7}$	0.85	$8x10^{-6}$	0,95
pilocarpine	$3x10^{-6}$	$2.1x10^{-6}$	1.0	$1.3x10^{-5}$	0.9
methylfurmethide	$4x10^{-6}$	$8.5x10^{-6}$	0.65	$5.3x10^{-5}$	1.0
carbamylcholine	$1x10^{-5}$	$2x10^{-5}$	0.55	$4.6x10^{-4}$	1.0

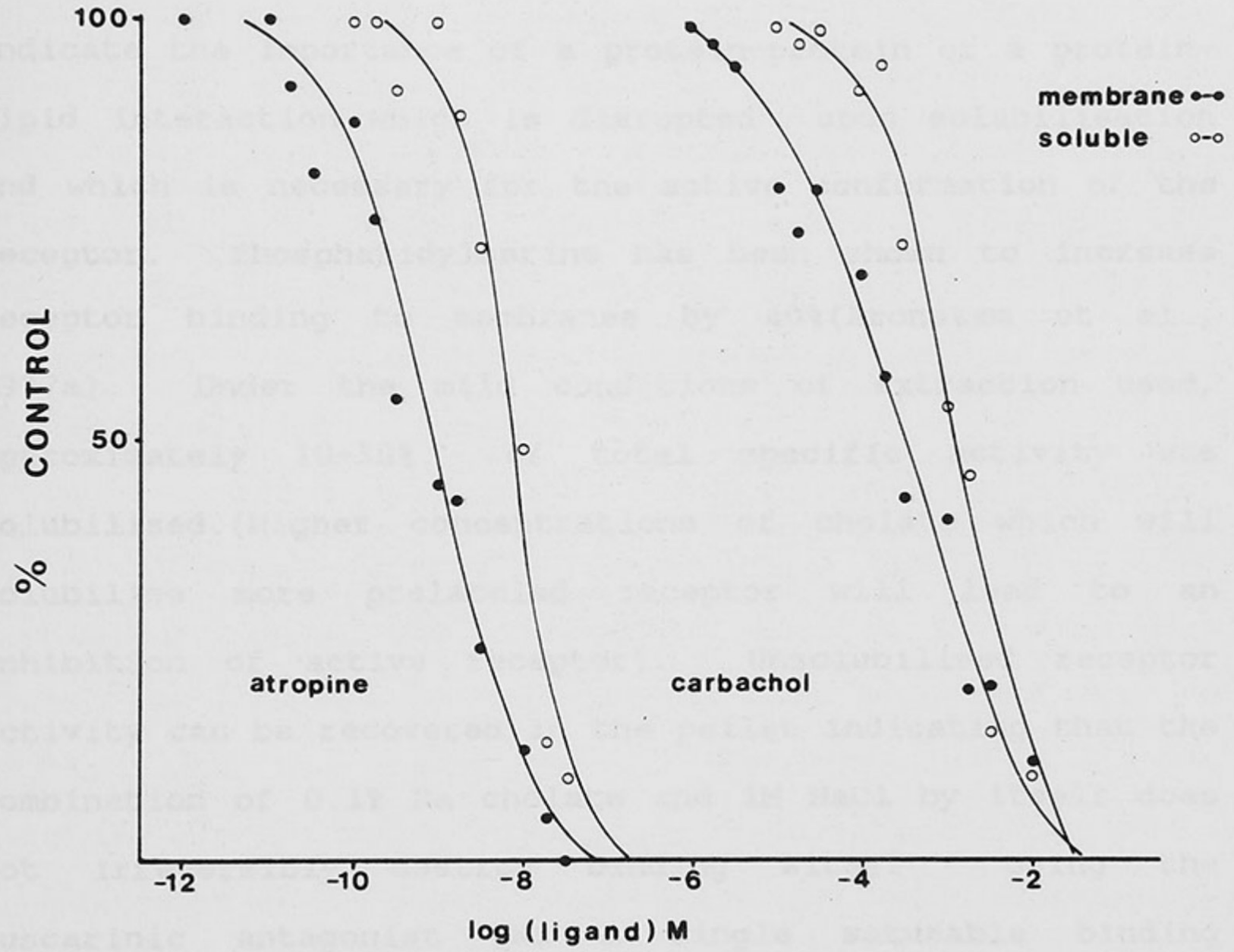
FIGURE 8

FIGURE 8

Comparison of Membrane and Soluble Inhibition Curves for a Typical Agonist(Carbachol) and Antagonist(Atropine).

Closed circles are membrane values taken from Chapter2,figure 3; open circles are soluble values taken from this chapter, figure 7. Note a parallel shift for the antagonist curves and a shift to the right combined with a loss of high affinity sites for carbachol binding to the soluble preparation.




100
50
% CONTROL
membrane
soluble
atropine
carbachol
-12
-10
-8
-6
-4
-2
log (ligand) M

This chapter has presented evidence that a combination of 0.1% Na cholate(2mM which is 6 times less the CMC(12mM) due to the presence of high counter ion concentration) and 1M NaCl will solubilise (^{3}H)-QNB binding sites from bovine caudate nucleus. The ability of a variety of detergents to solubilise prelabeled receptor(Table 1) but to inhibit active binding to the receptor appears to indicate the importance of a protein-protein or a protein-lipid interaction which is disrupted upon solubilisation and which is necessary for the active conformation of the receptor. Phosphatidylserine has been shown to increase receptor binding to membranes by 40%(Aronstam et al., 1977a). Under the mild conditions of extraction used, approximately 10-30% of total specific activity was solubilised.(Higher concentrations of cholate which will solubilise more prelabeled receptor will lead to an inhibition of active receptor). Unsolubilised receptor activity can be recovered in the pellet indicating that the combination of 0.1% Na cholate and 1M NaCl by itself does not irreversibly destroy binding sites. Using the muscarinic antagonist QNB a single saturable binding component was found with an equilibrium constant of 200pM, approximately 10-fold higher than the membrane receptor and 4-fold higher than the ratio k_{-1}/k_1 determined kinetically in the soluble material. This latter difference may indicate that binding of QNB to the solubilised receptor is not a simple second-order process. Thus, the possibility of isomerization of the R-L complex as described by Jarv et al.(1979) and in the work of Kloog and Sokolovsky(1977;

1978a,b) cannot be ruled out. Inhibition constants for a variety of muscarinic agonists and antagonists were 10 to 20-fold higher in the soluble material than in the membrane state and non-muscarinic ligands were without effect. The decrease in affinity of the soluble receptor for muscarinic ligands is probably a result of the high salt concentration present in the preparation as indicated by the work of Birdsall et al.(1979b). The Hill coefficients for the soluble material were all close to unity indicating the presence of a single binding site, as also described by Hurko using digitonin(1978). Whether the multiple binding sites of the potent agonists methylfurmethide and carbamylcholine were transformed to the low affinity state or whether the low affinity site was selectively solubilised cannot be decided at present.

It was of interest to more closely study the agonist binding of the soluble material in order to determine what indeed had happened to the high affinity sites and also to confirm that the change in affinity seen in the soluble material was due to the presence of high salt and not due to a conformational change undergone by the protein in solution. Both these aspects are studied in Part II of this chapter.

Part II: Agonist Binding to Cholate-Salt Soluble Material

Results and Discussion

1) Effects of Cholate and NaCl on Carbachol Binding to Membranes

Figure 9 shows the effect of carbachol on (^{3}H)-QNB binding to microsomal membranes in cholate or high salt alone. As can be seen, 0.5M NaCl decreases affinity by approximately 10-fold and interestingly 0.1% Na cholate increases slightly the affinity of the receptor for carbachol by about 4-fold. This could perhaps be due to the interaction of cholate with lipids in the membrane. Cholate in very low concentrations acts specifically(nM range) on the nicotinic receptor of electric organ(Changeux, 1975) but no specific interaction(increase or decrease in binding affinity) was seen with muscarinic receptors(data not shown). Therefore, the decrease in affinity for agonists and antagonists is mainly due to the presence of high salt , which perhaps interacts with the lipid environment of the membrane in much the same way as it reduces the CMC of Na cholate by reducing electrostatic repulsion between polar phospholipids or by directly interacting with the receptor protein. Analysis of Hill plots of the inhibition curves showed little change in the slope and therefore no selective change in agonist binding sites is due to the addition of NaCl itself.

It was known from solubilisation experiments that the combination of 0.05% Na cholate-0.5M NaCl would not

FIGURE 9

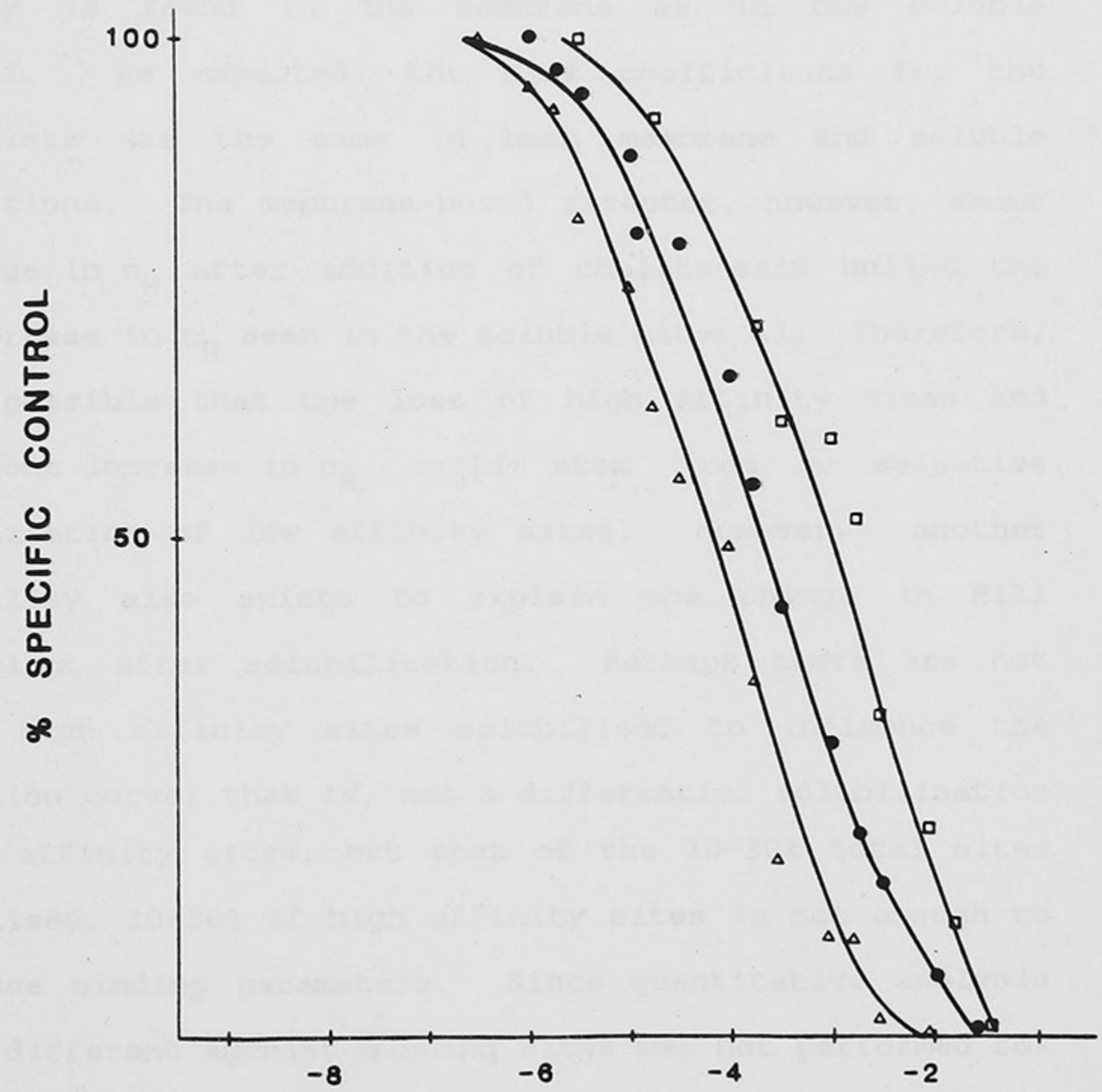
FIGURE 9

Inhibition of (^{3}H)-QNB Binding to a Membrane Preparation by Carbachol.

Bovine caudate nucleus microsomal membranes were diluted in 0.1% Na cholate-25mM phosphate buffer (△ - △); 0.5M NaCl-25mM phosphate buffer(□ - □); or
25mM phosphate buffer alone(● - ●) to a final protein concentration of approximately 30 µg/ml. Binding was performed as in Methods. The concentration of (^{3}H)-QNB was 100pM.

The data for Hill plots is not shown but the Hill coefficient was approximately the same under all conditions:
Phosphate alone n_H=0.55;
0.5M NaCl-phosphate n_H=0.6;
0.1% Na cholate n_H= 0.65.

ID_{50} were as follows: Phosphate = 3×10^{-4}M
0.1% cholate= 8×10^{-5}M
0.5M NaCl = 1×10^{-3}M


100
50
% SPECIFIC CONTROL
-8
-6
-4
-2
log (carbachol) M

solubilise muscarinic receptors. Therefore, addition of 0.05% Na cholate and 0.5M NaCl would affect binding by virture of its interaction with the receptor or its environment and not by any selective solubilising effect. Table 5 shows the effects of cholate-salt addition to microsomal membranes and its effects on agonist and antagonist binding. As can be seen, the same decrease in affinity is found in the membrane as in the soluble material. As expected, the Hill coefficients for the antagonists are the same in both membrane and soluble preparations. The membrane-bound receptor, however, shows no change in n_H after addition of cholate-salt unlike the the increase in n_H seen in the soluble material. Therefore, it is possible that the loss of high affinity sites and subsequent increase in n_H could stem from a selective solubilisation of low affinity sites. However, another possibility also exists to explain the change in Hill coefficient after solubilisation. Perhaps there are not enough high affinity sites solubilised to influence the inhibition curve; that is, not a differential solubilisation of low affinity sites, but that of the 10-30% total sites solubilised, 10-30% of high affinity sites is not enough to influence binding parameters. Since quantitative analysis of the different agonist binding sites was not performed for the bovine brain, a comparison with the extensively studied rat brain was necessary.

2) Rat and Bovine Brain Carbachol Inhibition Curves

A comparison between rat and bovine brain carbachol inhibition curves is shown in Figure 10. Rat forebrain receptors show a 3-fold higher affinity for carbachol than

TABLE 5

TABLE 5

Binding Data on Membrane and Soluble Muscarinic Receptors in 0.05% Na Cholate, 0.5M NaCl, 25mM Phosphate Buffer

Inhibition constants ($ID_{50\ cor}$) were determined as described in Methods. The table represents a summary of inhibition curves performed in duplicate(not shown). The data for soluble material is taken from Table 4. Microsomal membranes were diluted in 0.05%Na cholate, 0.5M NaCl, 25mM Phosphate buffer. Binding was performed at 100pM(^{3}H)-QNB as described in methods. Membranes were precipitated with ammonium sulphate in order to use exactly the same condition as soluble material and to preclude any possibility of solubilised membranes passing through the filter. However, if the membranes were filtered as previously described for membrane bound receptor, the results remained the same.

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passing
branes
embrane

TABLE 5

Binding Data on Membrane and Soluble Muscarinic Receptors in 0.05% Na Cholate, 0.5M NaCl, 25mM Phosphate Buffer

Ligand	Membrane		Soluble	
	K_d = 170pM		K_d = 200pM	
atropine	4.6×10^{-9}M	n_H=1.0	6.6×10^{-9}M	n_H=1.0
carbachol	4×10^{-4}M	n_H=0.55	4.6×10^{-4}M	n_H=1.0

is found in bovine caudate nucleus($1.5x10^{-5}M$ vs $5x10^{-5}M$). In both instances the Hill coefficient was 0.55 indicating the presence of more than one site. Birdsall et al.(1980c) found an even higher affinity of the receptor for carbachol with almost 35% of total sites in the super-high and high affinity states.Super-high,high and low agonist affinities determined in the rat striatum were $1.3x10^{-8}M$(5%), $4.5x10^{-6}M$(30%) and $1.2x10^{-4}M$(65%) respectively. (In parenthesis is shown the percentage of total sites). Figure 10 shows that for bovine caudate nucleus no inhibition of binding is seen until 1μM and an estimate can be made that there is perhaps only 1/3 the total number of sites in the high affinity stat(or 10%) in bovine brain as there is in rat. Therefore if only 10% of total sites were solubilised this would imply that only 10% of high affinity sites, or only 1% , were present in the soluble material. This may not be enough to influence the binding parameters or shape of the binding curve. If this were the case, then with an increasing percentage of total sites solubilised one should see a progressive increase in the number of high affinity agonist sites as indicated by a decrease in the Hill coefficient. This can be tested by studying the binding parameters of soluble material with different specific activities.

3) Agonist Binding to Different Soluble Preparations

Different specific activity preparations were obtained by varying initial solubilising conditions(see Chapter 4). A comparison of different soluble preparations appeared to show a decrease in n_H with increasing percentage of total sites solubilised. This is clearly seen in Figure

FIGURE 10

FIGURE 10

Rat and Bovine Carbachol Inhibition Curves

Microsomal membranes from rat forebrain or bovine caudate nucleus were prepared as already described.
Binding was performed at 100pM (^{3}H)-QNB and inhibition constants were determined as described in Methods.

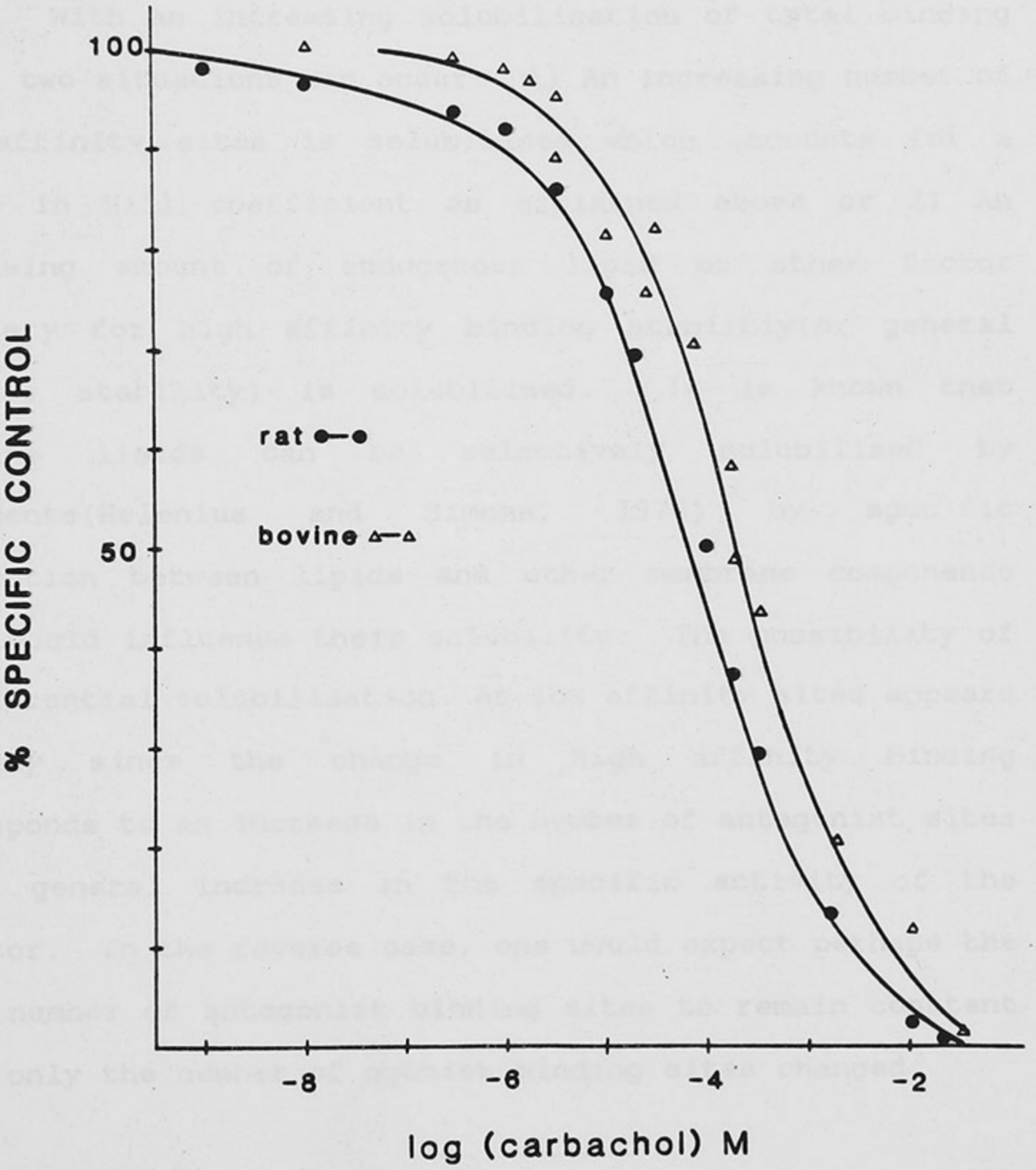
Rat(● - ●): $K_i = 1.5 \times 10^{-5}$M; $n_H = 0.55$

Bovine(△ - △): $K_i = 5 \times 10^{-5}$M; $n_H = 0.55$

% SPECIFIC CONTROL

bovine
already

and
scribed



11A-B. Figure 11A shows a carbachol inhibition curve for soluble preparations containing 25%, 10% and 5% of total membrane binding sites. As can be seen in Figure 11B and Table 6 there is a progressive decrease in the n_H parameter with increasing amount of total binding sites solubilised, although the inhibition constant remains the same.

With an increasing solubilisation of total binding sites, two situations can occur: 1) An increasing number of high affinity sites is solubilised which accounts for a change in Hill coefficient as explained above or 2) An increasing amount of endogenous lipid or other factor necessary for high affinity binding stabiltiy(or general receptor stability) is solubilised. It is known that membrane lipids can be selectively solubilised by detergents(Helenius and Simons, 1975) by specific interaction between lipids and other membrane components which would influence their solubility. The possibility of a differential solubilisation of low affinity sites appears unlikely since the change in high affinity binding corresponds to an increase in the number of antagonist sites and a general increase in the specific activity of the receptor. In the reverse case, one would expect perhaps the total number of antagonist binding sites to remain constant while only the number of agonist binding sites changed.

Conclusion

It has been shown that the decrease in agonist and antagonist affinity is indeed due to the presence of high salt as indicated by Birdsall et al.(1979b). It has also been shown that the presence of cholate-salt does not

FIGURE 11A

FIGURE 11A

Carbachol Inhibition Curves for Different Soluble Preparations.

Binding$_3$ was performed as described in Methods at 500pM (^{3}H)-QNB.

11A: Inhibition curves for the following preparations:

(● - ●):25% total membrane binding sites solubilised(413 pmoles/g protein).

(△ - △): 10% total membrane binding sites solubilised(150 pmoles/ g protein).

(□ - □): 5% total membrane binding sites solubilised(80 pmoles/g protein).

% SPECIFIC CONTROL

Soluble

ods at

llowing

embrane

embrane

embrane

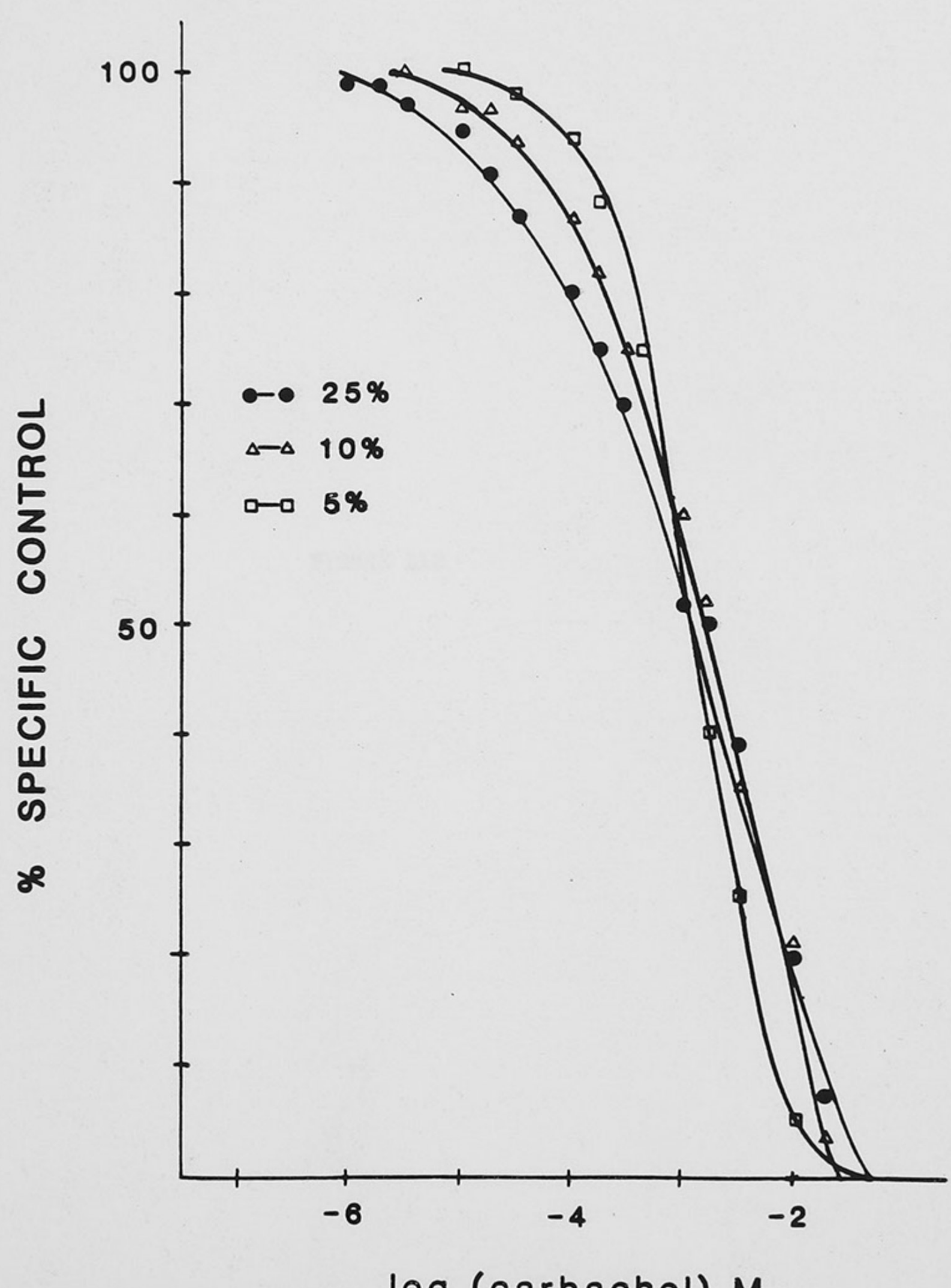


FIGURE 11B

FIGURE 11B

Hill Plots for the Inhibition Curves in 11A.

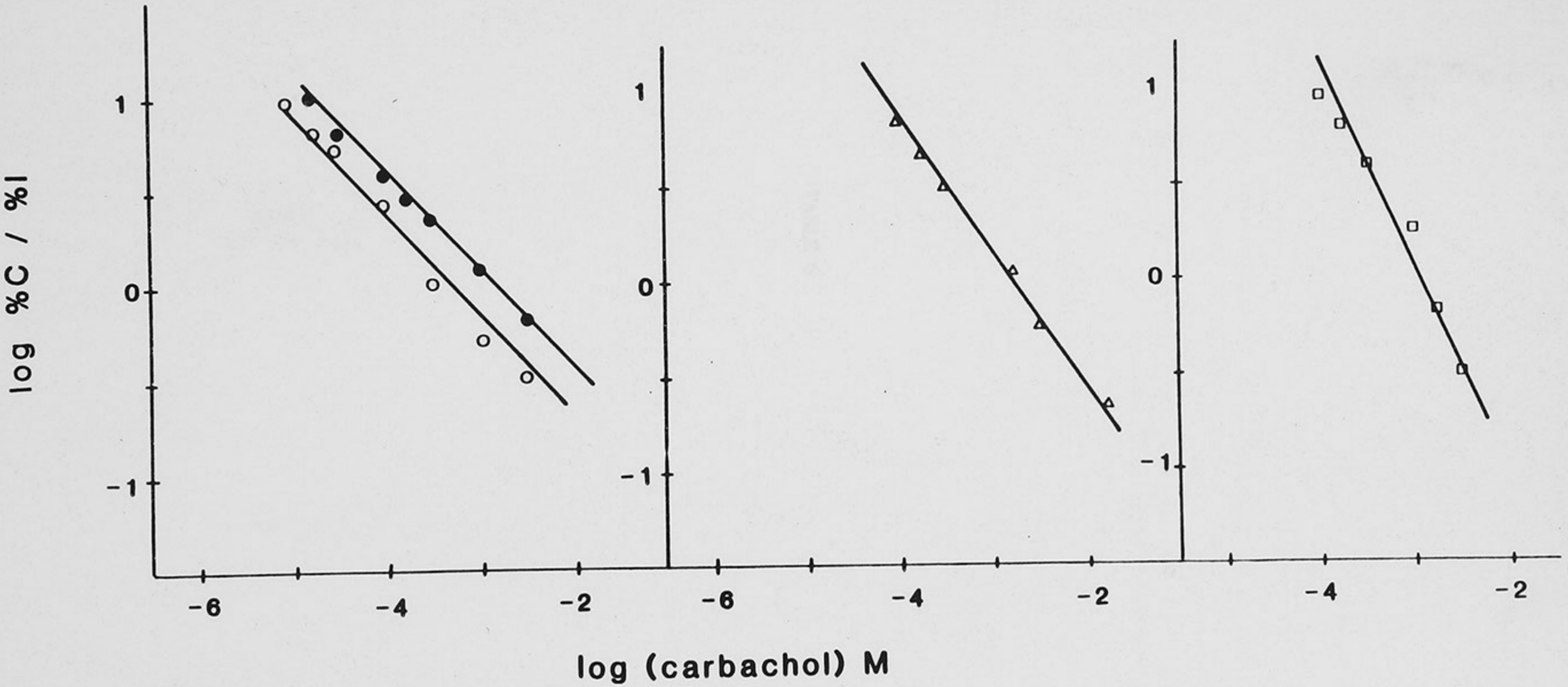
Data treated as in Methods. Microsomal preparation included for comparison.

(○ - ○): n_H= 0.55

(● - ●): n_H= 0.55

(△ - △): n_H= 0.75

(□ - □): n_H= 1.0



icrosomal

TABLE 6

TABLE 6

Summary of Carbachol Inhibition Constants and Hill Coefficients from Different Soluble Preparations

Hill coefficients were determined as in Methods. K_i (for membranes in phosphate buffer only) and ID_{50} cor for all other preparations were determined as already described.

TABLE 6

Summary of Carbachol Inhibition Constants and Hill Coefficients from Different Soluble Preparations

Preparation	K_i(M)	n_H	% Total Sites
Microsomal Pellet	$5x10^{-5}$	0.55	-
Microsomal Pellet (diluted in 0.05% Na Cholate-0.5M NaCl)	$4x10^{-4}$	0.55	-
Soluble(Specific Activity) pmoles/g protein			
80	$5.0x10^{-4}$	1.0	5
100	$4.6x10^{-4}$	1.0	6
150	$5.6x10^{-4}$	0.75	10
413	$5.6x10^{-4}$	0.55	25
430	$2x10^{-4}$	0.55	25

influence the n_H parameter in membrane preparations. Instead, there appears to be a critical percentage of total membrane sites which must be present in order to influence the shape of the binding curve (which is the only method used here for measuring high affinity sites). If we assume that a change in n_H refers to the number of high affinity sites present then from a membrane estimate of 10% total sites in the high affinity state, at least 2% are necessary for a Hill coefficient of 0.55. This line of reasoning may perhaps be too simplistic since other membrane components in addition to receptor sites are also solubilised which may in themselves be necessary for high affinity binding or general receptor stability.

An interesting point that this raises is why is there such a variation in the specific activity of the soluble preparations. Are there slight variations in procedure which give rise to more or less stable preparations? This question will be discussed in Chapter 4.

CHAPTER 4

CHARACTERISATION OF CHOLATE-SALT SOLUBILISED MUSCARINIC RECEPTORS

Introduction

It has been shown(Chapter 3) that a combination of 0.1% Na cholate and 1M NaCl is able to solubilise specific (^{3}H)-QNB binding sites from bovine caudate nucleus. The soluble material obtained was quite stable (up to one week) and the decrease in affinity for agonists and antagonists resulted from the presence of high salt in the preparation rather than any conformational changes which might have taken place during solubilisation, per se. In addition, the Hill coefficient was shown to depend upon the percentage of total muscarinic sites solubilised, in that the higher the percent total solubilised the closer the Hill coefficient was to the membrane state. This could be due to the greater percent of sites solubilised necessary to influence the binding parameters or it could perhaps be due to endogenous lipids or other factors necessary for stability or implicated in agonist binding.

The relation between lipid and detergent concentration and protein stability is well known. When the detergent to lipid ratio is small, the detergent can be

directly incorporated into the membrane without disrupting; however, with increasing concentrations of detergent the membrane will be disrupted and lead to: 1)protein-lipid-detergent complexes containing a single protein or many protein molecules; 2) lipid-free protein-detergent complexes and 3) lipid-detergent vesicles without any protein. Le Maire et al.(1976a) showed that active Ca^{2+}-ATPase complexes contained 0.2-0.3 umoles phospholipid/mg ATPase whereas inactive complexes were completely delipidated. Moreover, they found that this lipid was specifically bound to the protein and not merely an accessory factor in the retention of activity.

The fact that such a mild detergent as cholate and in such low concentrations (the presence of high salt reduces the critical micellar concentration from 14mM to 2mM), which would favour retention of protein-lipid interactions, solubilises the receptor in an active stable manner appears to INDICATE the importance of lipids to the activity of the receptor. Helenius and Simons(1972) suggested that bile salts(deoxycholate) and Triton X-100 bound hydrophobically by displacing endogenous lipids of the protein or lipoprotein. Lipid removal by the detergent would thus either lead to a conformational change of the protein or allow the detergent to directly interact with the protein. Le Maire et al.(1976b) showed that inactivation of Ca^{2+}-ATPase from sarcoplasmic reticulum was accompanied by delipidation and conformational changes of the protein. Further, Hebdon et al.(1981) showed that specific phospholipids (L-α-phosphatidyl choline) were necessary for the activity of adenylate cyclase solubilised from rat brain. The sensitivity of the muscarinic receptor to Triton

X-100 or to high concentrations of cholate(1%) which solubilise prelabeled receptor protein appears to indicate that lipid is important to receptor activity. Thus, the presence of 1M NaCl which lowers the critical micelle concentration allows less cholate to be used which conceivably displaces less of the endogenous lipid.

It therefore appeared of interest to study receptor stability under different conditions of solubilisation and to compare the different profiles of the material on gel filtration columns in order to see whether an aggregated or a monodisperse form of the receptor was more stable.

Methods

Soluble material and prelabeled (^{3}H)-QNB microsomal membranes were prepared as described in detail in Chapter 3. Binding was sometimes performed using (^{3}H)-N-methylscopolamine(gift from Dr. N.J.M. Birdsall, National Institute for Medical Research, London) at saturating concentrations: 1nM±10μM atropine, 30 minutes at 4°C.

Gel Filtration

Biogel 0.5m(agarose gel, exclusion weight 500,000) columns(1m x 1.5cm) were equilibrated in the same buffer as the soluble material to be run(which varied in cholate concentration). Flow rate was 4 mls per hour and 1 ml fractions were collected at 4°C. Columns were calibrated as described by Bon et al.(1973); the K_d being determined by the equation:

$$K_d = \frac{V_e - V_o}{V_f - V_o}$$

where V_e is the elution volume of the protein in question

V_o is the exclusion volume determined by S. aureus

V_f is the void volume determined by potassium ferricyanide

Plot of $(-\log K_d)^{1/2}$ vs. R_e(nm)(Stokes Radius) for a number of standard markers gives a linear plot from which the Stokes Radius for an unknown protein can be determined. The following enzymes were used as standards(from Bon et al., 1973):

Enzyme	R_e(nm)	S
B-galactosiadase(Z)		
(E. Coli)	8.2*	16
Ferritin(Horse Spleen)	7.9	65
Catalase(bovine liver)	5.2	11.3
Alcohol dehydrogenase		
(yeast)	4.6	7.4

The Stokes radius is calculated from the formula:

$$R_e = \frac{kT}{6n_o D^o}$$

where k is the Boltzman Constant
T is the absolute temperature
n_o is the viscosity
D^o is the constant of diffusion extrapolated to zero concentration

* Haga et al.(1977) calculated the R_e to be 6.84nm

Enzyme Assays

β-galactosidase(E.Coli, EC 3.2.1.23):

hydrolyses ONPG $\longrightarrow$ ONP (which gives yellow color)

Reaction Mixture:	Phosphate buffer pH 7.0	0.1M
	$MgCl_2$	0.001M
	$MnCl_2$	0.0002M
	β-mercaptoethanol	0.6ml/l

1ml of above mixture to 0.5ml 0.01M ONPG in 0.1M phosphate buffer, pH 7(0.15g/50 mls)

ONPG is o-nitrophenyl β-D-galactoside

0.5 mls of above mixture + 20-50 µl from column or 5-10µl from gradient.

Stop reaction with 1 ml 1M Na_2CO_3

Read OD at 420nm

Catalase(bovine liver, EC 1.11.1.6):

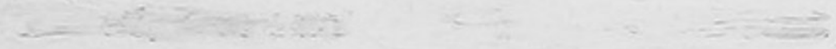
catalyses $2H_2O_2 \longrightarrow 2H_2O + O_2$

Buffer:	phosphate buffer	0.5M pH 6.7-7.0	5 mls
	H_2O		45 mls
	H_2O_2	30%	100 µl

1 ml of above mixture + 20-50 µl from column or 10-20 µl from gradient.

Amount of peroxide decomposed is proportional to the concentration of the enzyme. The decrease in absorption at 240nm is measured.

Alcohol dehydrogenase(yeast, EC 1.1.1.1.):



catalyses Ethanol + β-NAD ⟶ acetaldehyde + β-NADH + H

Absorption at 340nm increases with formation of β-NADH

Reaction Mixture: 1 ml Tris Buffer pH8.8

0.5mls NAD(nicotinamide adenine dinucleotide) at 35mg/ml

20-100 µl Ethanol(100%)

to 15 mls with H_2O

1 ml of above mixture + 20-50 µl from column or 10-20 µl from gradient.

All enzymes were added in amounts appropriate to give an easily read assay assuming that they would be eluted from the column or gradient within 10 fractions.

Ferritin, S. aureus and potassium ferricyanide were determined spectrophotometrically at OD_{420}.

Sucrose Gradients

5-20% linear sucrose gradients in the appropriate buffer were made according to the method of Martin and Ames(1961). Standard enzymes were used as markers and the gradients were run for 24 hours at 4°C in a Beckman rotor SW28.1 at 28,000 rpm(107,000xg).

SDS-Electrophoresis

SDS-PAGE as performed according to Laemmli(1970) either in 7cm. tube gels(for (^{3}H)-PrBCM-labeled membranes) or on slab gels(10 cm. in length).

Electrode Buffer: 0.025M Tris
0.192M glycine
0.1% SDS
pH 8.3

Stacking Gel: 3% acrylamide
0.125M Tris-HCl, pH 6.8
0.1% SDS

Separating Gel: 7% acrylamide
0.375M Tris-HCl, pH8.8
0.1% SDS

Polymerised by 0.025% w/v TEMED and Ammonium Persulphate

Sample Buffer: 0.0625 Tris-HCl, pH6.8
5% β-mercaptoethanol
2% SDS
10% glycerol
0.001% Bromphenol Blue

Staining: Coomassie Brilliant Blue 0.1%
5% Methanol
10% Acetic Acid

Samples were diluted in Sample Buffer 1:5 or 1:10.

1) Stability of Cholate-salt Soluble Material as a Function of Protein: Cholate Ratio of Soluble Preparations

The results from 100 soluble preparations were collated and analysed as a ratio of protein(in soluble material):cholate(w/w). A range of values was obtained by solubilisation at different initial protein concentrations. The absolute value of the specific activity of the soluble material varied, which was in part due to the different specific activities of the starting material(as shown in Chapter 2). Since because of this the activities of the different preparations could not be compared directly, a simple question was asked: Was specific binding present and did this vary as a function of the protein: cholate ratio. If it did it would imply a role for cholate-protein(and by extension endogenous lipid) interactions in the stability of receptor activity. Specific binding was determined by subtraction of non-specific binding in the presence of 10uM atropine from total binding. All soluble material with non-specific binding less than or equal to 30% of total binding in the presence of (^{3}H)-QNB(or 50% using (^{3}H)-N-methylscopolamine) were considered to contain specific binding(called active binding for clarity) to muscarinic receptors. The soluble material was analysed at two temperatures: 4^{o}C (using (^{3}H)-NMS at lnM saturation) or 37^{o}C(using (^{3}H)-QNB at saturation(500pM)). If binding was seen at 4^{o}C but not at 37^{o}C, the difference could be due to the stability of the material which could not survive assay conditions at 37^{o}C for one hour but could at 4^{o}C for 30 minutes.

Figure 1 shows the percentage of active binding preparations at each protein:cholate ratio. A value of 50% means that 50% of total experiments at this value showed active binding as previously defined and 50% did

not(completely inactive) or had a large non-specific binding component. It is clearly seen that there is a slow increae in the percent of experiments containing active binding until a ratio of 0.6 is reached at which value all preparations contain active binding. The ratio 0.6 represents 300 µg protein/ml at 0.05% Na Cholate or 600 ug protein/ml at 0.1% cholate. The 100% figure is attained at the lower ratio of 0.3 using (^{3}H)-NMS at 4^{o}C. Therefore, working at a lower temperature one can use much higher concentrations of cholate.

If lipids are important for receptor binding, since as Figure 1 shows higher cholate:protein ratios lead to a more heat-sensitive receptor possibly by displacing lipids, then one should expect to see a relation between the amount of lipid associated with the receptor and quantitative receptor binding. Preliminary experiments in collaboration with Dr. David Wing(University Department of Pharmacology, Oxford) indicate a role for phospholipids in receptor binding. Figure 2 shows that the higher the specific activity of the soluble material, the more phospholipid there is associated with the receptor. It is to be stressed that these experiments are preliminary and are included as added evidence that phospholipids play an important role in receptor binding.

2) Characterisation of Soluble Material

Gel filtration studies combined in Figure 3 indicate that as the ratio protein: cholate decreases a broad peak of receptor activity with a Stokes radius of 9nm is included in

FIGURE 1

FIGURE 1

Percentage of Active Binding at each Protein:Cholate Ratio

Percent active binding was calculated at each ratio as described in the text.

(^{3}H)-QNB at 37^{o}C: (● - ●)

(^{3}H)-NMS at 4^{o}C: (○ - ○)

each

each

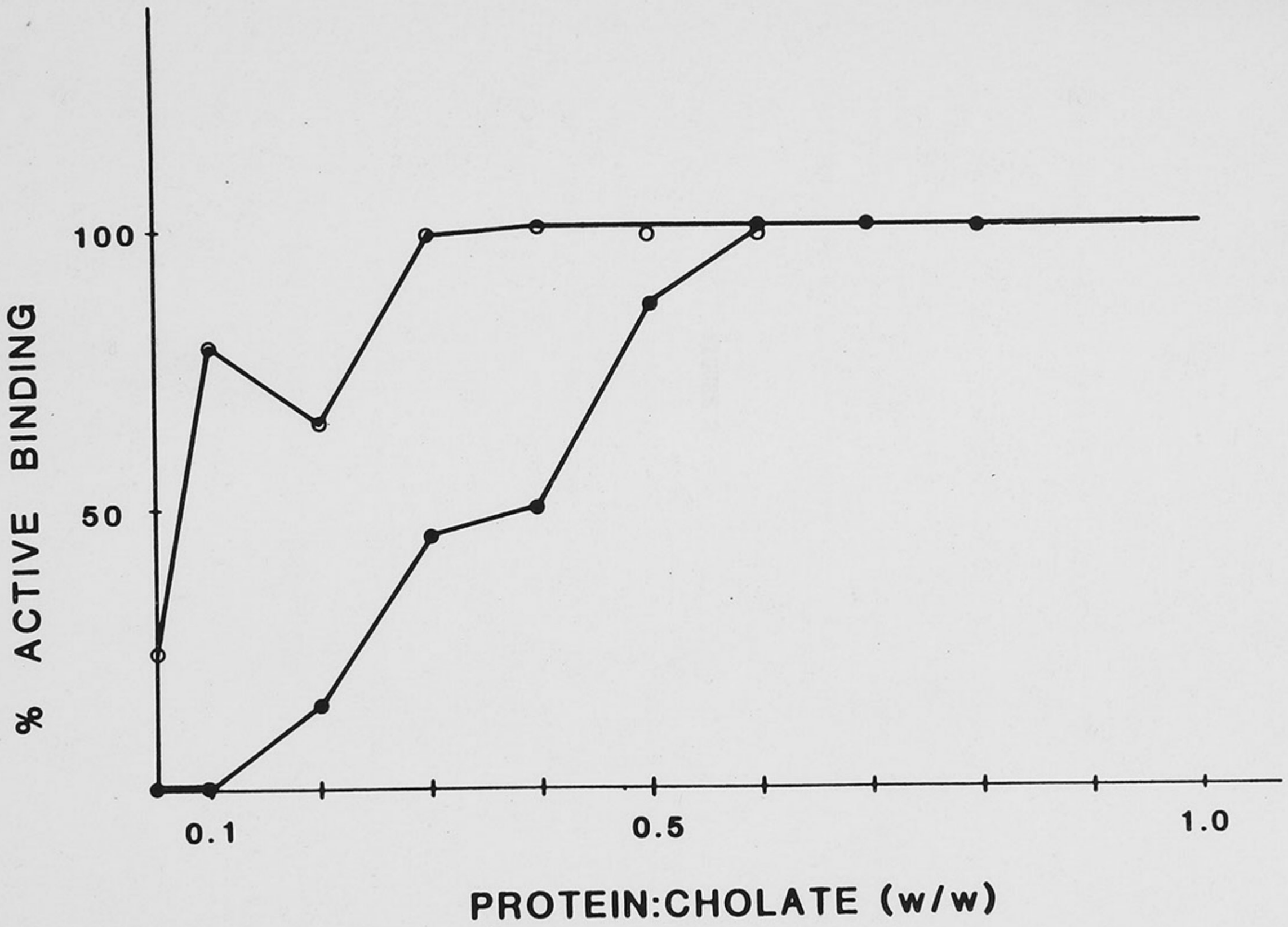


FIGURE 2

FIGURE 2

Amount of Phospholipid Associated with Receptor Activity

Soluble receptor(in Tris buffer) was prepared as described in Chapter 3. Initial protein concentration was varied to give a range of final specific activities (pmoles/g protein); each preparation was performed in duplicate. Receptor activity was assayed at 500pM (^{3}H)-QNB at 37^{o}C as described previously. Protein was determined by the method of Lowry et al.(1951).

Phospholipid (total) determinations were performed by Dr. D. Wing(University Department of Pharmacology, Oxford) briefly as follows: Phospholipids from the above soluble preparations were extracted with chloroform/methanol 2:1(v/v), evaporated and redissolved in chloroform/methanol 2:1(v/v). Total phosphate determination was by a modification of the technique described by Morrison(1964).

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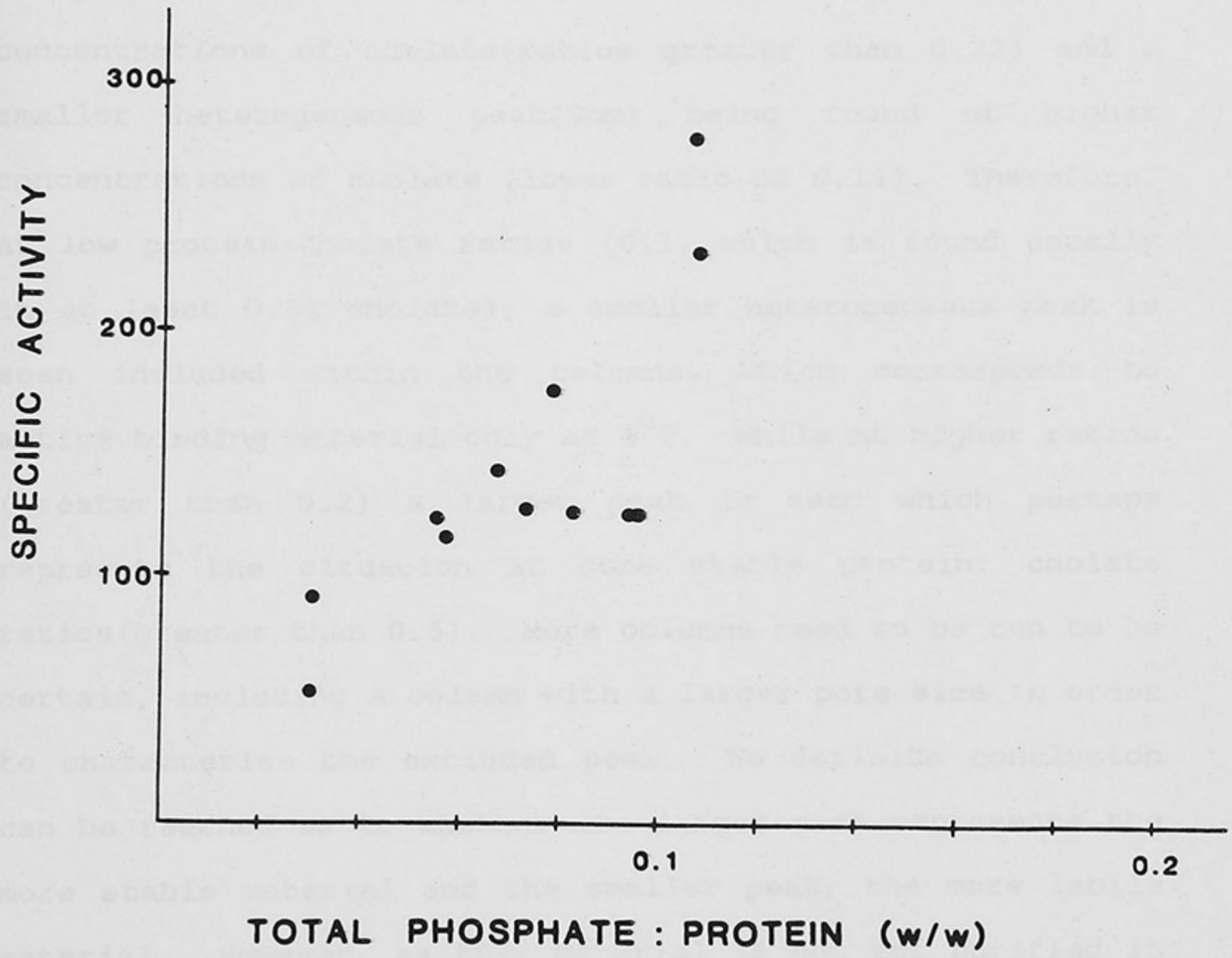


the column. Correlation with activity data(Figure 1) is difficult since at the low concentrations of cholate which are necessary to give a protein:cholate ratio greater than 0.6(where all binding is active at 37°C), not enough counts are solubilised which can be unambiguously detected after column chromatography. Approximately 20 columns were run at different cholate concentrations all indicating similar results with a larger peak being found at lower concentrations of cholate(ratios greater than 0.22) and a smaller heterogeneous peak(9nm) being found at higher concentrations of cholate (lower ratio of 0.14). Therefore, at low protein:cholate ratios (0.1, which is found usually in at least 0.3% cholate), a smaller heterogeneous peak is seen included within the columns, which corresponds to active binding material only at 4°C. While at higher ratios (greater than 0.2) a larger peak is seen which perhaps reprsents the situation at more stable protein: cholate ratios(greater than 0.5). More columns need to be run to be certain, including a column with a larger pore size in order to characterise the excluded peak. No definite conclusion can be reached as to whether the larger peak represents the more stable material and the smaller peak, the more labile material. However, as this material is not yet purified it appeared more important to find a stable preparation which could be manipulated biochemically without losing activity.

Sucrose gradients(not shown) showed a broad peak from 4S-10S indicating a heterogeneous population of labeled receptor.

Figure 4 shows the results of (^{3}H)-PrBCM-prelabeled microsomal membranes run on SDS gels after cutting and counting of gel slices. Several peaks(61,000, 38,000, and

FIGURE 3

FIGURE 3

Gel Filtration on Biogel 0.5m Columns at 4°C

Profiles are the combined runs of two soluble(prelabeled (^{3}H)-QNB) preparations at different protein:cholate ratios. Markers are as indicated.
Figure 3B shows the calibration curve as described in Methods.

(-) 0.22 (- - -) 0.12

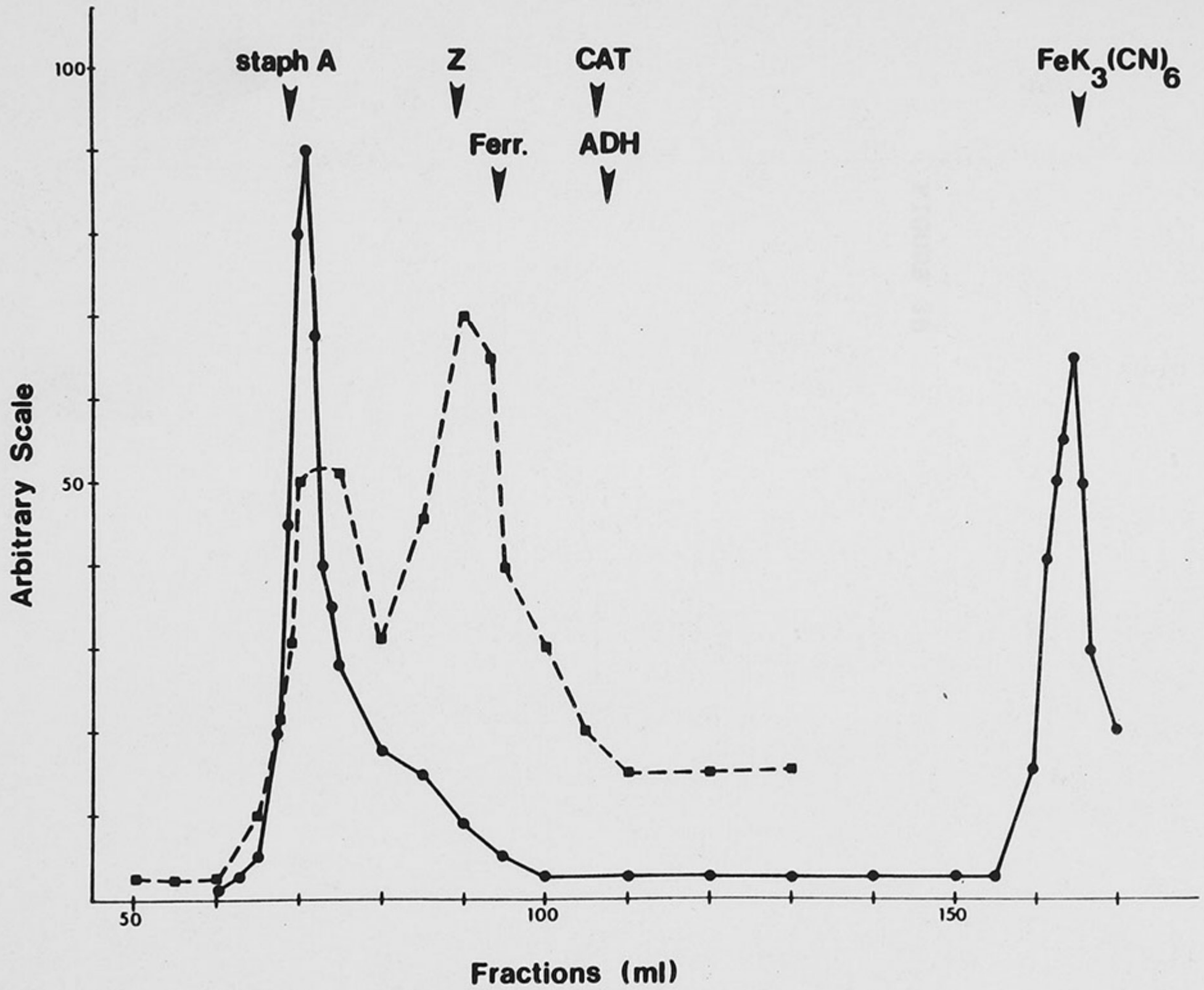

staph A
Z
CAT
Ferr.
ADH
FeK3(CN)6
100
50
Arbitrary Scale
50
100
150
Fractions (ml)

FIGURE 3B

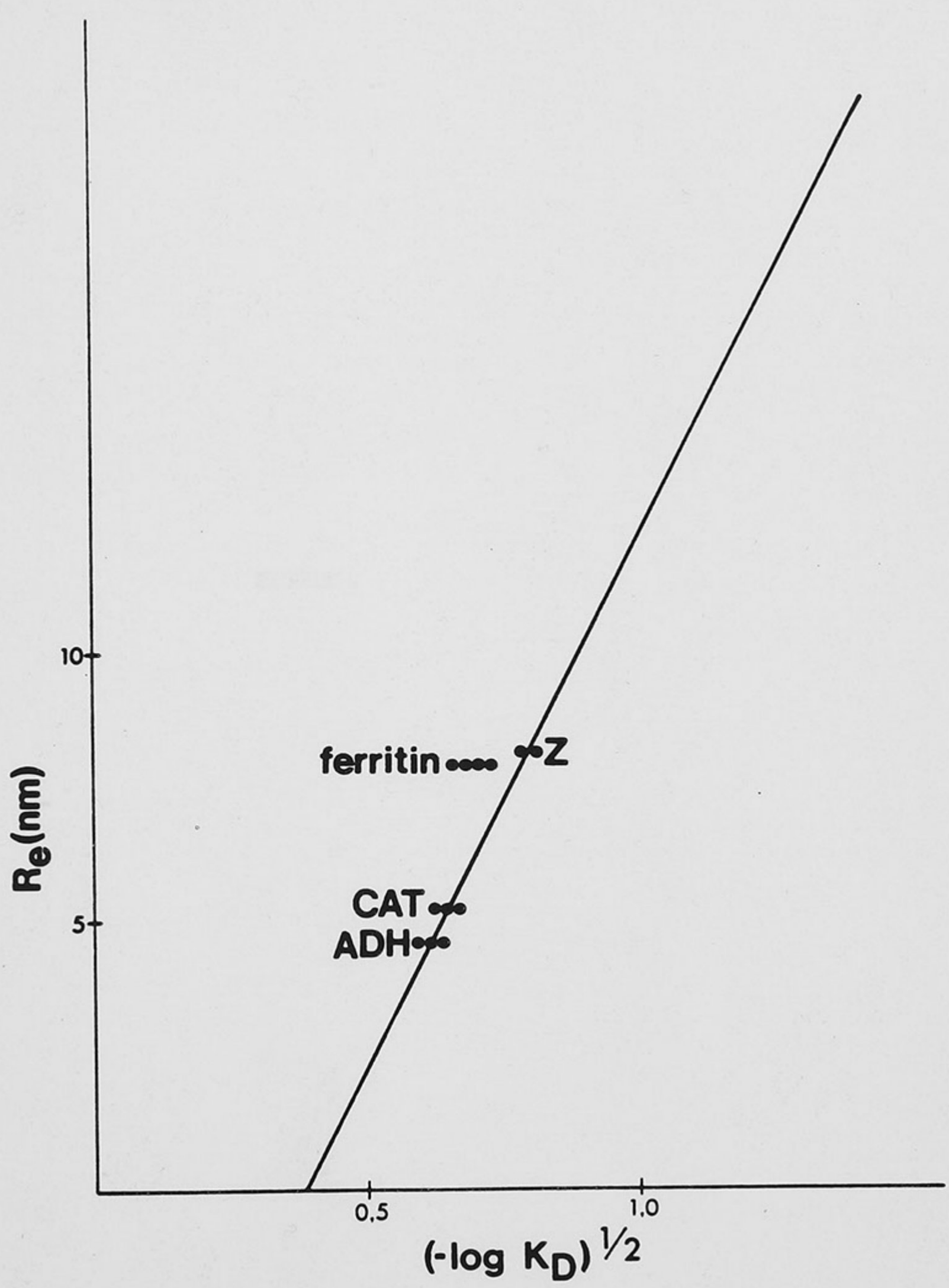

10
5
R_e(nm)
ferritin
Z
CAT
ADH
0.5
1.0
$(-\log K_D)^{1/2}$

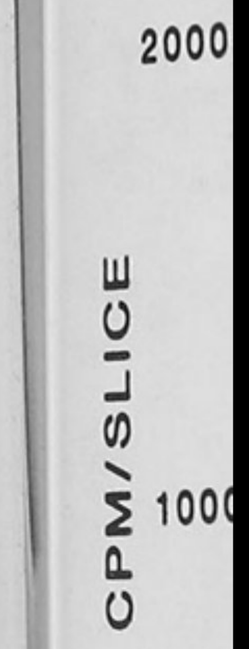
FIGURE 4

FIGURE 4

SDS-PAGE of (^{3}H)-PrBCM-prelabeled membranes

20,000 cpms were applied to each gel in a volume of 100ul. Electrophoresis was performed at 2mA/gel. Gels were sliced into 2mm sections and incubated with 200µl of 30% H_2O_2 and heated for one hour in a closed container at 60°C.
Membranes were incubated in sample buffer for 2 hours at 50°C which may have accounted for some proteolysis.

Peaks(from left to right): R_f= 0.56, 0.68, 0.78



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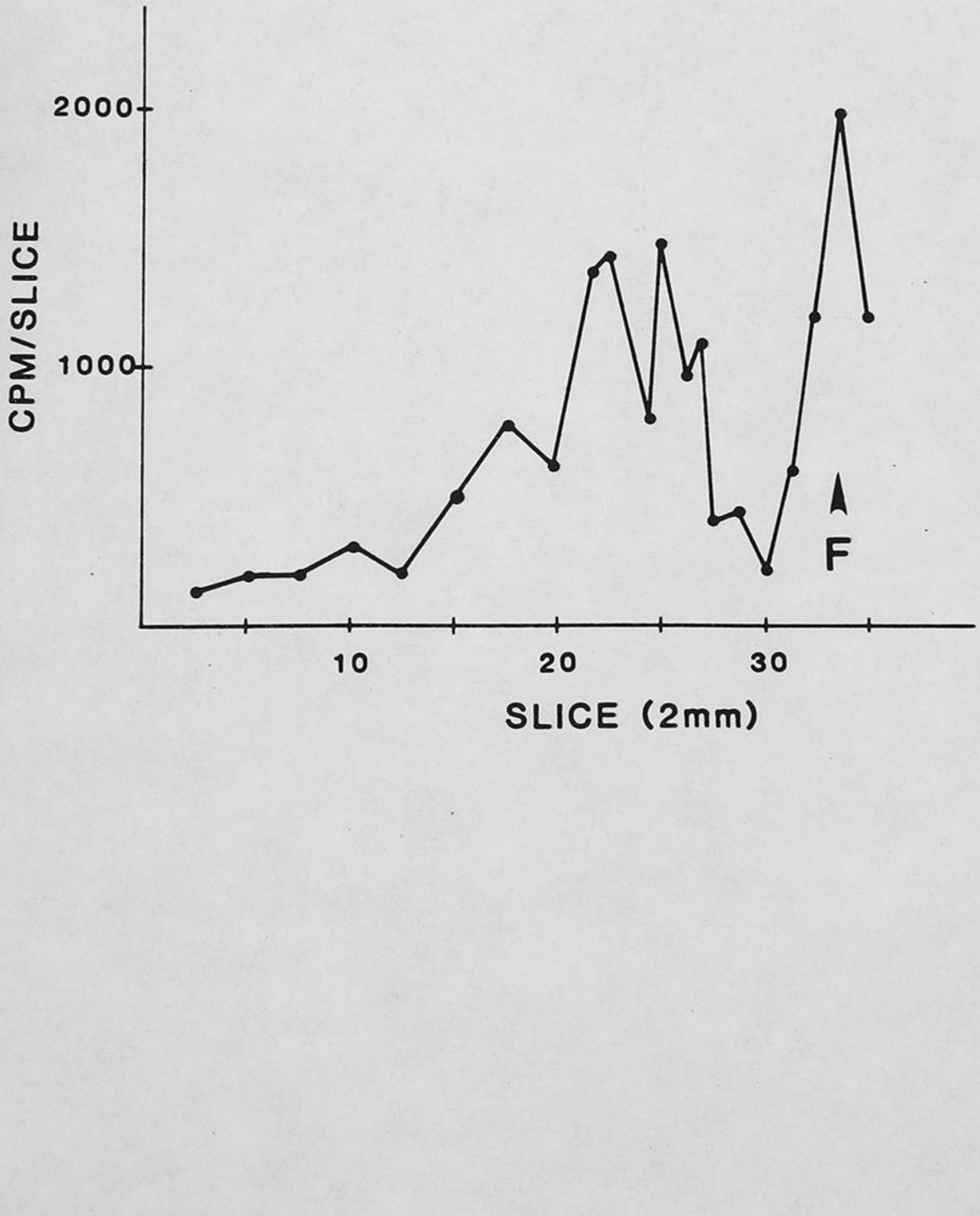


25,000 molecular weights) are seen ;however, earlier work by Birdsall indicated the presence of a single peak 80,000MW. The results here most probably indicate that some proteolysis had taken place, although the labeled peaks were still of possible use as antigens.

No estimate can be made of the molecular weight of the labeled receptor as a result of the column and gradient studies since in detergent the values obtained for a protein often do not reflect the native state of the protein, but instead reflect the number of hydrophobic areas of the protein which are available to bind the detergent, which in turn influences the behaviour of the protein.

Discussion

Stability of Cholate-Salt Soluble Material

Inactivation of protein can arise when the amount of detergent associated with membrane lipids reaches a critical level. If the detergent concentration is much less than the membrane lipid concentration, all the detergent is associated with the membrane phase and the detegent/lipid ratio dominates inactivation; if it is much greater than the lipid concentration then the detergent is mainly in the aquaeous phase and total detergent concentration is important and if it is approximately equal to the lipid concentration than the activation depends on both lipid and detergent concentration(Brotherus et al., 1979).

The stability of the cholate-salt soluble material is seen to depend on the ratio of protein:cholate(w/w) of the soluble material. As the ratio decreases(i.e. concentration

of cholate increases), the stability of binding activity at 37°C also decreases and specific binding can only be seen at 4°C. This is most clearly seen at a protein:cholate ratio of 0.3 (or 150 µg protein/ml at 0.05% cholate; 300µg/ml at 0.1% cholate or 600µg/ml at 0.2% cholate) where 100% active binding is seen at 4°C but only 40% is seen at 37°C.

Lipid:detergent ratio could also affect conformational changes necessary for receptor binding. The gradual increase to 100% active binding could mean that another factor other than protein:cholate ratio is changing and is causing the gradual increase in stability at 37°. It is only at higher ratios(0.6 or 300 µg protein/ml at 0.05% or 600 µg/ml at 0.1%) that binding is seen 100% of the time at 37°C. Preliminary work with Dr. D. Wing(University Department of Pharmacology, Oxford) indicates that there is a linear relation between the amount of phospholipid associated with the soluble material and the amount of specific activity seen.

Anholt et al.(1981) found a protective effect of endogenous lipids against ion channel inactivation which would could be greatly increased by the inclusion of soybean lipid(asolectin) in their experiments on nicotinic receptor reconstitution. Other groups have found that exogenous phospholipid is necessary for stability for a variety of proteins. Work on the Saxitoxin binding site(Na^+ channel)(Barchi and Murphy, 1980; Barchi et al., 1980) showed that the dependence of solubilisation efficiency on membrane protein concentration most likely reflected sensitivity to the ratio of endogenous phospholipid to detergent. Agnew and Raftery(1979) also studying the Na^+ channel, suggested that what was critical for stability was

the ratio of lipid:detergent and not membrane:detergent as was also found by Isambert and Henry(1981) for the noradrenaline uptake system in bovine chromaffin granules. Stability may be affected by micelle surface charge composition, local partitioning into lipid or detergent-enriched domains or by hydrocarbon fluidity or micelle geometry(Agnew and Raftery, 1979). Tanford and Reynolds(1976) suggested that exogenous phospholipids could be included into any detergent mixture to prevent dilution of lipid into protein-free micelles.

Endogenous and exogenous lipids could protect against inactivity or instability of proteins by 1) preventing displacement of lipids bound to specific sites on the receptor which results in a loss of binding activity directly or indirectly (eg. oxidation of SH groups) or 2) protecting against direct binding to sites necessary for functional activity(Popot, 1982; Heidman et al., 1980). Evidence for the first possibility was provided by Chang and Bock(1979) who had found a relationship between the retention of high affinity binding sites for the nicotinic receptor and the amount of endogenous phospholipids remaining in the purified preparation and suggested that the phospholipids were protecting receptor sulfhydryl groups from air oxidation. In addition, Anholt et al.(1981) showed a lowered susceptibility of nicotinic receptor disulfide bonds to reducing agents added during solubilisation of reconstituted membranes(with added asolectin present). It would also be of interest to see the effect of sulfhydryl and reducing agents on muscarinic soluble material to see if a change in the well-characterised response to NEM, pCMB

etc. is observed.

In the present work, the stability of the receptor has been shown to depend on the ratio of soluble protein: cholate(w/w) and this in turn probably implies a role for endogenous lipids. The difference in stability at 37°C and 4°C could reflect a conformational change taking place at the different temperatures due to the displacement of bound lipids by cholate. In addition, it has been shown that the use of higher cholate concentrations also leads to a less stable preparation.

In the recent work of Hulme et al.(1982), rat brain solubilised at 0.3% Na cholate and 1M NaCl and assayed at 4°C yielded a preparation with 30% of the total receptor a tivity solubilised with a $t_{1/2}$ of 60 hours at 4°C. The $t_{1/2}$ of 0.1% Na cholate-1M NaCl solubilised material was greater than 1 week at 4°C(Chapter 3). The stability of the rat brain material could be improved by the addition of egg lecithin (2.5mg/ml) in the solubilising medium ($t_{1/2}$ increased to 130 hours at 4°C). Interestingly, in the presence of lecithin the decrease in affinity for agonists and antagonists is not seen. Most groups now working on nicotinic receptor reconstitution have found that membrane binding characteristics can be retained(and no change to a low affinity binding site which is otherwise seen) by addition during solubilisation of exogenous lipids , usually asolectin(Epstein and Racker, 1978; Popot et al., 1981; Schiebler and Hucho, 1980; Wu and Raftery, 1981). However, as has been shown in Chapter 3, the addition of NaCl by itself will cause a decrease in affinity which is therefore not only a result of solubilisation. Therefore, the addition of lecithin in addition to increasing the stability

of the receptor also "reverses" the effect of salt on binding. NaCl at high concentrations could conceivably have an effect on membrane phospholipids in much the same way as it reduces electrostatic repulsion between the charged faces of the detergent molecules and thus perhaps causes a conformational change of the receptor situated in its membrane lipid environment. It would be of interest to see if the addition of lecithin to membranes in high salt would cause a change from lower affinity back to the original membrane affinity. It seems, then, that in combination with Na cholate-NaCl, lecithin has a dual role of increasing receptor stability and returning the affinity to its native state.

Using a combination of Na cholate and high salt, dopamine receptors from bovine caudate nucleus have been solubilised and the addition of asolectin led to an increase in receptor stability(Dr. P.G. Strange, personnel communication). This may indicate that the combination of low cholate(0.1-0.3%) and high salt may be useful for receptors which have in general been difficult to solubilise using non-ionic detergents. This may also indicate a key role for phospholipids in the functional activity of these receptors.

Characterisation of Muscarinic Cholinergic Receptors

A possible relationship between stability of receptor preparations and their profile on gel filtration columns was sought. Using high ratios of protein:cholate(greater than 0.25, 4 times as much cholate as protein) one large peak is seen just inside the exclusion

limit of a Biogel 0.5m column. This compares to the situation found at lower ratios(0.10, ten times as much cholate as protein) where a broad peak centering around 9nm(running just ahead of B-galactosidase) is seen preceded either by a larger excluded peak or by a peak just inside the exclusionlimit. This compares well with the data reported by Hulme et al.(1982), who found that in the absence of lecithin their material eluted in two peaks: one excluded and the other a very broad peak included in the column. Upon addition of lecithin, the labeled material eluted as a single sharp peak of Stokes radius 6.7nm(running just behind B-galactosidase). The authors suggest that the broad peak was due to hydrophobic interactions among a fraction of the receptor population. This could also be due to heterogeneity among the micelles formed: as increasing amounts of cholate are added the lipids are displaced and cholate-aggregates(Chapter 3, figure 4) are formed. Lower concentrations of cholate(by comparison to protein) may lead to many protein molecules being included in a single micelle while higher cholate concentrations whould lead to a smaller, more uniform population being obtained. The lower concentrations of cholate were difficult to work with, since the amount of receptor solubilised could not be reproducibly detected after gel filtration. Sucrose gradients also lead to the conclusion that a heterogeneity exists in the present preparations of solubilised receptors. It should, however, be noted that both Hulme's work and the present work were done with prelabeled receptor and may not accurately reflect the situation using unlabeled, soluble receptor. Further work by Haga et al.(1982, in preparation) on the muscarinic receptor indicated a sedimentation coefficient of 3.46 and a

Stokes radius of 7.05nm yielding a final MW of 111,000. a final answer will have to await further purification of the receptor.

From the present work, no estimate of the molecular weight of the receptor can be obtained. However, a stable preparation is perhaps the first important step to a purification. The next step is an efficient affinity compound. Chapter 5 describes our attempts at finding a muscarinic affinity compound.

CHAPTER 5

AFFINITY COMPOUNDS

Introduction

Affinity compounds should combine an affinity high enough to recognize and bind to the protein in question with a low enough affinity to allow the protein to be subsequently eluted without too rigorous a treatment. This delicate balance is often difficult to attain.

Muscarinic receptors have been difficult to study due to their sensitivity to a variety of detergents and their instability in solution. Work at characterisation of the receptor has been slow even where an adequate solubilisation exists due to the low specific activity of the available sources of receptor. Since the first solubilisation of the receptor was reported in 1975 groups have been searching for an appropriate affinity compound; with the reintroduction of digitonin as a possible detergent , efforts intensified still without success.

Muscarinic antagonists are the obvious first choice for an affinity compound. Initial experiments had been performed using derivatives of benzilylcholine(Figure 1) but these had low affinity and, being esters, could not withstand the extended alkaline treatment often necessary for affinity gel synthesis. Therefore, our efforts were

turned towards other drugs, in particular tricyclamol. Tricyclamol is the methachloride of procyclidine which is used as an anticholinergic and acts as an antispasmodic on guinea pig ileum. It decreases gastric and intestinal motility and the volume of gastric, intestinal and pancreatic secretions.

In an attempt to localise muscarinic receptors at the cellular level, we have made use of the biotin-avidin system(Bayer and Wilchek, 1980). The basic glycoprotein avidin has a very high affinity for biotin(Vitamin H). It is therefore possible to make a biotinylated compound(in our case a derivative of QNB) which could then be used as an affinity compound by attaching the compound to an avidin-sepharose gel vis its biotin moiety while leaving the QNB end free to react with receptor sites. The biotinylated compound could also be used in localisation studies on fixed tissue by allowing the compound to react with muscarinic receptors via the QNB portion of the compound, and then visualising its localisation by the addition of avidin-peroxidase which can be visualised at the light or electron microscope level.

This chapter reports on our efforts at obtaining an affinity compound(in collaboration with Dr. E.W. Gill, University Department of Pharmacology, Oxford) using these derivatives and the possible use of lectin affinity chromatography in the isolation of muscarinic receptors.

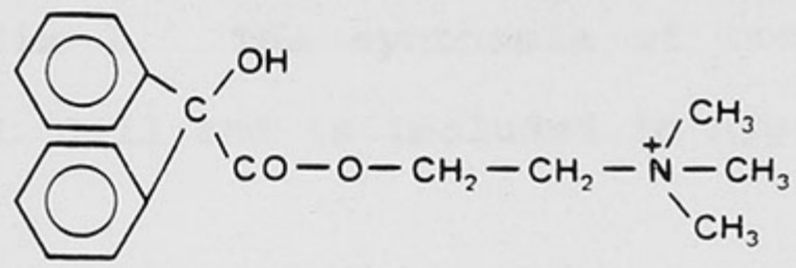
Methods

Synthesis of Affinity Compounds

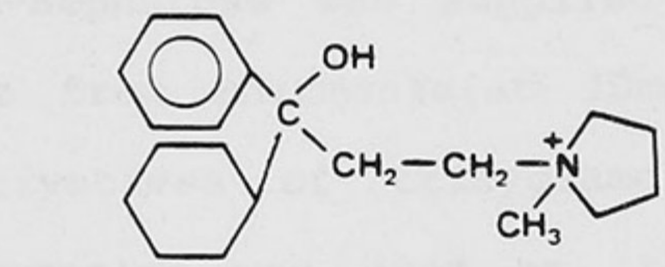
FIGURE 1

Figure 1

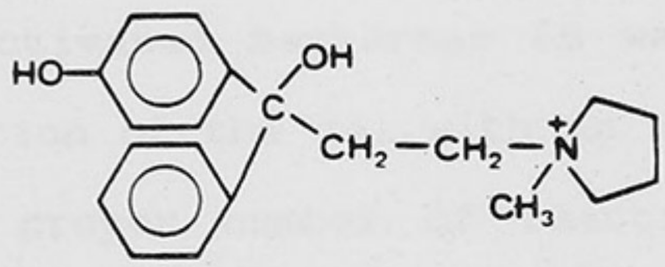
Structures of Compounds and their Derivatives used as Possible Muscarinic Affinity Compounds



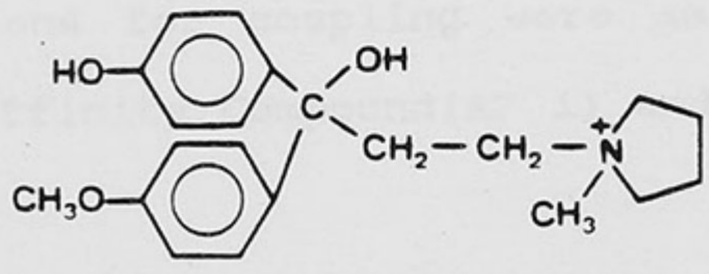
Benzilylcholine



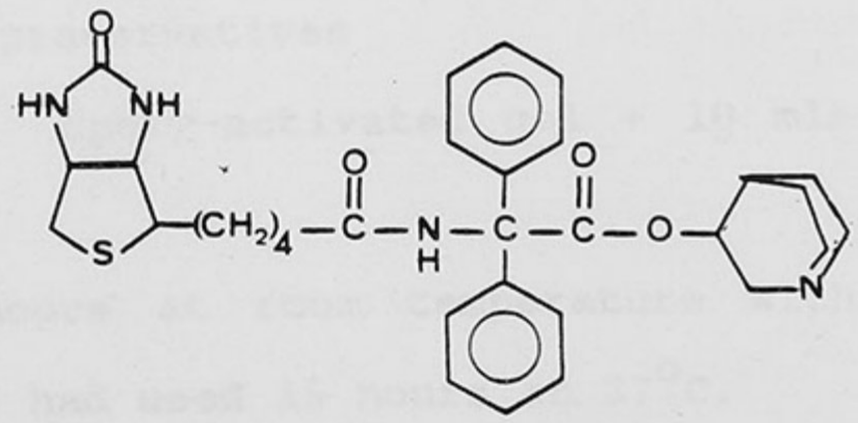
Tricyclamol



AF(1)



AF(2)



Biotinylated QNB

See Appendix 1. The synthesis of compounds was performed by Dr. E.W. Gill and is included in Appendix 1.

Coupling of Compounds to Affinity Gels

Avidin-Sepharose was supplied by Sigma. Con A-Sepharose 4B was from Pharmacia(at 10mg ConA/ ml packed gel). For derivatives of tricyclamol, Epoxy-activated Sepharose 6B(Pharmacia) was used as it will couple with hydroxyl-containing compounds via ether bonds under alkaline conditions.

Epoxy-activated Sepharose 6B was first tested by incubating a portion of the gel with an excess of phenol to ensure that the proper number of reactive oxirane groups were present(15-20 uequivalents per ml of swollen gel). This phenol gel was also used as a blank(or control) gel. Optimal conditions for coupling were as follows and were used for both Affinity Compound(AF 1) and Affinity Compound 2(AF 2):

1) 17.3mM AF(1) in 0.05M carbonate, pH11.0 200mM AF(2) in NaOH to give pH11.6(insoluble at lower pH)

2) Epoxy-activated gel was first washed for one hour with H_2O to remove preservatives

3) 1 gram Epoxy-activated gel + 10 mls AF(1) or 20mls AF(2)

4) 16 hours at room temperature with shaking. Earlier experiments had used 16 hours at 37^{o}C, but this caused the gel to turn a dark brown which made it difficult to calculate the exact amount of product on the gel.

5) Wash the gel thoroughly as follows:

carbonate,	pH11.0	200mls
H_2O		500mls
phosphate,	pH7.4	200mls
acetate,	pH4.0	200mls
H_2O		500mls

6) 0.03 grams/ 3 mls in 0.1N HCl; heat 100°C for 5 minutes

7) Read optical density at 200-400nm, specific peak at 275nm

Absorbance of AF(1)= 7.5OD/mM

0.466μmoles/ml/0.010 gram gel or 46.6 umoles/gram dry gel

8) Excess groups were blocked with 1M C_2H_6OS(B-mercaptoethanol) for 5 hours at RT

9) Gel was extensively washed with high and low pH and stored at 4°C

Incubation of Gels with Soluble Material

Soluble material in variable amounts was usually incubated batchwise with the affinity gels for 1 hour at RT. Gels were allowed to settle at 4°C and the supernatant was tested for binding activity using (^{3}H)-QNB as thoroughly described in Chapter 3; (^{3}H)-NMS was sometimes used but no difference was seen in the results.

Elution of Affinity Gels

Elution of gels for test of active receptor was as

described in the results section. Gels were thoroughly washed in cholate-salt buffer before specific elution was begun.

SDS-PAGE

SDS-electrophoresis was performed as described in Chapter 4 according to the method of Laemmli(1970). Some gels were stained following the silver nitrate method of Switzer et al.(1979). Approximately 100ul of material was applied to each lane and gels run at 40mA per slab gel.

Affinity Gel Eluates Prepared for SDS-PAGE

Elutates from the affinity gels were prepared in two ways: one method was to elute all protein which remained on the gel after extensive washing to remove non-specifically absorbed protein and the second method was to specifically elute the gel using the appropriate ligand.

Elution 1:

-Affinity gels(AF(1), AF(2), Con A -Sepharose, Biotinylated QNB-avidin-sepharose) were washed for 3 hours at RT with 0.05%-0.5M(cholate-Nacl).

-Gels were spun in microfuge to pellet Affinity gel and resuspended in 200µl Sample buffer(5% SDS)-see Chapter 4.

-Gels were heated for 2 hours at 50°C as were markers and soluble material.

-Gels were spun in microfuge, supernatant is SDS-eluate; 100µl was applied to each lane.

Elution 2:

-Affinity gels were washed for 3 hours at RT with 0.05%-0.5M (cholate-NaCl) and spun in microfuge to pellet.

-Gels were resuspended in 500µl of appropriate ligand:

AF(1,2):- 1M carbachol in 0.05%-0.5M(cholate-NaCl)

- 1 hour at RT. Spin to pellet affinity gel.

-Resuspend in 1M carbachol as above and incubate 16 hours at 4°C.

Con A: - a-D-methylmannoside added in increasing concentrations, microfuge between each addition: 1mM, 10mM, 100mM, 1M-1 hour at RT, final addition 16 hours at

4°C.

-50µl specific eluates + 100µl Sample Buffer 10 minutes at 100°C.

-Affinity gels were treated as in Elution 1, but incubated with Sample Buffer only 1 hour at 50°C.

Results

1) Test of Possible Affinity Compounds: Inhibition of Binding of (^{3}H)-QNB to Microsomal Membranes

Different derivatives of benzilylcholine were first made as possible compounds, but their affinity was low and the presence of ester linkages made working at high pH unsuitable. Several alternatives were tried with the most promising being tricyclamol. Two derivatives of this compound were made: AF(1), AF(2) as described in Appendix 1 and coupled to Epoxy-activated Sepharose as per Methods. The affinity of tricyclamol and its derivative was in the nM range(Figure 2). The final hydroxyl group was not added in the derivatives tested since this would reduce the affinity of the drug for the receptor and would not represent the true situation when the ligand was finally coupled to the gel. The biotinylated compound(Figure 2) had an affinity for the receptor close to 1µM, almost 10^6 times lower than the initial QNB affinity(Chapter 2).

2) Test of Affinity Gels

Table 1 gives a typical example of preliminary experiments with the affinity gels. The Blank gel(Phenol

FIGURE 2

FIGURE 2

Inhibition Curves of Possible Affinity Compounds

Binding was performed at 50pM (^{3}H)-QNB; microsomal membranes were used as described in Methods to Chapter 2.

Tricyclamol(● - ●); $K_i = 1.4x10^{-9}M$; soluble(not shown) $ID_{50} = 3x10^{-8}M$
AF(1) cor (○ - ○); $K_i = 1.4x10^{-9}M$

Biotinylated QNB (□ - □); $K_i = 4x10^{-7}M$

Off-time for tricyclamol binding to soluble material was 20 minutes($t_{1/2}$) at 37°C as determined by dilution of tricyclamol.

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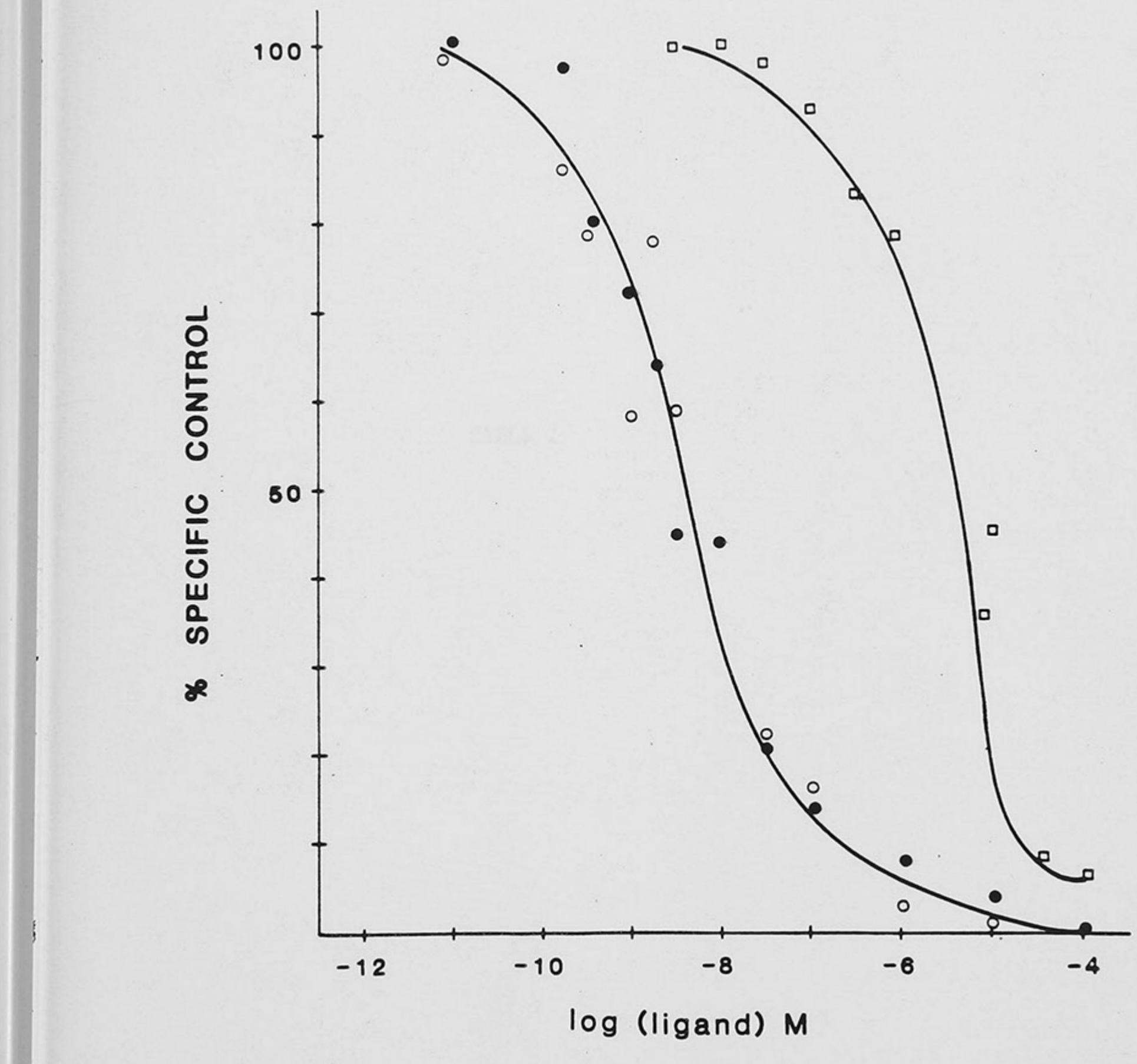


TABLE 1

TABLE 1

Affinity Gels

Binding to supernatants after incubation with affinity gels was performed at 500pM (^{3}H)-QNB as in Methods. See text for further description.

TABLE 1

Affinity

Gel

Control

Blank ge

AF(1)

AF(2)

TABLE 1

Affinity Gels

Gel	Specific cpms/ml	% on gel
Control	1305	-
Blank gel	1329	0
AF(1)	581	56
AF(2)	1208	10

treated) did not "pick-up" any receptor while AF(1) appeared to absorb 50% of the cpms in the supernatant(this often went as high as 90%). AF(2) appeared to absorb very little if any receptor from the supernatant. These results were very encouraging; however, all efforts to elute an active receptor from the gel failed. Small columns of affinity gels were eluted by the following ligands: 1) tricyclamol; 2) tricyclamol + BSA(1 mg/ml); 3) 10mM tricyclamol + BSA(10 mg/ml); 4) 1mM oxotremorine; 5) 1M carbachol; 6) 1M, 2M, 4M NaCl; 7) 10^{-4}M QNB + 10^{-6}M (^{3}H)-QNB. Approximately 80-90% of all protein applied to the gels was not absorbed to the gel; however, specific eluates from the affinity gels contained only approximately 10% of the protein remaining on the gel. This was always below the minimum required for detection by the filtration assay. It was therefore decided to try and "overload" the gels as much as possible and then examine the eluate from the gels on an SDS gel.

Only preliminary experiments were performed with the biotinylated QNB-avidin-sepharose gel and the Con A-sepharose; therefore, eluates from these gels were examined only by their profiles on SDS gels.

3) SDS-PAGE of Affinity Gel Eluates

Table 2 shows the " apparent" absorption of receptor from the supernatant by a series of affinity gels. Additions were made batch-wise at RT and active receptor remaining in the supernatant was assayed. The gel appeared to be "saturated" with receptor well below the calculated capacity of the gel. This could imply that the receptor was interacting with a secondary site on the affinity gel(eg.

hydrophobic). Earlier tests had shown that the compound was not leeching off the gel as addition of gel supernatant to soluble material did not inhibit binding. Con-A gel apparently absorbed all the receptor that was added, while AF(1) appeared to be specific for the receptor.

The results of elution of the affinity gels as described in Elution 1 in the Methods is shown in Figure 3(see legend for explanation of individual tracks). Figure 3A represents soluble material at different cholate concentrations, only an increase in intensity appears to occur without the appearance of new bands. Therefore, the instability seen at higher cholate concentrations does not reflect the presence of new protein components but rather components which are not stained by coomassie blue. Figure 3B represents the eluates from the affinity gels while Figure 3C shows a silver nitrate stained gel. Table 3 is a summary of the molecular weights of the soluble material and representative gel eluates. Con A eluates are shown in Table 5. As can be seen, most of the bands eluted from the affinity gels are similar to the bands of the original soluble material in size and in number. There are fewer higher molecular weight bands seen which could just reflect a lower concentration of protein. Interestingly, the high MW band appears clearly on the silver nitrate gel. In addition, the specific gel eluate from the Con A shows no staining on the coomassie blue stained gel while showing 5 bands on the silver nitrate gel, an indication of the latter's higher sensitivity. From this experiment, it appeared that the affinity gels were absorbing almost all of the soluble material, even though what remained on the gel resisted several washes to remove non-specific binding. It

TABLE 2

TABLE 2

Apparent Absorption of Muscarinic Receptor to Affinity Gels

Binding was performed at 500pM (^{3}H)-QNB to supernatants after incubation with the affinity gels for 1 hour at RT. Soluble material was added to the gels over a period of 3 days. Each value refers to the final total amount of receptor which had apparently absorbed to the gel in pmoles (^{3}H)-QNB binding sites. Blank columns imply that no soluble material was added that day. The different numbers refer to different affinity gel preparations. Note the difference between AF(1) and AF(2). An apparent saturation of gels appeared to be seen in some cases. The amount of soluble material added to gels varied from preparation to preparation.

TABLE 2

Apparent Absorption of Muscarinic Receptor to Affinity Gels

Affinity gel		Total pmoles/ gel		
		(1)	(2)	(3)
AF(1)	1	0.653	0.808	
(2)		0.012	0.187	
AF(1)	2	1.16	1.304	1.387
(2)		0.212	0.257	0.347
AF(1)	3	0.415	0.433	0.573
(2)		0.145	0.267	0.355
AF(1)	4	0.387	0.387	0.497
AF(1)	5	0.556	0.611	0.679
AF(1)	6	0.49	0.642	0.721
AF(1)	7	0.56	0.642	0.721
Con A		0.188		

FIGURE 3

FIGURE 3

SDS-PAGE of Affinity Gel Eluates(Table 2)

Affinity gels were eluted in Sample Buffer(5% SDS) as per Elution 1 in Methods. Table 3 summarises the MW of some of the bands shown here.

Figure 3A: Coomassie Blue Stained Cholate-salt solubilised material(bovine caudate nucleus):

Lane (1): 0.05%-0.5M(cholate-salt respectively)
(2): 0.05%-1M
(3): 0.1%-1M
(4): 0.2%-1M
(5): 0.3%-1M
(6): 0.4%-1M

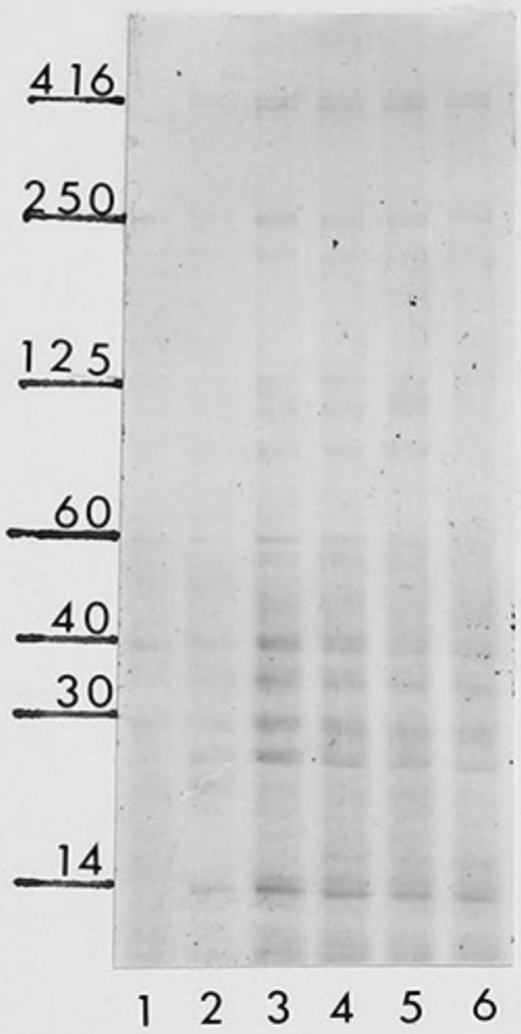
Numbers to the right refer to MW calculated from a standard curve $x10^{-3}$

Figure 3B: Affinity gels in Table 2 eluted with SDS(Elution 1)

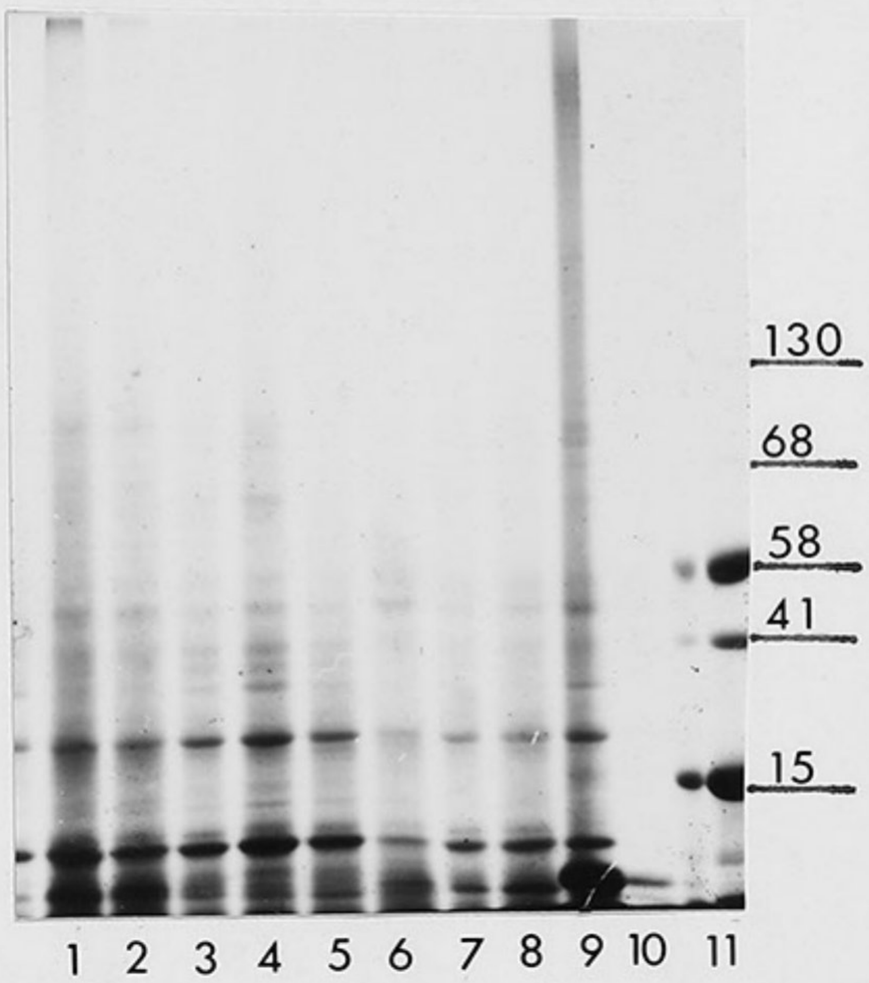
Lane (1): AF(1) 1
(2): AF(2)
(3): AF(1) 3
(4): AF(2)
(5): AF(1) 4
(6): AF(1) 5
(7): AF(1) 6
(8): AF(1) 7
(9): Con A
(10): Con A(eluted with 1M a-D-mannoside)
(11): Markers(B-galactosidase, BSA, Catalase, Alcohol dehydrogenase, hemoglobulin

Figure 3C: Silver nitrate stained gel
Lane (1): AF(1) 6; Lane(2): AF(1) 7
Lane (3): Con A eluted with mannoside

A


416
250
125
60
40
30
14
1 2 3 4 5 6

B


130
68
58
41
15
1 2 3 4 5 6 7 8 9 10 11

C

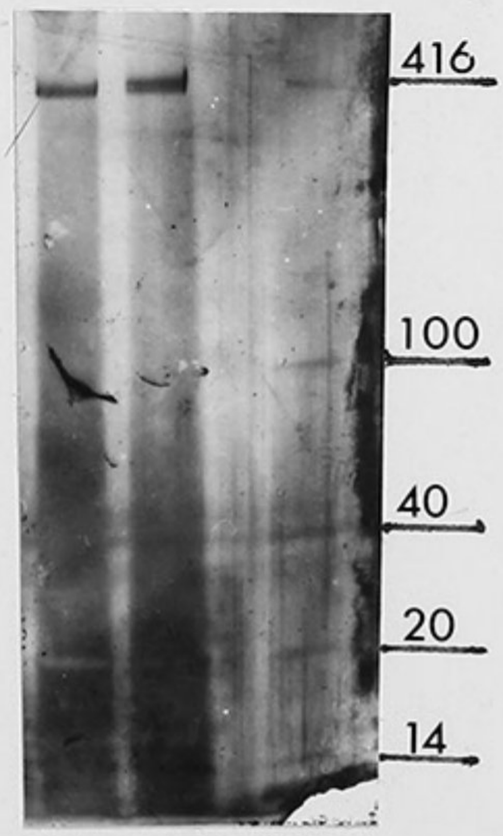

416
100
40
20
14
1 2 3

TABLE 3

TABLE 3

Molecular Weight of SDS-PAGE Bands

Representative soluble and affinity gel bands were calculated for MW according to the equation:

$$R_f = \frac{PB}{DM}$$

Where PB is the distance run by the polypeptide band
DM is the distance run by the dye marker
Standard proteins were as indicated in Figure 3B.

TABLE 3

Molecular Weights of SDS-PAGE Bands(from Figure 3)

Soluble(0.1%-1M)		AF(1)-1		Con A	
R_f	MW	R_f	MW	R_f	MW
0.1	416,900	0.07	467,700	0.07	467,700
0.108	398,100			0.14	354,800
0.225	251,200				
0.233	245,500				
0.26	213,800	0.28	182,000	0.28	182,000
0.33	162,200				
0.39	125,900				
0.43	107,200				
0.46	95,500	0.45	100,000	0.46	95,500
0.47	91,200	0.47	91,200	0.47	91,200
0.5	79,400	0.5	79,400	0.5	79,400
0.55	66,000	0.53	67,600	0.53	67,600
0.57	60,200	0.56	63,100		
0.58	58,900	0.57	60,200		
0.66	41,700	0.59	56,200		
0.7	35,500	0.62	50,100	0.63	47,800
0.74	30,200	0.65	43,600	0.65	43,600
0.78	25,100	0.7	35,500	0.74	30,200
		0.71	33,900		
		0.74	30,200		
		0.8	24,000	0.8	24,000
0.92	14,800	0.92	14,800	0.92	14,800
		0.97	11,800	0.98	11,200

could be that the affinity gels were binding the receptor by hydrophobic or other non-specific interactions.(Preliminary experiments with hydrophobic gels indicated that some activity was absorbed but not as much as that seen with the putative affinity gels described here.) Although it was known that active receptor could not be eluted(or more correctly could not be assayed in the eluates) from the affinity gels, it was of interest to examine the specifically eluted supernatants via SDS-PAGE.

The apparent absorption to affinity gels is shown by a second series of gels in Table 4. These affinity gels were subsequently treated as indicated in Methods(elution 2). Specific eluates(1M carbachol) from AF(1), AF(2) and biotinylated QNB-avidin gels did not stain on coomassie blue gels and silver nitrate gels were not available for this series of gels. The results from the Con A gel are shown in Figure 4(see legend for explanation). Figure 4A is soluble material included for comparison; 4B is silver nitrate stained gels and 4C coomassie blue stained gels. The gels were sequentially eluted with increasing concentrations of a-D-methylmannoside and the results are shown in Table 5. As can be seen from Figure 4, the Con A gel apparently absorbs a major band of 100,000MW that is eluted by the sugar. The Con A gel itself absorbs the soluble material in much the same manner as the affinity gels. These results can only be considered preliminary as no effort has yet been made to elute an active receptor from the Con A gels.

Discussion

TABLE 4

TABLE 4

Apparent Absorption of Muscarinic Receptor to Affinity Gels

AF(1) 1-5, AF(1) 6-7 and AF(2) 1-5 were combined and regenerated and used in a new series of tests. Con A(1 gram) was used fresh.
Approximately 50 mls of soluble material was added to each gel and shaken at RT for one hour. The gel was allowed to settle at 4°C and the binding was assayed at 500pM (^{3}H)-QNB as described in the legend to Table 2. These gels were subsequently specifically eluted as described in Methods(Elution 2).

TABLE 4

Apparent Absorption of Muscarinic Receptor to Affinity Gels

Affinity Gel	% on Gel	Total pmoles/gel
AF(1) 1-5	48	1.32
AF(2) 1-5	32	0.88
AF(1) 6-7	48	1.32
Con A	24	0.66

FIGURE 4

FIGURE 4

SDS-PAGE of Con A Gel Eluates(Table 4)

Figure 4A: See Soluble material included for comparison(Figure 3A)

Figure 4B: Silver nitrate stained gel
(see Table 5 for MW)
Lane(1): 1mM(concentrations refer to a-D-mannoside used in elution as described in Text)
(2): 10mM
(3): 100mM
(4): 1M
(5): 1M(16 hours at 4°C)
(6): Con A gel eluted with SDS(Elution 1)

Figure 4C: Coomassie Brilliant Blue Stained Gels
(as for Figure 4B)
Lane(1): 1mM
(2): 10mM
(3): 100mM
(4): 1M
(5): 1M(16 hours at 4°C)
(6): Con A gel eluted with SDS (Elution 1)

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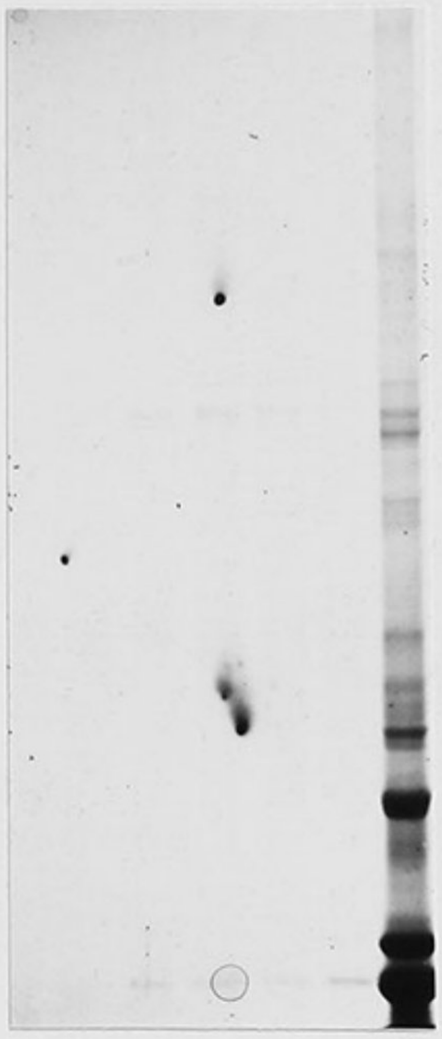


B

TABLE 5

TABLE 5

Molecular Weight of Con A Specific Eluates on SDS-PAGE

MW determined as described in the legend to Table 3.

TABLE 5

Molecular Weight of Con A Specific Eluates

Elution with a-D-mannoside	Silver Nitrate R_f	Silver Nitrate MW	Coomassie R_f	Coomassie MW
1mM	0.4	117,500		
10mM	0.39	125,900	0.45	95,500
	0.41	112,200	0.65	39,800
	0.95	11,500	0.95	11,500
100mM	0.37	138,000	0.43	104,700
	0.4	117,500	0.45	95,500
	0.41	112,200	0.65	39,800
	0.95	11,500	0.95	11,500
1M	0.37	138,000	0.43	104,700
	0.4	117,500	0.45	95,500
	0.41	112,200	0.65	39,800
	0.87	15,800	0.95	11,500
	0.95	11,500		
1M(16 hours at 4°C)	0.37	138,000	0.43	104,700
	0.4	117,500	0.45	95,500
	0.41	112,200	0.65	39,800
	0.87	15,800	0.91	13,500
	0.95	11,500	0.95	11,500
1M(Figure 3C,lane3)	0.10	416,900		
	0.45	95,500		
	0.66	42,000		
	0.81	23,000		
	0.94	12,000		

The results in this chapter indicate that modification of the aromatic end rather than the amino end of a muscarinic ligand will result in an affinity gel which will apparently absorb receptor. Efforts at eluting an active receptor from the putative afffinity gels were without success. This could be due to : 1) The low amount of protein present in the eluate or 2) Stripping of lipids from the receptor protein which as earlier chapters(3 and 4) and the results of Hulme et al.(1982) indicate , are important for receptor stability. Further development of the silver nitrate staining technique might help resolve the question of exactly what is present in the gel eluates. Addition of lipids during solubilisation and elution on columns might prevent the stripping of lipids from protein. AF(1) but not AF(2) appeared to preferentially bind the receptor. This could be due to the higher affinity of AF(1) for the receptor, but without further work no definite conclusion can be drawn.

A glycoprotein band of 100,000MW was specifically eluted from the Con A-Sepharose gel. The relation between this band and an active muscarinic receptor remains to be shown. Marquis et al.(1980) working with lysolecithin solubilised plasma membranes from lobster walking leg nerve bundles appeared to show that no QNB binding sites were specifically absorbed to Con A columns. Further work is needed to be done on this affinity compound but the results are encouraging. A second approach to affinity chromatography, the use of immunoabsorbant columns, is described in Chapter 6.

CHAPTER 6

MUSCARINIC ANTIGENS

Introduction

Affinity chromatography could also be accomplished by the use of antibodies directed against specific muscarinic determinants. In the case of impure antigens which give rise to polyclonal sera directed against a large number of determinants, the monoclonal antibody technique can be used for the selection of the antibody of interest. This in turn can be used for the preparation of an immunoabsorbant column which can be used to affinity purify the desired antigen.

The different muscarinic preparations studied in the previous chapters can all be considered as possible antigens. Ideally, a pure preparation is required where the protein of interest would make up at least 0.1%-1% of total protein present. Since this is not possible as of yet for the muscarinic receptor, a rich source of receptor could instead be used and "purification" would take place at the level of antibody screening. The caudate nucleus provides a rich source of receptor and various membrane fractions have been used as possible antigens. However, there are many receptors in the caudate nucleus and other very antigenic components which make screening for a particular component

present in very small quantities difficult.

This chapter describes our attempts at producing specific antibodies against different muscarinic preparations. Since no specific antibodies were selected, this chapter constitutes only a brief summary of the various techniques used.

Methods

Immunization

Animals were injected intradermally or subcutaneously once each week (in Complete Freund's adjuvant for the first week only) for two weeks followed by a third boost at 4 weeks(or as otherwise noted). They were then bled at different times after the final boost by the tail vein(rats), ear vein(rabbits) or orbital sinus(mice). Animals were injected interperitonally 3 days before a fusion. Rat sera was precipitated with 50% Saturated ammonium sulphate and IgG fraction was prepared.

Fusions

Fusions were performed as modified from the method of Kohler and Milstein(1975). This method will not be described in detail as specific antibodies were never obtained. Suffice it to say, that the method per se, worked well and that viable clones were obtained(80-100% frequency).

Screening of Polyclonal Sera

Classical methods were employed(see: Weir, 1978;

Methods in Enzymology, Vol. 70,73,74):

1) Plate-binding assays using ELISA or iodonated second antibodies

2) Hemagglutination

3) Direct inhibition or modification of binding activity

Screening of Monoclonal Sera

Classical methods were again tried:

1) Double-antibody precipitation of prelabeled soluble receptor

2) Inhibition of binding activity

Binding was performed as previously described using (^{3}H)-QNB.

Results and Discussion

1) Antigens

Series I: Rat(Wistar), total of 25 females

Caudate Nucleus: P3B1,P3B2 from subfractionation of microsomal pellet(see Chapter 2 for further details)>

A total of 2.4 pmoles of (^{3}H)-QNB binding sites in a total of 1 mg of protein was injected.

Cerebellum: P3, since the cerebellum contained low amounts of muscarinic receptor it was used to test non-specific interactions of sera in the different assays used.

A total of 0.05pmoles of (^{3}H)-QNB binding sites

was injected in a total of 1 mg of protein.

SDS-gels: The appropriate band was cut out(see Chapter 5), homogenised and injected.

Approximately 1 pmole of binding sites was injected in a total of 3 cm of polyacrylamide gel.

The rats were immunised as in Methods. They were bled from the tail vein after 1 month and once each week for 3 successive weeks. Polyclonal sera were screened against caudate nucleus and cerebellum membranes using iodonated Protein-A without any success. No difference was seen between caudate nucleus sera binding to caudate nucleus or to cerebellum membranes. No effect was seen on total (^{3}H)-QNB binding or affinity. Both antibody and antigen concentration was varied as were the times and lengths of incubation but no clear difference from control sera greater than 20% was ever seen.

Series II: Mouse(Balb/c), a total of 25 females

Caudate nucleus: as above, a total of 3 pmoles was injected.

Cerebellum: as above, a total of 0.05pmoles was injected.

Soluble Receptor: a total of 30 fmoles was injected.

AF(1): a putative affinity gel with receptor apparently absorbed was injected, approximately 10pmoles was injected.

The mice were immunized intradermally(if possible) once each week for three weeks and bled after 1, 2 and 8 weeks with a further boost at week 11. Iodonated Protein-A and $F(ab')_2$ fragments were used in a plate-binding assay: different brain regions were used containing varying amounts of muscarinic receptor(Cerebellum, brain stem, hippocampus, cortex and caudate nucleus). A direct binding assay was also used but no clearcut effect was ever seen. Antibodies were also absorbed with liver membranes in an effort to reduce the number of non-specific interactions but without success.

Series III: Mouse(Balb/c), a total of 25 females

Bovine caudate nucleus: a total of 5 pmoles was injected.

Caudate nucleus absorbed with cerebellum antibodies(to reduce non-specific interactions): a total of 2 pmoles was injected.

Rat caudate nucleus: a total of 2 pmoles was injected.

SDS-gel bands: a total of 1 pmole was injected.

Mice were immunized as described: once a week for 2 weeks and boosted 3 weeks after the final boost. Mice were bled after 2 weeks and tested using a direct binding assay or using the double-antibody precipitation method using pre-labeled soluble receptor. No difference from controls greater than 20% was seen.

It was decided that any antibodies against

FIGURE 1

FIGURE 1

Effect of Supernatant from Clones on (^{3}H)-QNB Binding at 500pM to Membranes

Distribution of clones from B(Bovine caudate nucleus P3), BA(Bovine P3 caudate nucleus absorbed with cerebellum) , R(Rat caudate nucleus P3) fusions. Hatched figures are control values. Supernatants from a blank fusion(using a non-immunized animal) were used for this purpose. 50ul of each supernatant from each clone were incubated with 1 ml of bovine microsomal membranes(or 1 ml of rat microsomal membranes for the rat caudate nucleus antigen). Binding was performed as per Methods(Chapter 2). The x-axis represents the number of cpms/ml of membranes and the y-axis represents the number of clones with each value.

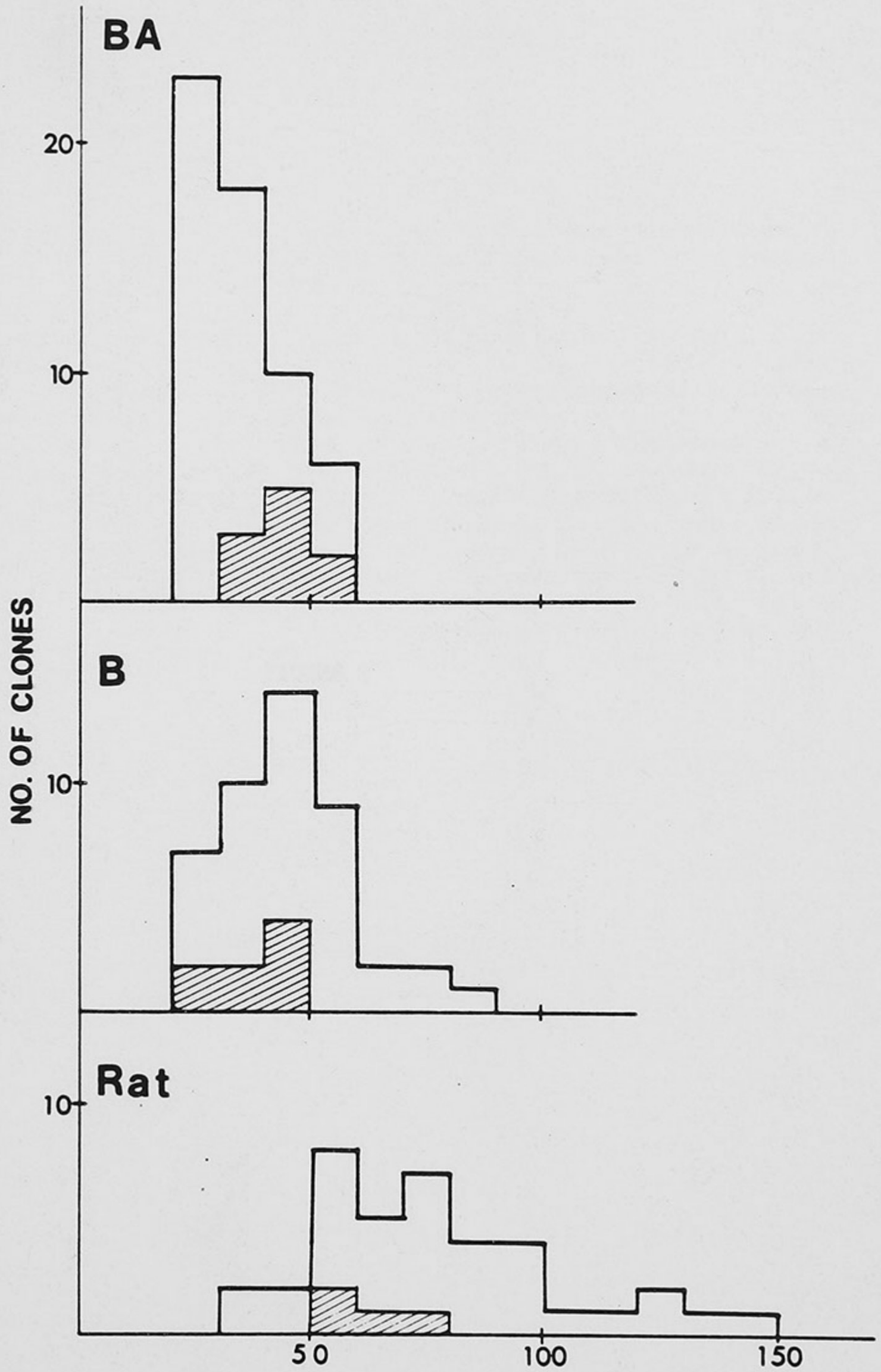
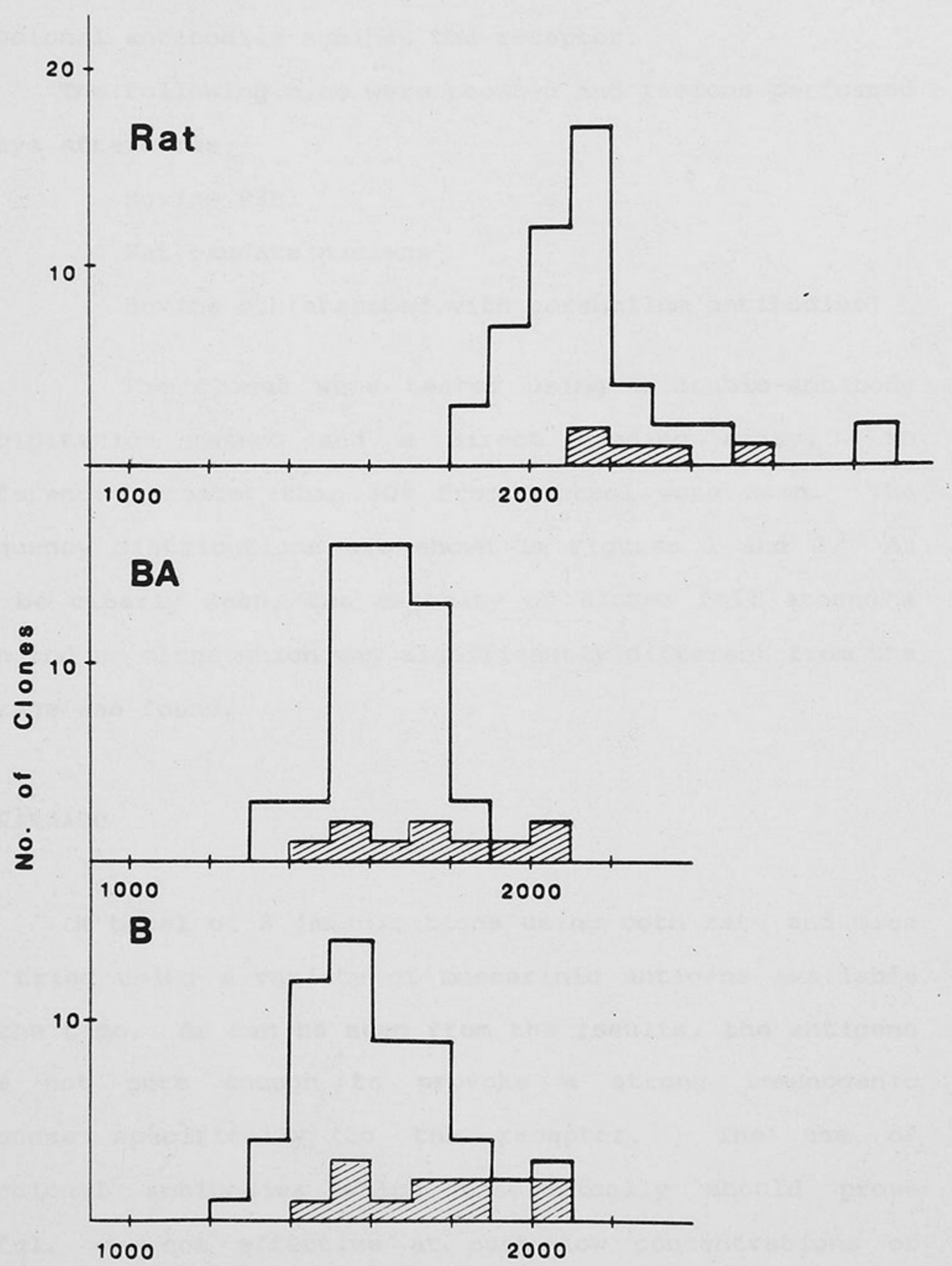

BA
20
10
NO. OF CLONES
B
10
Rat
10
50
100
150
CPM/TUBE

FIGURE 2

FIGURE 2

Supernatants from Fusions Tested using a Double-antibody Precipitation Assay

50ul of supernatant from each clone was incubated with 100 µl of solubilised prelabeled receptor(1000cpms) for 16 hours at 4°C. 20ug of IgG-anti-IgG precipitate(Rabbit-anti-mouse) were incubated for a further 5-8 hours at 4°C with the above mixture. The mixture was microfuged and the pellet washed once with 200µl of 0.1%Na cholate-1M NaCl; the final pellet was brought up in a final volume of 200ul of the cholate-salt buffer and 100ul was counted. The x-axis represents the total number of cpms per each assay and the y-axis the number of clones at each value.


Rat
20
10
1000
2000
BA
No. of Clones
10
1000
2000
B
10
1000
2000
CPMS / mi

receptor(if present at all) were present in too low a concentration to affect binding and so a fusion was tried in an effort to obtain a higher concentration of specific monoclonal antibodies against the receptor.

The following mice were boosted and fusions performed 3 days afterwards:

Bovine P3B1

Rat caudate nucleus

Bovine P3B(absorbed with cerebellum antibodies)

The clones were tested using a double-antibody precipitation method and a direct binding assay. No differences greater than 30% from control were seen. The frequency distributions are shown in Figures 1 and 2. As can be clearly seen, the majority of clones fall around a mean and no clone which was significantly different from the average was found.

Conclusion

A total of 3 immunizations using both rats and mice was tried using a variety of muscarinic antigens available at the time. As can be seen from the results, the antigens were not pure enough to provoke a strong immunogenic response specifically to the receptor. The use of monoclonal antibodies which theoretically should prove useful, was not effective at such low concentrations of receptor. It was therefore decided that it would be more useful if further purification of the receptor itself was undertaken before future immunizations were attempted. The conclusion to be reached(as indicated in the General

Introduction) is that even the monoclonal antibody technique has its limitations.

CHAPTER 7

GENERAL CONCLUSION

For a better understanding of the muscarinic cholinergic receptor and its relation to other membrane systems it is necessary to study the receptor in an isolated and purified state. This thesis has investigated the necessary first stage of any membrane-protein purification, that is, its solubilisation. Throughout the course of this work , the importance of phospholipids to receptor stability has become increasingly evident.

A novel solubilising agent (0.1% Na cholate-1M NaCl) was developed which will solubilise 10-30% of specific (^{3}H)-quinuclidinyl benzilate(QNB) binding sites from bovine caudate nucleus. As discussed in Chapter 3, the abiltiy of a variety of detergents to inhibit active binding to the receptor apppeared to indicate the importance of a protein-protein or a protein-lipid interaction which became disrupted upon solubilisation and which was necessary for an active conformation of the receptor. Using the muscarinic antagonist QNB a single saturable binding component was found with an equilibrium constant of 200pM, approximately 10-fold higher than the membrane receptor and 4-fold higher than the ratio k_{-1}/k_1 determined kinetically in the soluble material. This latter difference may indicate that the binding of QNB to the solubilised receptor is not a simple second-order process. Inhibition constants

for a variety of muscarinic agonists and antagonists were 10 to 20-fold higher in the soluble material than in the membrane state and non-muscarinic ligands were without effect. The Hill coefficients for the soluble material were all close to unity indicating the presence of a single binding site; whether the multiple binding sites of the potent agonists methylfurmethide and carbachol were transformed to the low affinity state or whether the low affinity state was selectively solubilised required further investigation.

It was shown(Chapter 3, Part II) that the decrease in affinity for muscarinic agonists and antagonists by the soluble receptor was due to the presence of high salt as was also indicated previously by the work of Birdsall et al.(1979b). The presence of high salt alone or the combination of high salt and cholate (when added to membrane preparations) did not, however, result in an increase in the Hill coefficient as seen for the soluble material. This latter difference was explained after a comparison of soluble preparations of varying specific activities, where there appeared to be an inverse relation between the Hill coefficient and the percentatge of total sites solubilised. As the total number of binding sites increased, the Hill coefficient decreased(less than 1) indicating the presence of multiple binding sites. This implied that there might be a critical percentage of total binding sites and/or other components(such as lipids) which must be present in order influence the binding parameters.

Evidence for this was shown in Chapter 4, where a link was established between receptor stability and the protein:cholate(w/w) ratio. As the proportion of cholate

increased, the stablity of binding activity at 37°C decreased and specific binding could only be detected at 4°C. This could imply that detergent is displacing endogenous lipids which are important for receptor activity either by direct association with the receptor or by a role in maintaining the receptor in an active binding conformation; thus making the receptor more temperature sensitive. That phospholipids are important for receptor activity is shown by preliminary experiments with Dr. David Wing(University Department of Pharmacology, Oxford) where a close relation is shown between the amount of phospholipid associated with the receptor(w/w) and quantitative receptor activity. These findings are in keeping with evidence found by other groups working on reconstitution of different membrane proteins who found that endogenous lipids were important in protecting against protein inactivation(see Chapter 4).

In addition, the work of Hulme et al.(1982) on cholate-salt (0.3% and 1 M NaCl respectively) solubilised muscarinic receptor showed that addition of lecithin could increase the stability of the receptor. The fact that working at higher concentrations of cholate led to a decrease in receptor stability appeared to indicate that the added cholate(0.3% vs 0.1%) did indeed displace endogenous lipid which was necessary for receptor activity. Addition of lecithin also prevented the decrease in affinity for agonists and antagonists which was otherwise seen; this has also been seen by groups working on nicotinic receptor reconstitution(see Chapter 4 for references).

Preliminary work on characterisation of muscarinic receptors compared well with the data reported by Hulme et al.(1982). Using high ratios of protein: cholate, a single

peak just inside the exclusion limit of a Biogel 0.5m column was seen; however, upon increasing the amount of cholate present, a smaller heterogeneous peak centering around 9nm was found. It might be supposed that the larger peak corresponds to the more stable of the preparations due to the presence of endogenous lipids which could lead to the formation of larger micelles; however, no definitive answer can be given until the muscarinic recpetor is in a purer state.

Efforts at eluting an active receptor from the putative affinity gels developed in Chapter 5 met with failure due either to the low amount of protein present in the eluate or to the striping of lipids(which appear to be important for activity) from the receptor protein. All of the preparations studied were also used as possible antigens for the receptor. After extensive immunizations and assays however, no specific muscarinic receptor antibody was detected. This is most probably due to the low amount of receptor present in the membrane preparations(0.005%) used as antigens.

In summary, this thesis has presented evidence that the combination of 0.1% Na cholate-high salt could reproducibly solubilise muscarinic receptors from bovine caudate nucleus. The change in affinities seen after solubilisation could be explained by the presence of high salt, and the apparent loss of high affinity agonist sites could be accounted for by the total percentage of specific sites which were present in the final soluble preparation. The sensitivity of the muscarinic receptor to a variety of detergents and the temperature sensitivity of the receptor as a function of the protein:cholate ratio indicated a role

for phospholipids in receptor stability. This was confirmed by preliminary experements which showed a relation between the amount of phospholipid present and the amount of specific activity present.

FUTURE PROSPECTS

The work presented in this thesis has provided a basis for further study on soluble muscarinic receptors. The most important question raised in this work is the exact role of phospholipids for receptor stability. Further work on analyzing the lipids present in receptor preparations and their effect on receptor stability is essential. Addition of exogenous lipids during affinity and column chromatography appears a necessary precaution. The development of sensitive SDS-PAGE staining techniques(such as the silver nitrate method) would also appear useful in order to analyze the specific eluates from affinity gels. Finally, the use of antibodies as a way of purifying proteins should be exploited, but only on preparations where the protein of interest makes up greater than 0.1% of total protein. The application of recombinant DNA technology to the problem of receptor purication is a possibility. Here one can use mRNA from different brain areas known to contain varying amounts of muscarinic receptor and screen for cDNA probes to brain regions of interest. The limiting feature in this technique would be an in vitro assay for expression of the muscarinic receptor.

The combination of Na cholate and high salt has also been used to solubilise dopamine receptors from bovine brain caudate nucleus(Dr. P.G. Strange, personnel communication); upon addition of asolectin to this preparation an increase in stability was seen. This may indicate that the

combination of low cholate (0.1%-0.3%) and high salt may prove to be useful in solubilising receptors which have in general been difficult to solubilise using non-ionic detergents and which may also signal a key role for phospholipids in the functional activity of these receptors.

CHAPTER 8

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APPENDIX 1

The following syntheses were described and performed by Dr. E.W. Gill(University Department of Pharmacology) and are included for possible future reference.

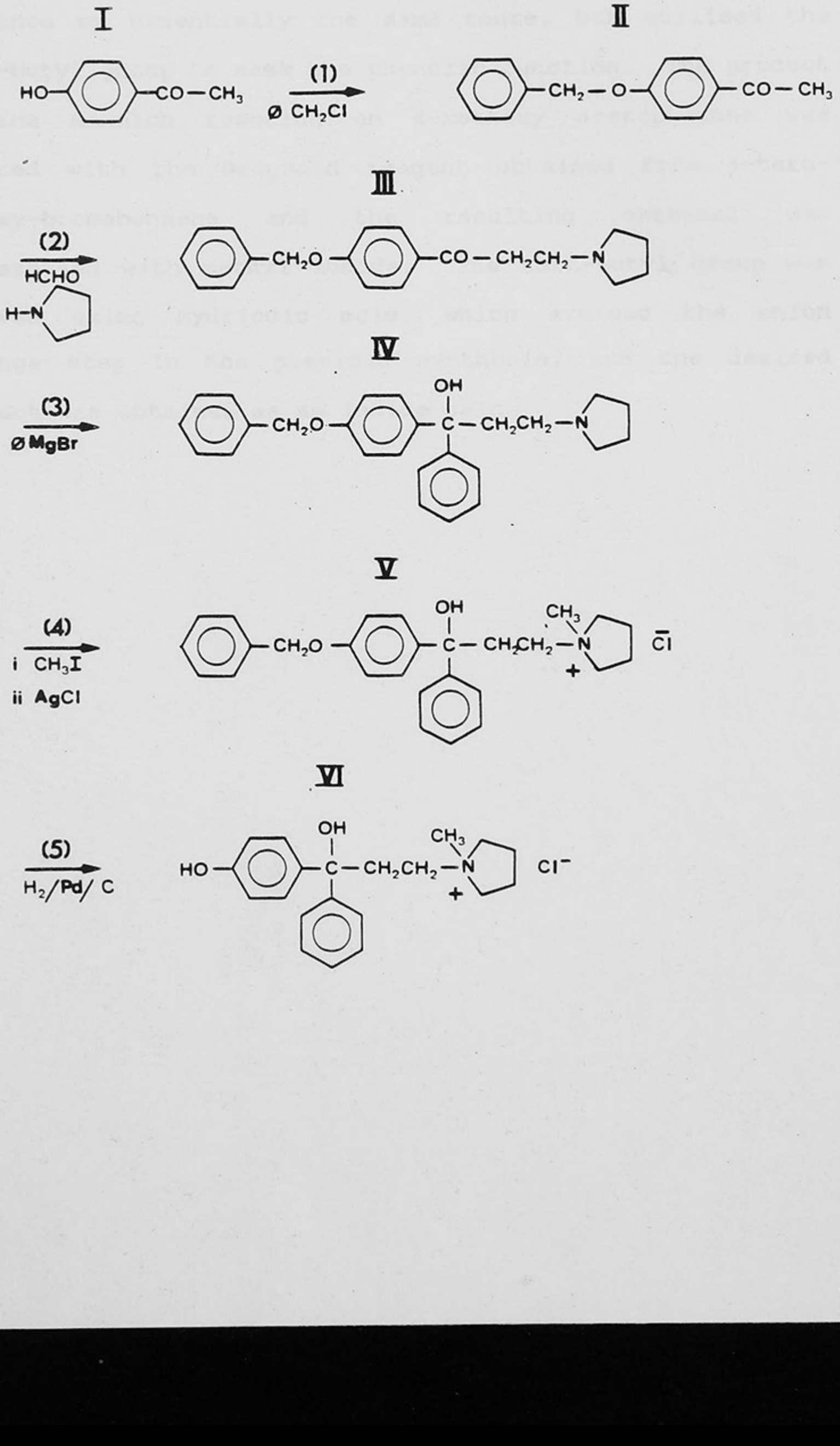
Synthesis of AF(1)

The objective of this synthesis was to produce a tricyclamol derivative that could be coupled to an epoxy-activated gel via a phenolic hydroxyl group.

The hydroxyl group of the starting material(see Figure 1), 4-hydroxy acetophenone, was blocked using benzyl chloride and base, and the resulting compound(II) was converted to an a-keto amine by the Mannich reaction, using formaldehyde and pyrrolidine. Reaction with phenyl magnesium bromide yielded the diphenyl carbinol derivative(IV), which was converted to its corresponding methiodide(V). The iodide ion was found to inhibit catalytic debenzylation so it was exchanged for chloride by shaking with solid silver chloride and the blocking benzyl group was then smoothly cleaved by hydrogenation over a palladium charcoal catalyst.

FIGURE 1

AF(1)

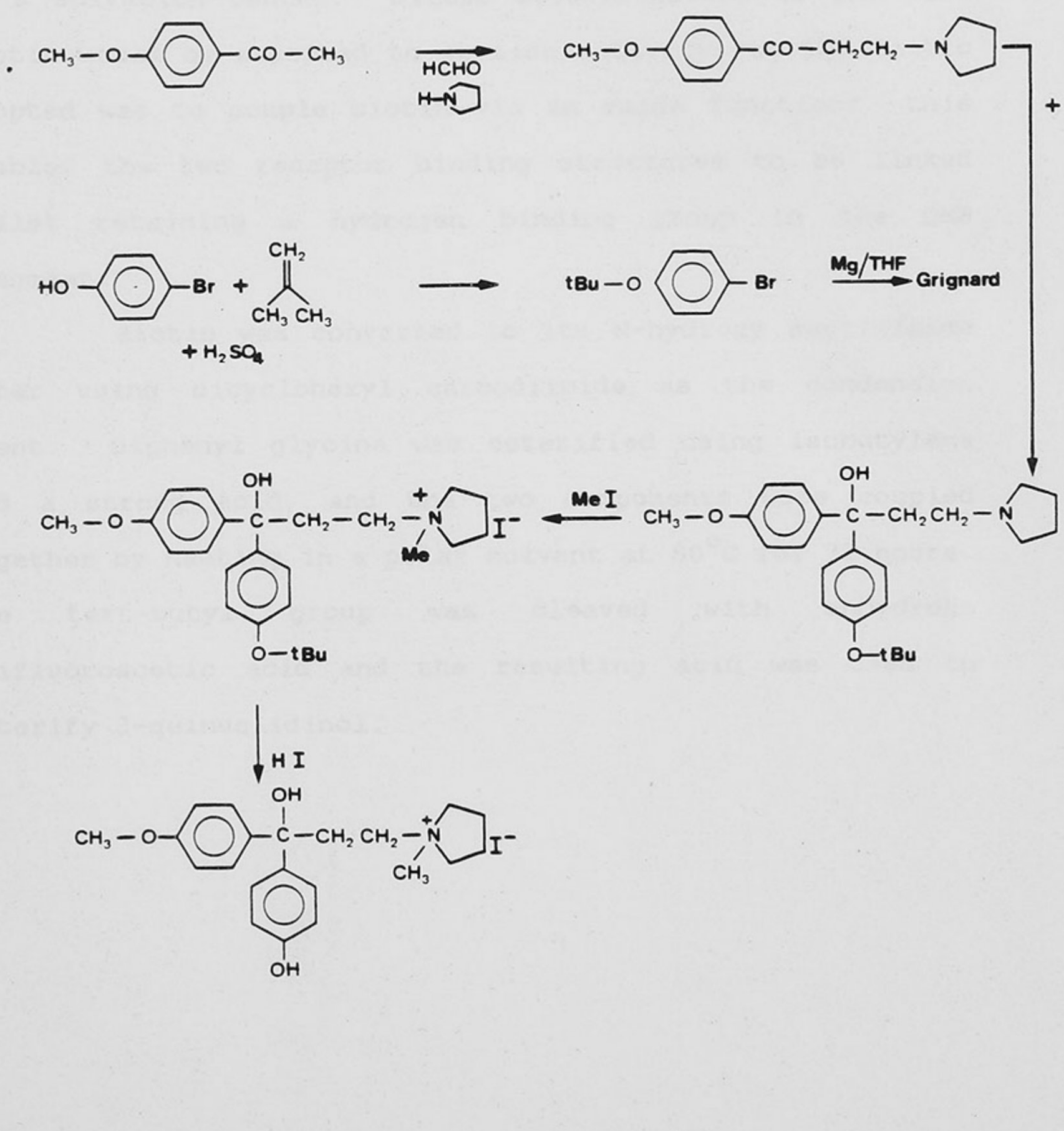

I
II
HO
CO — CH3
(1)
Ø CH2Cl
CH2 — O
CO — CH3
III
(2)
HCHO
H—N
CH2O
CO — CH2CH2 — N
IV
(3)
Ø MgBr
OH
C
V
(4)
i CH3I
ii AgCl
CH3
+
Cl
VI
(5)
H2/Pd/ C
HO
Cl⁻

Synthesis of AF(2)(Figure 2)

The corresponding methoxy substituted compound was obtained by essentially the same route, but utilised the tert-butyl group to mask the phenolic function. The product of the Mannich reaction on 4-methoxy acetophenone was reacted with the Grignard reagent obtained from 4-tert-butoxy-bromobenzene and the resulting carbinol was quaternised with methyl iodide. The tert-butyl group was cleaved using hydriodic acid, which avoided the anion exhange step in the previous synthesis, and the desired product was obtained as an iodide salt.

FIGURE 2

AF(2)


CH3 — O
CO — CH3
HCHO
H—N
CH3—O
CO — CH2CH2 — N
+
HO
Br
+
CH2
CH3 CH3
+ H2SO4
tBu — O
Br
Mg/THF
Grignard
OH
CH3 — O
C — CH2 — CH2 — N
+
Me
I-
MeI
O—tBu
HI
CH3

Biotinylated-QNB(Figure 3)

It is well known that the hydroxyl group in QNB is an important structural feature, acting either by hydrogen binding directly to the muscarinic receptor, or by serving as a solvation centre. Direct esterification of QNB with biotin would be expected to abolish activity, so the tactic adopted was to couple biotin via an amide function: this enabled the two receptor binding structures to be linked whilst retaining a hydrogen binding group in the QNB fragment.

Biotin was converted to its N-hydroxy succinimide ester using dicyclohexyl carbodiimide as the condensing agent. Diphenyl glycine was esterified using isobutylene and a strong acid, and the two components were coupled together by heating in a polar solvent at 60^{o}C for 72 hours. The tert-butyl group was cleaved with anhydrous trifluoroacetic acid and the resulting acid was used to esterify 3-quinuclidinol.

FIGURE 3

BIOTINYLATED QNB