BETA-ADRENOCEPTORS AND INTRAOCULAR PRESSURE

BY

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Date of Submission: June 1987

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ACKNOWLEDGEMENTS

I would like to thank Professor W.S. Foulds, Dr. B. Clark, Dr. Robert Logan, Dr. John Dudgeon, Professor W.S. Lee and Dr. C. Mortimer for their help and support throughout this research project. I would like to thank Sir Steven Miller and the Frost Charitable Foundation London for both their interest and financial support. I would like to thank Dr. Hazariwala, Dr. Liu, Mr. I. Finkelstein, Barbara Bednarczk and Mr. S. Niewojt for technical assistance. I would like to thank the various drug companies who kindly donated drugs toward this research project. Finally, I would like to thank Maria Cabral for typing this thesis.

The work reported in this thesis is entirely that of the author.

Material from this thesis has been published and presented at conferences and meeting as indicated on following pages.

This thesis is dedicated to my parents, Robert and Adele Trope.

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1. TROPE, G.E. ET AL.

   Effect of B_2 specific and dopaminergic drugs on rabbit I.O.P. recovery rate and buphthalmic rabbit I.O.P.

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Identification of beta-adrenergic receptors in the pigmented mammalian iris-ciliary body diaphram.

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PRESENTATIONS

1. Recent advances and adverse drug reactions in glaucoma.

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2. Effect of B_2 specific and dopaminergic drugs on rabbit I.O.P. recovery rate and buphthalmic rabbit I.O.P.

   Association for Research and Vision in Ophthalmology (A.R.V.O.), Florida, U.S.A., May 3, 1987.

3. The role of the sympathetic nervous system in intra-ocular pressure control.

   Department of Ophthalmology, Southern General Hospital, Glasgow, July 1983.

4. Adrenergic drugs and intra-ocular pressure control.

   Glaucoma Up-date Course, University of Toronto, Canada, February 1983.

5. The sympathetic nervous system and intra-ocular pressure control.

   Tennent Institute of Ophthalmology, January 1983.

6. Detection of beta-2 adrenergic receptors in the ciliary processes.

   Opthamological Society of the United Kingdom, Glasglow, April 1983.

7. Characterization of beta receptors and the affinity of Timolol to bind to these receptors in the pigmented iris-ciliary body diaphragm.

   Association for Eye Research, St. Andrews, Scotland, 10 September 1981.

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SUMMARY

Primary open angle glaucoma (P.O.A.G.) is a major cause of blindness throughout the world. This disease is characterised by raised intraocular pressure (I.O.P.) leading to progressive vision loss. Topical beta blockers decrease aqueous production and therefore lower I.O.P. How beta blockers decrease aqueous production is not known. It is presumed they exert their I.O.P. lowering effect by binding to ciliary process beta-adrenoceptors.

The purpose of this study was to determine whether beta-adrenoceptors exist in pigmented ciliary process; to determine the subtype (Beta-1 or Beta-2) of receptor present in the ciliary processes; to determine which beta blocker binds most potently to the receptors in vitro and finally to determine whether specific beta blockers inhibit aqueous production and lower I.O.P.

The results of the radioligand binding portion of this study indicate that beta-adrenoceptors do exist in pigmented animal ciliary processes (B_{max} = 98.0 ($\pm$ 7.30) fmol/mg protein, K_D = .363 ($\pm$ 0.01) nM) and they are of the Beta-2 subtype. The Beta-2 blockers LI 32-468, ICI 118551 and IPS 339 bind potently to these receptors (mean K_i values 2.91 x 10^{-8}M, 4.65 x 10^{-8}M, 8.16 x 10^{-8}M respectively).

The results of I.O.P. recovery rate experiments (aqueous humour index) indicate that Beta-2 specific blockers significantly inhibit rabbit aqueous humour production. The results of studies on rabbits with glaucoma revealed that Beta-2 blockers lower intra-ocular pressure.

In conclusion, this is the first study implicating Beta-2 adrenoceptors as the major receptor subtype controlling aqueous production and I.O.P. Furthermore, the results of this study indicate that Beta-2 blockers may prove to be a valuable new treatment for patients with glaucoma.

INTRODUCTION

I. Primary Open Angle Glaucoma and Beta-Adrenoceptors

Primary open angle glaucoma (P.O.A.G.) is a major cause of blindness throughout the world (Ghafour et al., 1984). The disease is characterised by progressive vision loss, abnormally high intraocular pressure (I.O.P.) and glaucomatous optic neuropathy probably due to failure of circulation at the optic nerve head (Hayreh, , 1972). The abnormally high I.O.P. is due to inadequate drainage of aqueous humour through the trabecular meshwork (Grant, 1958, Ashton, 1960; Tripathi, 1969; Krasnov, 1972; Segawa, 1979; Moses et al, 1981, Fine, 1981).

Intra-ocular pressure is directly related to both aqueous production and outflow (Shields, 1982). An increase in outflow resistance increases I.O.P., while a decrease in aqueous production lowers I.O.P. It is believed that lowering I.O.P. prevents glaucomatous optic neuropathy and visual field loss (Podos, 1980; McLaughlin et al, 1985).

Topical beta blockers decrease aqueous production and lower I.O.P. (Higgins and Brubaker, 1980; Zimmerman and Kaufman, 1977). The obvious assumption is that these drug induced effects are modulated by beta-adrenoceptors. This view however has been challenged (Smith, 1987; Langham, 1979; Kriegelstein, 1980; Strempel, 1982). There is little evidence of beta-adrenoceptors in ciliary process epithelium (which is the major aqueous secreting tissue (see later)) and little evidence as to whether the receptors are of the beta-1 or beta-2 subtype.

The work reported in this thesis supports the view that beta-adrenoceptors control I.O.P. Specifically we report:

1) That beta-adrenoceptors exist in pigmented ciliary processes.

2) That B_2 receptors are the major subtype.

3) That B_2 blockers inhibit aqueous production in normal animal eyes.

4) That B_2 blockers lower I.O.P. in rabbits with glaucoma.

II. The Sympathetic Nervous System and Adrenoceptors

A) General Review

The human efferent autonomic nervous system is divided into two divisions:

1) The parasympathetic nervous system.

2) The sympathetic nervous system.

The sympathetic nuclei are mainly found in the hypothalmus and medulla oblongata. Descending fibres pass down the spinal cord to innervate preganglionic cell bodies located in the intermedio-lateral column of the thoracolumbar segments of the spinal cord (T_1 - L_2). These preganglionic neurons synapse with post-ganglionic neurons in the sympathetic trunk or other specialized collateral ganglia. The post ganglionic neurons terminate in nerve endings at responsive organs. Most post-ganglionic sympathetic fibres release catecholamines from their nerve endings. These fibres are designated adrenergic neurons. Adrenergic neurons elicit adrenergic responses by liberating catecholamines that bind to end organ receptors. The end organ receptors for adrenergic transmitters are known as adrenoceptors.

In vivo adrenergic responses are ellicited by;

1) Catecholamine transmitters (especially noradrenaline).

2) Circulating catecholamines from the adrenal medulla (Adrenaline).

3) Topical systemic or parenteral administration of catecholamines or related compounds.

In vitro adrenergic responses are ellicited by exogenous catecholamines in denervated or innervated preparations. This indicates

that nerve endings per se are not required for a response. As indicated above the adrenoceptor is the specialized part of the effector cell through which catecholamines and adrenergic compounds act to evoke a characteristic response.

Briefly, the biosynthetic pathway for catecholamines is as follows (Blaschko, 1973):

L-Tyrosine
↓ Tyrosine hydroxylase
L-DOPA
↓ L-Dopa decarboxylase
Dopamine
↓ - Dopamine B - hydroxylase
Noradrenaline
↓ Phenylethanolamine N-methyltransferase
Adrenaline

In most sympathetic ganglionic fibres this synthesis stops with noradrenaline production, while in the adrenal medulla it continues on to adrenaline. In some central nervous system (C.N.S.) pathways synthesis stops at dopamine. Dopamine, noradrenaline and adrenaline are liberated as neurotransmitters in the C.N.S. (Livett, 1973; Fuxe et al., 1975; Ungerstedt, 1971).

Adrenoceptors are divided into alpha and beta subtypes (Ahlquist, 1948). Beta-adrenoceptors have been further subdivided by Land et al., into Beta-1 and Beta-2 adrenoceptors (Land et al., 1967). Alpha receptors are classified as alpha-1 and alpha-2 receptors (Hoffman and

Lefkowitz, 1980).

Table 1 illustrates the autonomic responses. It shows that adrenergic responses are mediated by alpha and beta-adrenoceptors.

Beta adrenoceptors ellicit intracellular responses by activating a membrane bound enzyme, adenylate cyclase. This results in the intracellular production of adenosine 3'-5'-monophosphate (see later).

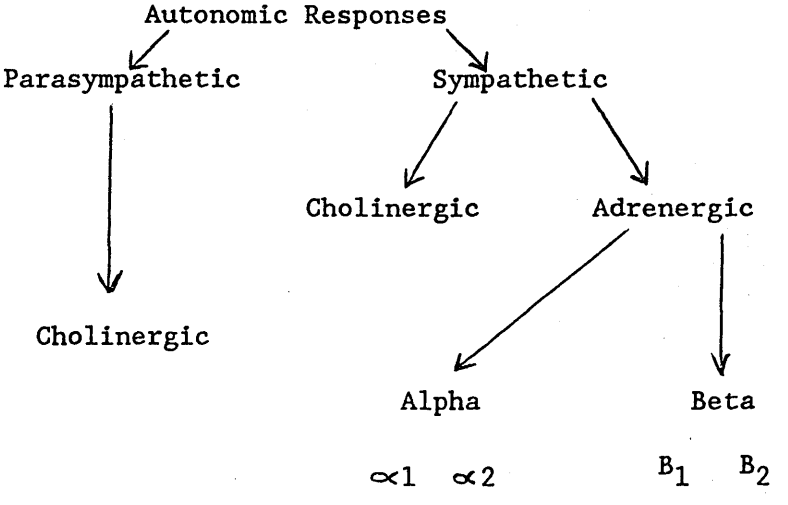


Table 1: Autonomic responses, transmitters and receptors.

Figure 1 illustrates the events that occur at the synapse of a typical sympathetic neuron and its effector cell. It shows pre-synaptic and post-synaptic receptor sites.

Alpha receptors and beta receptors on the post-synaptic membrane elicit responses when stimulated by noradrenaline or circulating adrenaline. Pre-synaptic $alpha_2$ receptors are believed by some workers to inhibit noradrenaline release from the nerve terminal (Hoffman and Lefkowitz, 1980; Lees, 1981), while pre-synaptic beta-2 receptor stimulation may cause neurotransmitter release (Majewski and Rand, 1981).

Noradrenaline is released from vesicles in the nerve terminal by a process of exocytosis initiated by the influx of calcium ions during each nerve action potential (Langer and Hicks, 1984; Lees, 1981). Once released noradrenaline diffuses into the synaptic cleft to stimulate alpha or beta receptors on the effector cell membrane. Depending on the frequency of discharge of the nerve action potential and the junctional width, presynaptic alpha-2 receptors may reduce further release of noradrenaline (negative feedback) (Hoffman and Lefkowitz, 1980).

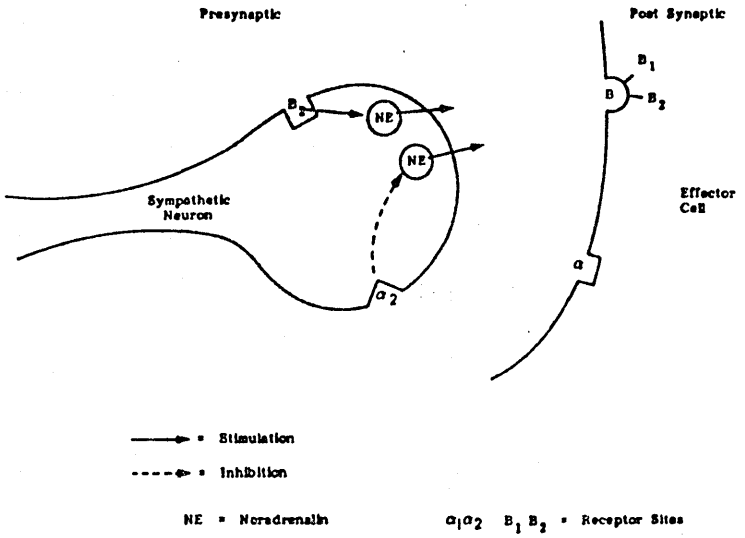
It is believed by some workers that adrenaline may be taken up from the circulation into the sympathetic nerve terminal vesicles for release with the neurotransmitter noradrenaline. Nerve action potentials and pre-synaptic beta-2 receptor stimulation by circulating adrenaline may therefore cause both adrenaline and noradrenaline release into the synaptic cleft (Majewski and Rand, 1981).

Figure 2 summarizes this positive feedback mechanism in diagramatic form (Majewski and Rand, 1981). Blockade of beta receptors at the pre

and post synaptic junctions not only prevents catecholamines from stimulating the effector cell but also may decrease the release of transmitter agents. Pre-junctional beta-2 receptor blockade is believed by some workers to be of some importance in the mechanism of action of beta blockers in hypertension (Majewski and Rand, 1981). Prejunctional dopamine (DA-2) and muscarinic receptors are believed to lead to inhibition of noradrenaline release when stimulated (Langer and Hicks, 1984).

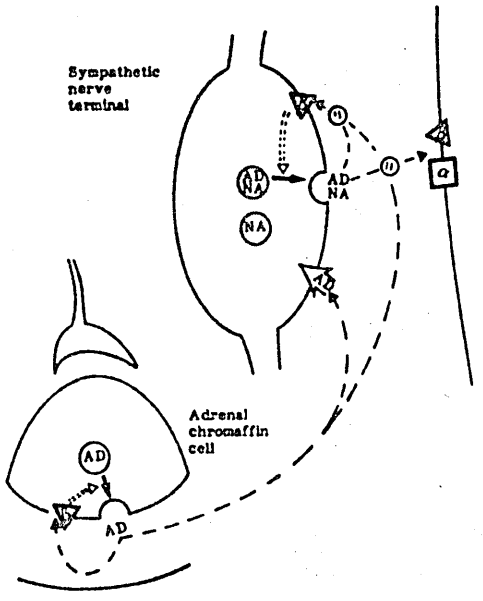
Figure 3 illustrates the fate of norepinephrine after binding to receptor sites. It can be taken up by the presynaptic neuron (uptake 1), or be taken up and metabolized by the post-synaptic cell (uptake 2), finally norepinephrine can simply diffuse away into the extracellular compartment.

Table 2 is a schematic representation of the range of action of agonists and antagonists at adrenoceptor sites. Timolol, pindolol and propranolol are shown to block beta-1 and beta-2 receptor sites, while atenolol, metoprolol and practolol mainly block beta-1 receptor sites. IPS-339, ICI-118551 and LI 32-468 are all beta-2 adrenoceptor blockers. Betaxolol, not shown on this table is also a potent beta-1 blocker. Isoprenaline stimulates both beta-1 and beta-2 receptors while salbutamol stimulates beta-2 receptors. Phenylephrine is an alpha-1 stimulant.



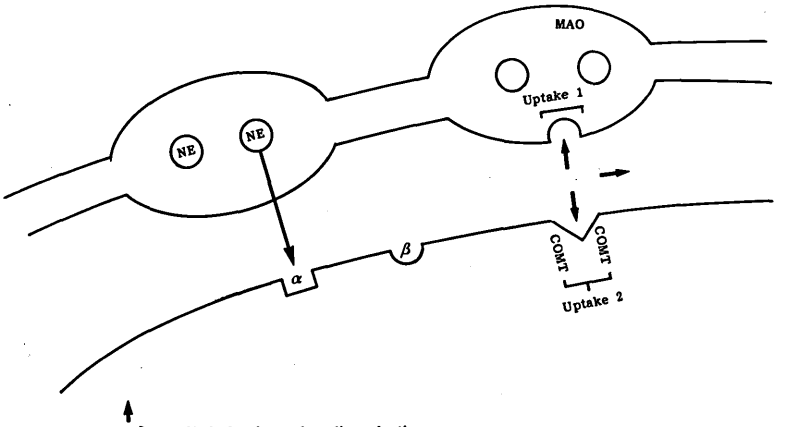
DIAGRAMMATIC REPRESENTATION OF EVENTS AT NORADRENERGIC NEUROEFFECTOR JUNCTIONS

FIGURE 1



Diagrammatic representation of possible adrenaline (AD) activation of facilitatory β-adrenoceptors. Adrenaline released from the adrenal chromaffin cells may modulate its own release by activation of facilitatory β-adrenoceptors. Adrenaline in the circulation could activate facilitatory prejunctional β-adrenoceptors at sympathetic nerve endings to increase noradrenaline (NA) release directly or alternatively, adrenaline could be incorporated into sympathetic transmitter stores and then be subsequently released by nerve stimulation to activate facilitatory prejunctional β-adrenoceptors, thus completing a facilitatory feedback loop.

(B) Beta Blockade



= Methods of noradrenalin reduction

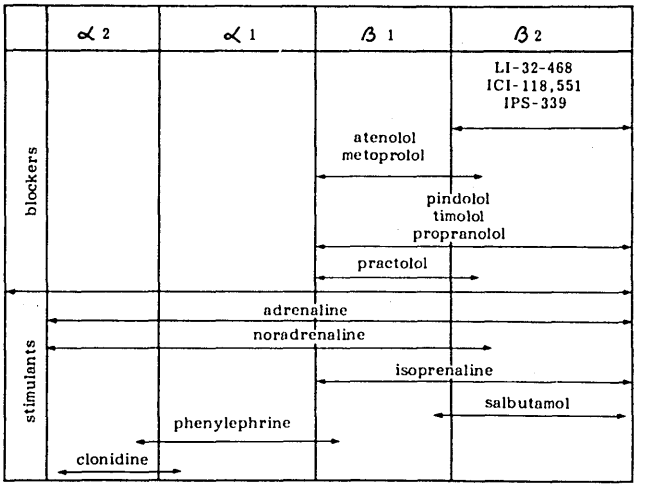
MAO = Monoamine Oxidase

COMT = Catechol-O-Methyltransferase

NE = Noradrenalin

FATE OF NORADRENALIN

Figure 3



Schematic representation of the range of action of agonists and antagonists at adrenergic receptor sites

Table 2

B) The Sympathetic nervous system and Intra-ocular pressure

Considerable evidence exists to suggest that the sympathetic nervous system plays a significant but complex role regulating eyefluid (aqueous humour) production and intra-ocular pressure. A short review of the evidence appears below.

1. Adrenergic drugs (both stimulants and blockers) influence intraocular pressure by affecting aqueous humour production (Thomas et al., 1981; Yorio, 1985; Brubaker, 1986; Podos, 1980 and Neufeld, 1979).

2. Sympathetic nerve fibres innervate the ciliary processes and lie in intimate contact with the ciliary epithelium (Ehinger, 1964). Histochemical work shows that most of the adrenergic neurones in the ciliary processes lie in a sub-epithelial plexus. Adrenergic innervation to the blood vessels supplying the ciliary processes is limited (Ehinger, 1964).

3. Stimulation of the cervical sympathetic chain affects both uveal blood flow and aqueous humour formation (Best et al., 1972; Alm, 1977).

4. Cyclic AMP is produced by the ciliary processes on adrenergic stimulation (Neufeld, 1973). Cyclic AMP is not produced by the ciliary processes in the presence of beta blockers (Neufeld, 1979).

5. Aqueous humour contains all three catecholamines (Trope and Rumley, 1985; Cooper et al., 1984; Trope et al., 1987).

The role of the sympathetic nervous system on aqueous production and I.O.P. control will be further discussed in detail later.

III) Review of Beta-Adrenoceptors

A. Beta-adrenoceptor: Structure and Size

Beta adrenoceptors are presently believed to be membrane bound glycoproteins (Lefkowitz et al., 1984; Stadel et al., 1981). The size of the beta receptor varies according to tissue, species and methodology used to detect it. The two methods usually used to study receptor structure are affinity chromatography and photoaffinity labeling (Lefkowitz et al., 1984). Other techniques however, have been described (Venter and Fraser, 1983). Mammalian beta-adrenergic receptors are reported to consist of a single glycoprotein with a molecular mass of 58,000 to 64,000 daltons (Lefkowitz et al., 1984; Venter and Fraser, 1985).

B. Beta-adrenoceptors and the Hormone-Sensitive Adenylate-Cyclase System

Beta-adrenoceptors are functionally coupled to an enzyme known as adenylate cyclase. Binding of beta stimulants to the beta-adrenoceptor adenylate cyclase complex accelerates the rate of Adenosine 3'-5' cyclic monophosphate (cAMP) synthesis. cAMP mediates and activates many intracellular reactions. All tissues with beta-adrenoceptors react by producing cAMP (Sutherland and Rall, 1980). Alpha-1 adrenoceptors are not clearly linked to the adenylate cyclase system while alpha-2 adrenoceptors inhibit adenylate cyclase activity (Michael et al., 1982).

Figure 4 illustrates the schematic model and components of the receptor hormone sensitive adenylate cyclase system (Stiles et al., 1984). Adrenergic receptors can stimulate or inhibit this system. Beta receptors are stimulatory in action whereas Alpha-2 and muscarinic

receptors are inhibitory. An important second component of the system is the catalytic moiety of the adenylate cyclase system (C). This moiety converts adenosine 5'-triphosphate (ATP) to adenosine 3':5'-cyclic monophosphate (cAMP). Details of the molecular structure of adenylate cyclase remain elusive (Ross, and Gilman, 1980). The third important component of this system is the guanine nucleotide regulatory protein (N protein, also called N, G/F or G) (Rodbell, 1980). Adenylate cyclase requires guanine nucleotides in vitro for activation. The regulatory protein is present in a stimulatory and inhibitory form. The stimulatory N protein is composed of 3 subunits known as alpha beta and gamma subunits. The inhibitory N protein is believed to be similar to the stimulatory protein except that the alpha subunit has a different size. The alpha subunit contains the guanosine-5'-triphosphate (GTP) binding site (Stiles et al., 1984).

The N protein involved in coupling stimulatory beta-adrenergic receptors contains a guanosine triphosphatase (GTPase) that stops each cycle of adenylate cyclase activation by hydrolyzing GTP to guanosine-5'-diphosphate (GDP) (Lefkowitz et al., 1985) (Cassel, and Selinger, 1974). Cholera Toxin activates cyclic AMP by inactivating the GTPase portion of the alpha subunit of the Ns protein. This inactivation inhibits the physiological turn off mechanism for adenylate cyclase and makes GTP an irreversible activator i.e. the Ns protein is unable to stop adenylate cyclase activation (Cassell and Pfeuffer, 1978).

Figure 5 further elucidates this complex reaction (Stiles et al., 1984). The initial event is the binding of agonist H to receptor R to form a low affinity H.R. complex. This binary complex then forms a high

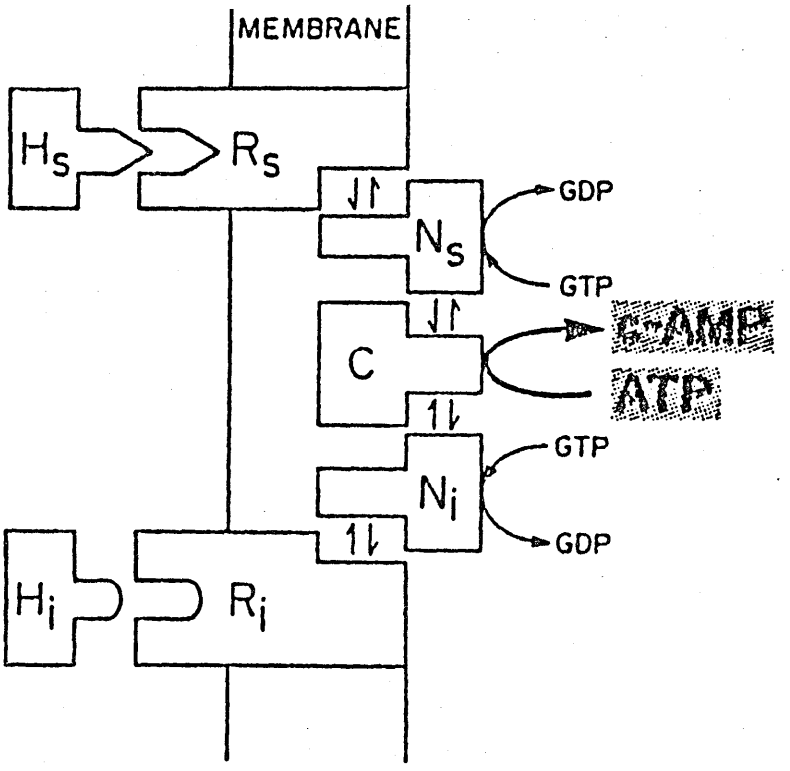


FIG. 4 Schematic model of hormone-sensitive adenylate cyclase system. ATP, adenosine triphosphate; C, catalytic unit; cAMP, adenosine 3':5'-cyclic phosphate; GDP, guanosine 5'-diphosphate; GTP, guanosine 5'-triphosphate; H, hormone; i, inhibitory; N, guanine nucleotide regulatory component; R, receptor; s, stimulatory.

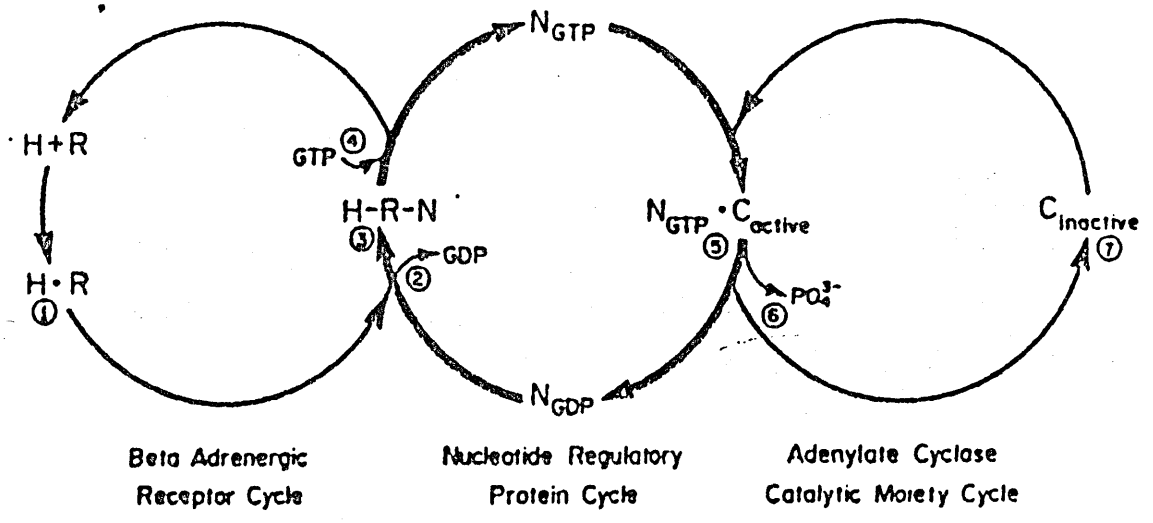


FIG. 5. Working model for adenylate cyclase regulation by β-adrenergic agonists and guanosine 5'-triphosphate (GTP). H, β-adrenergic agonist; R, β-adrenergic receptor; N, guanine nucleotide regulatory protein; C, catalytic moiety of adenylate cyclase.

Above two figures produced from Stiles et al., 1984 with permission from the editor.

affinity tertiary complex with the N protein HRN. A change occurs in the N protein such that its subunits dissociate. GTP binds to the alpha-subunit. This N-GTP unit complexes with the catalytic moeity of adenylate cyclase (C) thereby activating and forming the $N_{GTP}C$ complex which is assumed to be the active form of the enzyme. The GTPase moeity on the N protein rapidly cleaves GTP to GDP thereby destabilizing the $N_{GTP}{}^{C}$ complex reverting its activity to base level. If hormone is removed or its antagonist is added, the entire chain of events is blocked because there is no impetus to the formation of the crucial intermediory HRN (Ross, 1977; Stiles et al., 1984; Lefkowitz, 1984).

The intracellular biological effects of cAMP are mediated [illegible] protein kinase. (Greengard, McAfee and Kebabian, 1972). cAMP is metabolized by cyclic nucleotide phosphodiesterase to 5'AMP and inorganic phosphate. (Thompson and Applemon, 1971).

C) Modulation of Beta-adrenoceptor adenylate cyclase activity

Two types of mechanisms exist to regulate beta-adrenoceptor function namely; 1) Homologous regulation, 2) Heterologous regulation.

Homologous regulation is the term sometimes used to denote the regulation of numbers or concentration of receptors by hormones or drugs that usually combine with the receptor (Su and Harden, 1980), e.g., adrenaline and propranolol. The term heterologous regulation is used to denote receptor regulation by drugs or hormones that do not ordinarily bind to beta-receptors, for example steroids and prostaglandins.

1) Down Regulation

Homologous and heterologous regulation are believed to be able, at

least in the short term, to cause translocation of receptors from the plasma membrane to the intracellular compartment (Stiles et al., 1984) (Chuang and Costa, 1979) (Stadel et al., 1983) (Su et al., 1980). Short term decrease in receptor numbers from agonist stimulation is thought to be due to this endocytic process (Stadel et al., 1983). Once internalized, the receptors seem to reside in vesicles. On removal of the agonist, receptor numbers increase probably via two mechanisms, namely a recycling process and in some cell lines new protein synthesis (Doss, Perkins and Harden, 1981). In this latter situation it is believed that the internalized receptors are degraded (Stiles et al., 1984).

In addition to this rapid down-regulation of beta-adrenoceptor density, long term exposure to adrenergic drugs in vivo induces a gradual alteration in receptor density that persists for several weeks (Vitulani and Salser, 1975). Stiles et al and others have suggested that the effects observed in receptor numbers after agonist use over the long term may be controlled by beta-receptor gene transcription affecting both the rate of receptor synthesis and perhaps degradation as well (Stiles et al, 1984) (Chiu, 1978) (Dax, Partilla and Gregerman, 1981).

Another method of regulating beta-adrenoceptor function involves receptor uncoupling to the adenylate cyclase system. It is believed that there maybe a number of different kinds of processes that lead to uncoupling (Su et al., 1980) (Harden et al., 1980). Phosphorylation reactions may be involved in this uncoupling reaction (Stiles et al., 1984). In some cases, cAMP is possibly a mediator. In heterologous

desensitization N-protein alterations may also occur which could be responsible for this effect. To date little information exists on receptor uncoupling (Stiles et al., 1984).

In summary multiple mechanisms are involved in modulation of catecholamines induced desensitization of adenylate cyclase. Agonists induce down regulation by promoting receptor internalization and also sequestration away from the other components of the adenylate cyclase system. The receptors are then degraded or recycled to the cell surface. In other cases the receptors can become functionally uncoupled from adenylate cyclase.

2. Up-Regulation

Long-term reduction of circulating catecholamines or adrenergic denervation induces "up regulation" i.e. an increase in receptor numbers, e.g. after adrenalectomy there is a marked increase in both number of liver beta-adrenoceptors and adenylate cyclase activity (Wolfe et al., 1976). The exact mechanism controlling this phenomenon is not clear. Up regulation has however been confirmed by a variety of workers (Kajiyana and Acgawa, 1980) (Glaubiger and Lefkowitz, 1977) (Glaubiger et al., 1978) (Tenner et al., 1982). Whether up regulation is responsible for the well known supersensitivity that occurs after propranolol withdrawal however is uncertain (Boudoulas et al., 1977; Stiles et al., 1984; Kennedy and Connelly, 1982). Upregulation of beta receptors has been suggested as a reason for the slight decrease in

efficacy of beta blocker eye drops in patients with glaucoma (Boger, 1979).

Heterologous beta-adrenoceptor regulation does occur. Thyroid hormones for example regulate beta-receptor density and possibly affinity as well. Hyperthyroidism is associated with an increase in receptor numbers, while hypothyroidism is associated with a decrease in receptor numbers. The exact mechanism responsible for thyroid regulation of the receptor numbers remains unclear (Stiles et al., 1984).

D) Receptor Identification (Burt, 1985)

A binding site is identified as a receptor for a drug or transmitter if it meets certain criteria. The binding site should

1) be present in FINITE numbers and have high affinity (saturability).
2) have acceptable kinetics
3) be stereoselective and bind drugs in a predictable rank order of potency.
4) be found only where receptors are expected (distribution)
5) be occupied by drugs effective at the receptor at concentrations in proportion to those needed to produce a biological response (Pharmacology).
6) be destroyed by high temperature (temperature sensitivity) and influenced by other factors, (for example ph and ions) (Burt, 1985; Motulsky and Insel, 1982).

1. Saturability

To determine whether receptors exist in a tissue there must be a minimal requirement of a finite number of binding sites of fairly high affinity (i.e. with a dissociation constant in the nanomolar range or lower).

a) Saturation:

Indicative of receptors is the phenomenon of saturation, that is as one adds increasing concentrations of radioligand, binding to receptor sites increases to a point until all sites are occupied. Thereafter additional concentration of hot ligand binds only to non-receptor sites. Binding to non receptor sites is known as non-specific binding. Total binding is determined for each concentration of radioligand. Total binding is determined by measuring the radiation emitted from each sample. It represents the amount of radioligand bound to both receptor sites and non-specific binding sites (Motulusky and Insel, 1982).

It is essential to determine exactly how much non-specific binding occurs. This is done by incubating samples ("Blanks") of the radioligand membrane preparation together with a fixed concentration of a non radioactive drug which is known to bind only to the receptor sites, e.g. 1 uM (-) propranolol for beta-adrenoceptors (Mukherjee et al., 1975). This "cold ligand" competes with radioactive ligand for the receptor sites and displaces radioligand off these sites. The residual amount of radio-activity bound non-specifically to membrane preparations is then measured. Specific binding is determined for each concentration of radioligand from the formula: Specific binding = Total binding -

Non-specific binding (Motulusky and Insel, 1982). Non-specific binding is therefore potentially infinite while specific binding characteristically shows saturation (Burt, 1985).

b) Number and Affinity of Sites

It is well known that too large a number of receptors with low affinity suggests binding is not to a receptor. Receptor density (B_{max}) in brain for example falls in the range of 1 - 100 pMoles/g weight of tissue (Burt, 1985). Rat cerebral cortex beta adrenoceptors have a Bmax of 12 pmol/g and a K_D of 0.1 nM. Rat heart beta-adrenoceptors have a Bmax of 5-10 pMol/g with a K_D of 1-2 nM.

Mathematical analysis of saturability data, allows one to determine both affinity and receptor numbers and will be considered in the next chapter. Graphical analysis of binding data is often performed using micro-computer assisted programs e.g. ligand (Munson and Rodbard, 1980) or ligand (Applesoft) (Jackson and Edwards, 1984). For this study, we used an Apple II micro-processor with a program developed by Clark et al. (Clark et al., 1983).

c) Competition by Non-Radioactive drugs

In the presence of a finite number of bindings sites, addition of enough non-radioactive drug molecules of the same or different drug, results in competition with radioactive molecules for occupation of the limited number of receptor binding sites. This competition, also known as displacement, reduces the number of radioactive bound molecules. The Pharmacology of binding refers to the effectiveness of different drugs acting as competing agents. Some form of competition experiment using

one or more drugs is often among the initial experiments used when trying to identify a receptor.

2. Kinetics

Binding should occur with speed and should be rapidly reversible. The equations describing simple binding reactions predict that the equilibrium dissociation constant should be given by the ratio of the rate constant for dissociation to the rate constant for association (Lefkowitz, 1976; Insel and Stoolman, 1978). Kinetic studies are an important aspect of receptor identification and will be considered further in the next chapter.

3. Distribution

On detecting a high affinity saturable binding site, further experiments should be performed on tissues from different areas of the body to ensure the results are in keeping with known data on that specific receptor type. It is important to note however that large differences in receptor numbers or characteristics exist in various species and sub-cellular fractions. Receptors should also be of the type innervated by the appropriate nervous system.

4. Pharmacology (Burt, 1985)

It is important to relate the data derived from binding experiments to data derived from biological responses (Pharmacology). Drugs that are effective in mimicking or blocking the effects of a neurotransmitter should be shown to compete for binding at low concentrations whereas ineffective drugs should compete at high concentrations if at all.

a) Stereospecificity

A most useful pharmacological criterion for identifing receptor binding is stereospecificity which may involve either the radioligand itself or non-radioactive drugs used as competing agents. Many responses of biological systems distinguish between stereoisomers. Clearly, binding to receptors mediating these responses should be the same (Synder, Pasternak and Pert, 1975; Aurbach et al., 1974; Motulusky and Insel, 1982).

b) Choice of Blank

With multiple binding sites a critical factor in identifying the receptor of interest involves using the correct choice of blank. Use of a large excess of the same drug, in non-radioactive form is often inappropriate as it competes for all classes of binding sites. Rather a very carefully chosen excess of the neurotransmitter itself is often a good choice, although other highly specific drugs are reasonable alternatives e.g. 1 uM (-) propranolol for beta adrenoceptors (Mukher, J. et al., 1975). In many cases the concentration of drug used as the blank (non-specific binding) in saturation experiments is 100 times the concentration needed to inhibit specific binding by 50% (IC_{50}).

c) Agonist and Antagonists

Apparent binding affinities typically differ according to whether an agonist or antagonist is used as radioactive ligand. A variety of explanation exists for this effect including preferential binding of agonist and antagonists to different receptor conformational states, desensitized receptor binding to agonists and multiple classes of

agonist binding. A theory of spare receptors exists to explain the increased biological effect seen with agonists compared with the agonists in vitro binding potency (Takeyasu, 1979). The net result of these considerations is that only the affinities of antagonists competing for antagonist binding regularly match biological potencies (Burt, 1985).

d) Additivity with Blank

When 2 or more drugs are available as blanks an important criterion for identifying the portion of total binding representing receptor binding involves showing that maximally effective concentrations of both drugs compete for the receptor sites whether they are added individually or in combination.

Other aspects of pharmacology of binding include non-competitive agents, use of antibodies to identify receptors and response of the tissue under study to radioactive ligand used in a binding experiment (Burt, 1985).

5) Other Important Points

a) Tissue Linearity

Tissue linearity is important in binding studies to demonstrate the absence of artifacts such as receptor or ligand degradation during incubation and/or unrecognized endogenous ligand binding. Although a slight degree of downward curvature in a graph plotting binding against added tissue is unlikely to lead to wrong receptor identification, it will yield incorrect or ambiguous values for such parameters as density of binding sites. It is therefore desirable to find conditions that

give linearity of binding with tissue concentrations, even if this entails one or more membrane purification steps and a lower recovery of receptors. The unusual condition of upward curvature in tissue linearity graphs, which is equally undesirable, may indicate an inappropriate blank or loss of binding sites during the separation from free ligand (Burt, 1985).

b) Temp. Dependence

Temperatures of greater than 40 ^{o}C decrease or destroy receptor binding. Binding at higher temperatures suggests the possibility of a covalent chemical reaction of the radioactive ligand or breakdown product rather than reversible receptor binding.

c) Effect of ph and Ions

Receptors operate under physiological conditions usually at pH 7.4. Extremes of pH decrease or eliminate binding as the receptor structure disrupts.

Effects of ions are less predictable on binding but can aid receptor identification when the effects of receptor activation on ionic conductances are known and there is close or direct coupling between the receptor binding site and "ionic conduction modulation" (Burt, 1985).

d) Identity of Bound Radioactivity

If a new radioligand is used it is important to demonstrate that most or all bound radioactivity still represents the original ligand. Verification of the identity of bound radioactivity remains an important

part of receptor identification.

Final proof of receptor identification requires purification of the binding site and demonstration that adding the purified sites to model membrane systems results in these systems gaining receptor properties (Burt, 1985).

E) LIGAND RECEPTOR BINDING EXPERIMENTS: Practical and Theoretical Considerations.

1) **Binding Experiments (Bennet and Yamamura, 1985)**

a) General Mathematical Considerations

Ligand receptor binding studies follow kinetics similar to classic enzyme substrate interactions. For reversible ligand receptor interactions where

[L] = concentration of ligand

[R] = concentration of occupied receptors

[LR] = concentration of ligand receptor complex

$$a[L] + b[R] \underset{K_{-1}}{\overset{K_{+1}}{\rightleftharpoons}} c[L.R.] \qquad (1)$$

This formula represents a reversible binding phenomenon, with a, b and c representing stoichiometry of the reaction. At equilibrium, the rate of the forward reaction equals the rate of the reverse reaction namely; $K_{+1}\ [L]^a\ [R]^b = K_{-1}\ [LR]^c$ (2)

The equilibrium binding constant can be therefore defined as an association binding constant (K_A)

$$K_A = \frac{K_{+1}}{K_{-1}} = \frac{[LR]^c}{[L]^a\ [R]^b} \qquad (3)$$

or as a

dissociation binding constant (K_D)

$$K_D = \frac{K_{-1}}{K_{+1}} = \frac{[L]^a\ [R]^b}{[LR]^c} \qquad (4)$$

Therefore to determine the equilibrium dissociation constant for a reversible reaction requires that the experiment be performed under steady state conditions.

An important property of ligand receptor interaction is saturability; that is only a finite number of specific receptors exist per unit of tissue. This maximum number of specific receptors is designated B_{max}

$$[LR] + [R] = B_{max}$$

multiply by [L]

$$[LR]\ [L] + [L]\ [R] = B_{max}\ [L]$$

$$[LR]\ [L] + \frac{[LR]}{[LR]}\ [L]\ [R] = B_{max}\ [L]$$

Substitute equation 4 with a = b = c = 1. This gives

$$[LR]\ ([L] + K_D) = B_{max}\ [L]$$

$$[LR] = \frac{B_{max}\ [L]}{[L] + K_D} \qquad (5)$$

which is the classic law of mass action for enzyme substrate interactions adapted to ligand - receptor interactions.

If we now define [LR] as bound ligand = B, and L as free ligand = F, from equation 5

$$B = \frac{B_{max}F}{F + K_D}$$

$$BF + BKD = B_{max}F$$

divide by F

$$\frac{B + B\ KD}{F} = B_{max}$$

Transferring fields

$$\frac{B}{F} = \frac{B_{max} - B}{K_D} \qquad (6)$$

which is the Scatchard Equation (Scatchard, 1949). Thus knowing the concentration of ligand bound and free at equilibrium allows the determination of both the equilibrium binding constant K_D and the maximum number of binding sites B_{max} (Bennett and Yamamura, 1985).

b) Separating Bound from free ligand

Filtration and centrifugation are the major techniques used to separate bound from free ligand. Other techniques used usually on soluble binding sites include dialysis, column chromatography, precipitation of ligand receptor complex and adsorption of free ligand.

During washing, i.e. the process used to separate bound from free ligand, some ligand will dissociate from the complex. This rate of dissociation of the ligand receptor complex is the major constraint on separation techniques. Loss of binding due to dissociation usually increases linearly through the first half-life for dissociation of the receptor ligand complex. To avoid losing more than approximately 10% of bound ligand it is recognized that the separation procedure should be complete in less than 0.15 $t_{1/2}$ (assuming an association rate constant of $10^6 M^{-1}$ Sec^{-1}). Because it is desirable to wash the ligand receptor

complex as thoroughly as possible to remove free unbound ligand, the time of washing (which forms part of the total separation time, i.e. the total time in which the ligand receptor complex can dissociate during separation) plays a crucial role in increasing non-specific bound ratio or decreasing the specific bound ratio. As indicated studies have determined that 0.15 $t_{1/2}$ is a desirable maximum separation time. The approximate upper limit for separation time has been calculated for various steady state dissociation binding constants, for example a K_D of 10^{-6}M has an allowable separation time of only 0.01 sec. 10^{-8} M has an allowable separation time of 10 sec. 10^{-10} M has an allowable separation time of 17 mins. 10^{-11} M has a separation time of 2.9 hours.

1) Filtration

Filtration techniques allow for rapid and efficient separation of bound from free ligand especially for particulate receptor studies. As indicated above filtration works only for ligand receptor binding processes with steady state dissociation constant (K_D) values of $\pm 10^{-8}$ M or less due to time constraints.

A potential disadvantage of filtration is non-specific binding of radioligand to the filter material (Pasternak and Snyder, 1975). Thus it is important to select a filter that minimally binds radioligand or to include anti-adsorbants that minimize this non-specific binding. Filtration was used during this project.

2) Centrifugation

This method is useful for particular ligand-receptor studies under two conditions 1) where rapid dissociation occurs especially in association with low affinity receptor binding (10^{-7} M or greater), 2) where non-specific binding to filters cannot be reduced.

This technique involves surface washing of the pellet. This is less efficient than filtration techniques in separating bound ligand from free ligand. Unbound radioligand is often trapped in the pellet water space (Streeten, 1982). With centrifugation rapid careful rinsing of the tube and pellet with ice-cold buffer is performed to remove as much unbound radioligand as possible without allowing the rinsing buffer to remain in contact with the pellet for more than a few seconds as this may result in dissociation of the ligand receptor complex or else disrupts the integrity of the pellet with mechanical loss of ligand receptor complex. Conical microcentrifuge tubes containing dibutylphthalate-dinonylphthalate oil mixtures are used for such procedures. If mixed in a 3:2 ratio the oil is denser than isotonic or hypotonic aqueous buffers but less dense than for example brain preparations. A small quantity of oil is placed at the bottom of the tube and the tissue preparation in buffer containing ligand is layered on top. After incubation and washing samples are centrifuged in microcentrifuge tubes. The tip of the microcentrifuge tube containing the pellet in oil is then cut off and bound radioactivity measured. This procedure allows for rapid separation of the particulate bound matter from unbound and incubation buffer. The disadvantage as previously mentioned is related to insufficient washing of the pellet.

Also, very lipophilic radioligands can potentially diffuse into the oil layer giving elevated inconsistant non-specific binding.

3) Equilibrium Dialysis

This technique is suitable for receptors with low affinity ligands (10^{-7} M or greater) and thus very rapid dissociation rates. This technique involves the use of small dialysis cells of less than 1 ml volume (separated by semi-permiable membranes). Rapid equilibration of radioligands occurs across the semi-permiable membrane between the receptor and the buffer sides of the dialysis cell. A major disadvantage of the dialysis technique is the small difference in radioactive counts usually found between the receptor and the buffer side of the dialysis membrane. Large amounts of receptor protein must be added to one side of the dialysis chamber to achieve significant binding. It is also necessary to determine if the dialysis membrane itself contributes to radioligand binding (Bennett and Yamamura, 1985).

4) Gel Filtration Chromatography

This is a sensitive but tedious method of detecting bound radioligand in solubilized or physiologically soluble receptor preparations. There are 2 methods presently used both performed at low temperatures to retard receptor ligand dissociation (Bennett and Yamamura, 1985).

Gel filtration techniques have been used to study soluble cytoplasmic steroid hormones from brain and other tissues. The major limiting constraint is the dissociation rate of the ligand-receptor complex as it passes through the column. Also, the resin bed offers the

possibility of non-specific binding of radioligand, a condition that must be ruled out.

5) Precipitation of ligand-receptor complex and adsorption of free ligand.

The precipitation technique depends on a decrease in solubility of large proteins resulting from the addition of structural perturbing agents, e.g. ammonium sulfate, salts acids or polyethylene glycol. Successful studies using this technique include solubilized insulin, adrenergic, and dopamine receptors.

The adsorption of free ligand technique uses the selective adsorption of unbound radioligand to a slurry of inert support such as talc or charcoal. The inert support is often coated with a carbohydrate polymer (dextran). Small unbound molecules rapidly penetrate the dextran shell and are bound by the inert support, whereas large ligand receptor complexes are excluded from access to this adsorbent. The adsorbent is isolated by centrifugation or filtration and the amount of unbound ligand is measured. The difference between total radioligand and the unbound ligand adsorbed to the support represents the concentration of bound ligand (Bennett and Yamamura, 1985).

6) Non-Specific Binding: General Points (Bennett and Yamamura, 1985)

As previously indicated specific binding is considered as the difference between total binding and the binding that occurs in the presence of an excess concentration of unlabeled ligand. At this point non-specific binding may be composed of 1) true non-specific binding to the tissue, that is ligand not bound to receptors, 2) free radioligand

not effectively washed away, 3) non-specific binding to separation materials such as filters.

It is necessary to wash the preparations to get rid of free radioligand. This washing procedure should be optimized by comparing various numbers of washing and volumes of washing buffers.

Radiolabelled chemicals used as ligands in receptor-binding studies possess the ability to bind non-specifically to both biological and non-biological substances, for example, radiolabelled insulin binds to talc with affinities in the nanomolar range (Chatrecasas and Hollenberg, 1976) which is similar to the affinity of insulin for its biological receptor. Significant non-specific binding to separation materials can often be reduced by including anti-adsorbants in the assay mixture. Albumin or collagen are useful for radio-iodinated peptides and 0-catechol for catecholamine binding studies. Anti-adsorbants themselves can paradoxically bind significant amounts of radioligand thereby reducing available free ligand concentration. As low a concentration of anti-adsorbant as possible should be used to lower non-specific binding.

It is generally accepted that for binding studies, the competitive ligand should be chemically different from the radio-ligand used in the study. The use of a disimilar drug that binds potently to the receptors increases the probability of obtaining specific receptor binding.

7) Metabolism of radioligand (Bennett and Yamamura, 1985)

Radioligand bound to a receptor or free in solution can be metabolized or degraded. Analysis of both bound and free radioligand

can be performed by both conventional analytical procedures such as HPLC, TLC and electrophoresis and by rebinding studies. The principle of the chromatographic assays involves comparing the migration of radioligand after exposure to the tissue receptor to migration of radioligand not exposed to tissue receptor, but analyzed with homologous tissue extract. Rebinding studies involve eluting radioligand off the receptor site under mild acidic or denaturing conditions and rebinding it to a fresh receptor preparation alongside fresh previously unexposed radioligand. At equal concentrations of exposed and unexposed radioligand the two results should be equal if no metabolism or structural changes has taken place to the previously exposed radioligand. In addition to the above, the original unbound or free (radioligand) can be examined by rebinding studies as well. If the ligand is a natural product or a derivative of one (such as catecholamines) catabolism can be prevented by reducing the incubation temperature or via the addition of various drugs (e.g. MAO). Nonenzymic oxidative degradation or light degradation is inhibited by using antioxidants or dark incubation using a sodium lamp for visualization.

2) ANALYSIS OF BINDING DATA

a) Saturation Experiments

To determine the equilibrium dissociation constant K_D and apparent number of binding sites (B_{max}) for a radioligand and tissue receptor the following factors are involved:

1) Various concentrations of radioligand are incubated with fixed amounts of tissue.

2) Bound ligand concentration is determined by filtration or other techniques as previously described.

3) The resulting data is analyzed according to the Scatchard equation.

1) Dependence of apparent K_D on tissue receptor concentrations.

The apparent K_D can be influenced by receptor concentration. Estimation of K_D from standard binding isotherms can overestimate the magnitude of K_D as the total receptor site concentration (RT) is increased. One method to overcome this problem is to perform saturation studies using different concentrations of tissue. The results are then analyzed by nonlinear least square regression analysis. True K_D can then be determined from apparent K_D (Bennet and Yamamura, 1985).

2) Scatchard Plots.

The Scatchard equation

$$\frac{B}{F} = \frac{B_{max} - B}{K_D}$$

can also be written as

$$\frac{[LR]}{[L]} = \frac{[RT] - [LR]}{K_D}$$

This allows for linear transformation of the binding data so that K_D and B_{max} may be readily determined from standard radioligand saturation experiments (Bennet and Yamamura, 1985).

During saturation experiments radioactivity is increased while specific activity is held constant. The amount of radioligand specifically bound at each radioligand concentration is determined. Bound radioligand is expressed as molar concentrations of radioligand bound per unit weight of tissue protein and fitted to the Scatchard equation. Conventional linear regression techniques are used to calculate the line of best fit. After the best fit data is obtained the equilibrium dissociation constant (K_D) is estimated as the negative reciprocal of the slope of the line of best fit. The apparent maximum number of binding sites (B_{max}) is estimated from the intercept of the line with the abscissa. In this thesis a micro-processor was used to analyze data from saturation experiments. As previously indicated this program was developed by Dr. B. Clark et al (1983) for use on an Apple II computer. The major advantage of the Scatchard equation compared to other methods of plotting saturation data is that all points from the graph are given equal statistical weight. The experimental error in determining the major variable, namely bound radioligand is mathematically eliminated from the line fitting procedure because the variable is present on both axes. Assumptions in the derivation and the use of Scatchard analysis include the following; 1) the binding process must obey simple reversible mass action laws, 2) data must be obtained at steady state conditions, 3) binding of ligands to receptor site must not affect subsequent ligand receptor site interactions, (i.e. no co-operativity), 4) ligand and receptor sites must be homogenous species, i.e. radiolabelling can cause the ligand to become damaged producing a radioligand population composed of heterogenous species with different

binding affinities to the receptor sites. Heterogeneous receptor populations also exist. Detection of receptor subtypes requires either a ten fold variation in the affinity of the ligand for each receptor subtype or, if the radioligand has equal affinity for the subtypes, the availability of unlabeled drug with at least a 10 fold variation in binding potency at the individual receptor sub-population. 5) Concentration of free unbound ligand must be accurately determined. 6) Non-specific receptor binding must be properly defined (Bennett and Yamamura, 1985).

As previously discussed it is best to define non-specific binding using chemically dissimilar unlabeled drug to the radioligand at concentrations 100-300 times the K_i value. Before attempting a ligand saturation study, previously described criteria (radioligand purity and metabolism and minimization of non-specific binding) should be satisfied. In addition, linearity of radioligand binding with respect to the tissue of interest should be demonstrated and the appropriate incubation time and temperature to reach the steady state determined. With these precautions in mind some workers intially proceed using a small concentration of radioligand to obtain a binding constant by generating a ligand competition curve. The concentration of unlabelled ligand at which the maximum specific binding labeled ligand is inhibited by 50% (IC_{50}) can be taken as a first approximation of the apparent equilibrium dissociation binding constant (K_D). A binding saturation study can then be done over a range of radioligand concentrations from 10-20% of this K_D to 4-5 x this value (Bennett and Yamamura, 1985).

3) Competitive Inhibition Studies

The IC_{50} (the concentration of unlabelled ligand displacing 50% of specific binding) is used to determine the affinity constant for unlabelled drugs (K_i).

A fixed concentration of radioligand is incubated with increasing concentrations of unlabeled (cold) ligand. The concentration of unlabeled ligand displacing 50% of specific radioligand (IC_{50}) is then determined. As the concentration of radioligand [L] utilized in inhibition studies is increased, the difference between the IC_{50} of unlabeled ligand and the binding affinity constant of unlabeled ligand (K_i) progressively widens at fixed tissue concentrations according to the following equation.

$$K_i = \frac{IC_{50}}{1 + [L]/K_D}$$

This equation applies only to binding where labeled and unlabeled ligand interact competitively at the receptor site (Cheng and Prusoff, 1973).

Jacobs has reported that the concentration of receptor sites in the incubation medium can influence the relationship between IC_{50} and K_i.

$IC_{50} = n\ (K_D + LT + RT - 3/2\ LR)$ (Jacobs et al., 1975).

$= K_i + n\ (LT + RT - 3/2\ LR)$

LT = total concentration of radiolabeled ligand.

RT = total concentration of receptor sites.

LR = concentration of receptor - radioligand complex formed in the absence of unlabeled ligand.

$K_i = n\ K_D$

If LT+ RT is less than the K_D then the IC_{50} approximates K_i. In practice the total number of receptor sites should not exceed 10% of the K_D for a given radioligand when estimating affinity constants by competitive displacement.

Computer aided curve fitting programs are available to aid analysis of competition data and generation of graphs (McGonigle, 1984). In this study competition data analysis was performed with a computer aided curve fitting program using a mathematical model described by Wenke (Wenke, 1971). Details of this program entitled "Recfit" have been published elsewhere (Clark et al., 1984). All displacement data and receptor subtype estimation was analysed using "Recfit" on an Apple II microcomputer.

4) Hill Plots (Bennett and Yamamura, 1985)

In some binding studies displacement of radioligand from its receptor by unlabeled compounds structurally different from the radioligand yields behaviour different from classic mass action laws most commonly as negatively cooperative interactions (Hill plot significantly less than one). These situations arise primarily in instances of a radiolabeled antagonist being displaced by an unlabeled agonist or vice-versa. Hill plots are used to detect and quantitate such anomalous effects. The Hill equation is:

$$\frac{B}{B_{max}} = \frac{[L]^n}{K_D + [L]^n}$$

n = Theoretical number of ligand binding sites per receptor molecule.

K_D = Dissociation constant.

This equation can also be expressed in Log form as:

$$\text{Log}\ \frac{B}{(B_{max} - B)} = n\ \text{Log}[L] - \text{Log}\ K_D$$

or

$$\text{Log}\ \frac{f}{1-f} = n\ \text{Log}[L] - \text{Log}\ K_D$$

$$f = B/B_{max}$$

Therefore fitting ligand binding data is straight forward for either saturation or competition experiments. Methods to derive the above formulae are reviewed by Bennett and Yamamura (Bennett and Yamamura, 1985).

5) Rate Constants (Bennett and Yamamura, 1985)

As previously indicated K_D is the equilibrium dissociation constant which is the ratio of the binding reactions reverse and forward rate constants,

i.e.
$$K_D = \frac{K_{-1}}{K_{+}1}$$

For this reason it is desirable to estimate the rate constants for association (K_{+1}) and dissociation (K_{-1}) so that the binding constant derived from saturation data can be compared to the binding constant resulting from the ratio of the rate constants (K_{-1}/K_{+1}). If the binding reaction under study obeys mass action laws, the binding constants derived by these two independent means should be equal within experimental error.

$$L + R \xrightarrow{K_{+1}} LR$$

This follows second order kinetics and unimolecular dissociation

$$LR \xrightarrow{K_{-1}} L + R$$

which follows first order kinetics.

For many neurotransmitter receptor studies to date, the binding constants calculated from such a model are usually equal, within error, to the binding constants derived from equilibrium saturation studies. This is not however always the case.

a) Association Rate Constant (Bennett and Yamamura, 1985)

The simple rate equation for bimolecular association

$$\frac{d\,[LR]}{dt} = K_{+1}\,[L]\,[R] \qquad (7)$$

when integrated yields

$$K_{+1} = \frac{2.303}{t\,[L - R]}\ \text{Log}\ \frac{R\,[L - x]}{L\,[R - x]} \qquad (8)$$

with L + R equal to free ligand and unoccupied receptor respectively at time 0, x is equal to the amount of each consumed in time t. Theoretically equation 8 allows calculation of K_{+1} for any bimolecular binding reaction when L, R and X are known. However, for binding studies where 10% or less of the total available ligand is bound at equilibrium, K_{+1} estimated from equation 8 is subject to large error because x never exceeds 10% of L. Furthermore the problem is compounded because experimentally it is desirable to measure initial ligand association rates where ligand - receptor dissociation causes minimal error on estimation of K_{+1}. Thus in practice it is not unusual to

estimate K_{+1} based on data where 5% maximum of initial free ligand is bound to receptor (Bennet and Yamamura, 1985).

More theoretical and experimentally practical methods are used to estimate K_{+1}. If an initial free ligand concentration of 1/2 the K_D or less for that ligand is used and binding data only up to the first half-life of association are analysed then at most approximately 1/8 of the total receptor sites are occupied and [R] can be assumed to be equal to B_{max} x [Tissue]. Likewise, if the tissue concentration is controlled so as to be in zone A behaviour (less than 10% of [L] bound) then [L] does not change appreciably and can also be considered constant. Equation 8 then reduces to

$$\frac{d\,[LR]}{dt} = K'_{+1} \qquad (9)$$

where $K'_{+1} = K_{+1}\,[L]\,[R]$

Therefore, $d\,[LR] = K'_{+1}\,dt$.

To estimate K_{+1} specifically bound ligand is plotted versus time. The best line fit through, at most, the <u>first</u> half-life is constructed. The slope of this line, K'_{+1} is then divided by the initial concentration of free ligand [L] and receptor ([R] = B_{max} x [tissue]) to yield K_{+1}.

A second method takes into consideration the contribution of ligand-receptor dissociation to the eventual reaching of a steady state. Bound ligand is assayed at various time intervals upto steady state level (B_{eq}).

Ln [B_{eq}/ (B_{eq} - B_t)] is plotted versus time where B_t is the amount of specifically bound ligand at time t. The slope of the line

(K_{obs}) is related to the association (K_{+1}) and dissociation (K_{-1}) rate constants and free ligand concentrations [L] as:

$$K_{+1} = \frac{K_{obs} - K_{-1}}{[L]} \quad \text{(Insel and Stoolman, 1978).} \qquad (10)$$

This equation is valid only when the binding reaction is performed with no more than 10% of initial free ligand bound at steady state. Estimating K_{+1} by this method requires knowledge of the dissociation rate constant (K_{-1}) as described in the next section.

The use of equation 10 obviates the need for an independent determination of B_{max} which would be required to use equation 9, also, equation 10 can be rearranged to:

$$K_{obs} = K_{+1}\,[L] + K_{-1}.$$

Thus a plot of K_{obs} versus [L] should yield a straight line with a slope of K_{+1} and an ordinate intercept of K_{-1} if the reaction obeys mass action laws and is performed under pseudo-first order conditions with respect to [L].

K_{+1} can be determined from experiments in which both [L] + [R] are varied in order to confirm the bimolecular nature of the process. Also, K_{+1} can be calculated at various values of [L] from K_{obs} and the independently measured K_{-1}, and compared to values obtained from a plot of equation 11. In this way errors in the independent estimation of K_{-1} will become apparent.

In this study K_{+1} was determined using a computer program developed by Steinberg et al (Steinberg et al., 1985) - see below.

b) Dissociation Rate Constant

The dissociation rate constant (K_{-1}) is estimated by incubating radioligand and receptors for a given time period, usually to steady state and then infinitely diluting the radioligand concentration. After various time intervals specifically bound radioligand is then measured. The half-life ($t_{1/2}$) for loss of specifically bound radioligand is determined from a plot of log [LR] versus time. K_{-1} is calculated as $0.693/t_{1/2}$. Alternatively the slope of log [LR] versus time multiplied by $-2.303 = K_{-1}$.

Infinite dilution of radioligand can be done in two ways 1) receptor ligand solutions are incubated to steady state and then diluted 100 x or more with incubation buffer. Aliquots are then assayed for bound radioligand at various times, 2) receptor preparations and radioligand are incubated to steady state and 1,000 x molar excess of unlabeled drug is then added. Receptor-bound radioligand, is assayed at various time intervals thereafter (Bennet and Yamamura, 1985; Insel and Stoolman, 1978).

In this study, K_D was determined from kinetic experiments using a computer aided curve fitting program developed by Steinberg et al (Steinberg et al., 1986). This program analyses data using a mathematical model described by Moelwyn and Hughes (Moelwyn and Hughes, 1971).

3) RADIOLIGAND USED IN THIS STUDY

During this study the radioligand [^{125}I]-iodohydroxybenzylpindolol [^{125}I]-HYP was used. This ligand emits gamma radiation. Tritiated compounds such as [^{3}H]-dihydroalpranolol (Lefkowitz et al 1974) (beta radiation emitters) have low specific radioactivity (less than 80 Ci/mmol) (Engel et al., 1981). Because of this low specific activity, large amounts of tissue in the form of protein are required. In view of radioactive iodine's high specific activity (1500 - 2000 Ci/mmol) and higher affinity ($\pm$ 10 times) [^{125}I]-HYP has generally been accepted as a ligand of choice in beta-receptor studies when only small amounts of tissue protein are available (Brown et al., 1976). The high specific activity makes it ideal for use in experiments involving ocular tissues as they are not readily available in large amounts. [^{125}I]-iodocyanopindolol ([^{125}I]-CYP) also has high specific activity and high specificity for beta-adrenoceptors (Engel et al., 1981). This newer radio-ligand is said not to bind to alpha and other non-beta-adrenoceptor sites as may occur with [^{125}I]-HYP (Engel et al., 1981). [^{125}I]-CYP became available only towards the end of this study and as a result preliminary binding data derived with this ligand have not been included in this study.

Figure 6 reveals the structure of Pindolol and its two radioactive derivatives.

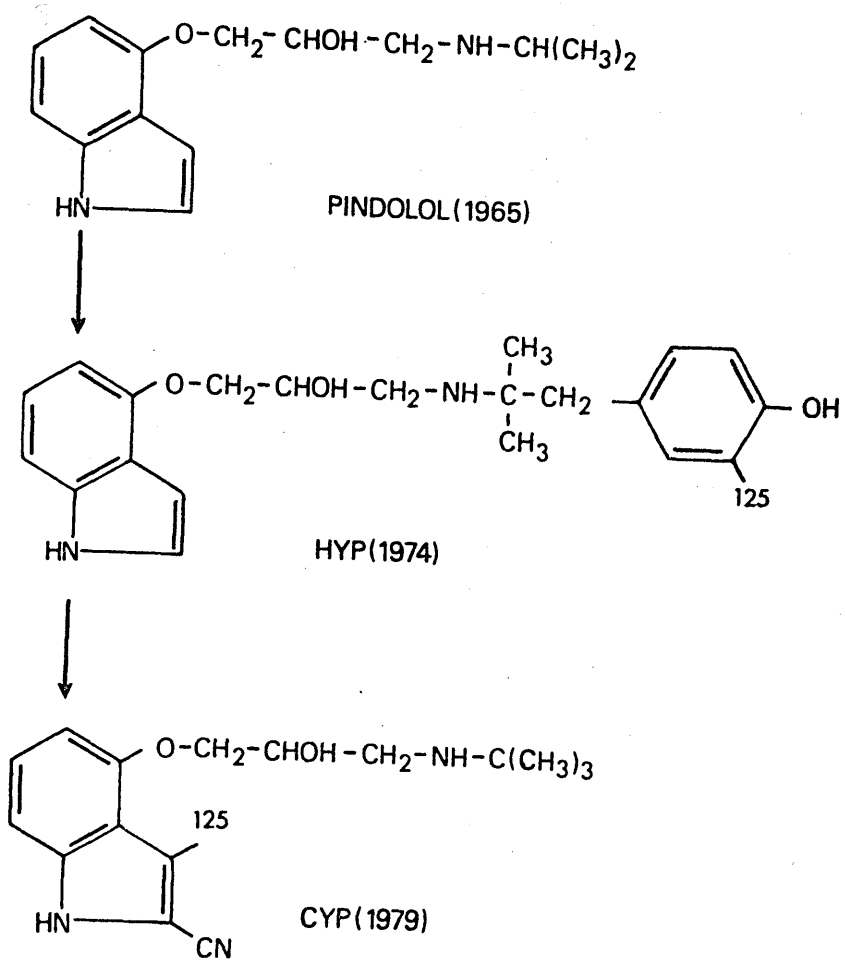


FIGURE 6 : Radioligand derivatives of Pindolol
^{125}I-HYP* is a radio-labelled Pindolol derivative

^{125}I-CYP is prepared by iodination of CYP** as described by Engel et al 1979.

* HYP is a trivial name for hydroxybenzylpindolol

** CYP is a trivial name for cyanopindolol

IV. Production of aqueous humour by the ciliary processes

As previously indicated I.O.P. depends on aqueous production. If aqueous production is inhibited (as occurs with beta-blocker therapy) I.O.P. drops. This section reviews the theories regarding aqueous production.

It is now a well established fact that the ciliary processes are the site of aqueous humour production (Cole, 1977) The exact mechanism of aqueous humour production however remains controversial, (Cole, 1977; Macri et al., 1978; Sears et al., 1986). Most authors agree however that the epithelium of the ciliary processes and, in particular, the inner non-pigmented layer, controls aqueous humour production (Cole, 1977).

The ciliary processes have a high rate of blood flow (15-25 ml/min/gram tissue) and a surface area of 6 sq/cm in man. The rate of aqueous flow is about 2 ul per minute.

Aqueous production depends on four processes:

A) Sodium potassium activated adenosine triphosphatase [(Na^+:K^+ ATPase]. Sodium potassium ATPase is present on the basal infoldings of membranes of both the outer pigmented and inner non-pigmented ciliary epithelial cells (Rich et al., 1980). Sodium potassium ATPase is considered synoymous with the sodium pump. This sodium pump is important as it is sodium transport into the lateral intracellular channels that creates the standing osmotic gradient. This gradient leads to osmotic waterflow down the channels in a unidirectional manner towards the posterior chamber (Nagasubramanian, 1982; Krupin et al.,

1986). The sodium pump is also important as it establishes an electrochemical gradient across the blood epiphelial cell/aqueous humour interface.

In vitro studies have shown that ciliary process sodium potassium ATPase activity can be inhibited by ouabain and vanadate (ouabain prevents potassium binding to the enzyme at the extracellular surface, while vanadate acts at the intracellular surface of the membrane bound enzyme molecule). The exact relationship between adrenergic drugs used to treat glaucoma and sodium potassium ATPase however has not been defined. Noradrenergic stimulation can inhibit or excite membrane bound sodium potassium ATPase depending on the organ or specie studied (Krupin, 1986; Swann, 1983; Lingham and Sen, 1982). It is believed that norepinephrine and epinephrine may stimulate sodium potassium ATPase in ciliary processes to increase aqueous production but this has as yet to be proved (Krupin, 1986).

B) Carbonic anhydrase.

The enzyme carbonic anhydrase is responsible for the catalytic hydration and dehydration of carbon dioxide

$$CO_2 + OH^- \xrightarrow[\text{anhydrase}]{\text{carbonic}} HCO_3^-$$

This enzyme is found in nature as one of four isoenzymes. Type II (formerly called type C) is the isoenzyme present in the ciliary processes (Kumpulainen, 1983). Carbonic anhydrase is localized both to the cell membrane and cytoplasm of pigmented and non-pigmented

epithelium. Inhibition of this enzyme decreases bicarbonate production which inhibits sodium ion movement by 30%. Water entry into the posterior chamber is therefore decreased (Maren et al., 1975; Maren, 1984).

Cole has suggested that acetazolamide, a carbonic/anhydrase inhibitor reduces short circuit currents across the in vitro ciliary body causing a fall in sodium transport. This reduction in short circuit current may be due to a drop in intracellular pH (effecting sodium potassium ATPase activity) or possibly due to a reduction in the amount of sodium entering the cell by way of the hydrogen sodium exchange system (Cole, 1966).

C) Adenylate cyclase.

It is now well established that catecholamine sensitive adenylate cyclase exists in the ciliary process of animal and man (Waitzman and Woods, 1971; Sutherland et al., 1966). Furthermore, it has been shown that beta-adrenoceptor stimulation of this tissue results in increased cyclic AMP production (Neufeld et al., 1973). Beta-adrenergic stimulation also increases aqueous production (Brubaker, 1984). It therefore follows that beta-adrenoceptors appear to mediate adenylate cyclase control of aqueous production through adenylate cyclase stimulation. The exact process involved however, is not well understood. In view of the ocular hypotensive effects elicited by Forskolin (an adenylate cyclase activator) Sears has suggested that increased cAMP activity lowers I.O.P. by stimulating aqueous humour outflow through the apices of the ciliary processes. He bases this

theory on the embryonic development of the ciliary processes (See Discussion). This theory however is controversial particularly inlight of recently published data by Brubaker et al., in which no effect on intra-ocular pressure or aqueous production was observed in human eyes treated with forskolin drops (Brubaker et al., 1986). Nathanson has published evidence that beta-adrenoceptor coupled adenylate cyclase control of aqueous production is epithelial rather than vascular in origin and is mediated by beta-2 receptors (Nathanson, 1981). (See Discussion).

D) Ultra-Filtration

Ultra-filtration is also believed to be an important factor regulating aqueous humour production (Pederson and Green, 1975). Infact, upto 20% of aqueous humour production is believed to be due to this process. (Nagasubramanian, 1982). Sears however, believes that ultra-filtration has a minor role to play in the production of aqueous humour (Sears, 1986).

E) Chloride secretion.

Chloride secretion, dependent on sodium presence, inhibited by ouabain and enhanced by intracellular cAMP, has been shown to occur in a number of cells across apical and mucosal surfaces (Frizzell et al., 1979). It is not known whether chloride secretion plays an important role in aqueous production but Sears has recently speculated that it may (Sears, 1986).

In summary, aqueous humour production seems primarily to be controlled by sodium potassium ATPase. Other important factors

influencing this process include adenylate cyclase, ultra-filtration, carbonic anhydrase and perhaps chloride secretion.

V. History of Adrenergic drugs and intra-ocular pressure

In 1895, Oliver and Schafer isolated adrenaline from the adrenal medulla (Oliver et al., 1895). It was not long before it was tested on the eye. Darier reported that topical adrenaline both dilated the pupil and lowered intra-ocular pressure (Darier, 1900). This effect on intra-ocular pressure caused much controversy in later trials, as an increase in intra-ocular pressure was noted to occur in some patients (MacCallan, 1903). Undoubtedly this paradoxical increase in pressure was due to acute and chronic angle closure glaucoma precipitated by pupillary dilatation in patients with narrow angles, a fact not understood until the gonioscopy lens was introduced during the 1930's. However, it is interesting note that J.H. Parsons in 1903 realized that there was a connection between the rise in intra-ocular pressure and pupillary dilatation (Parsons, 1903). Wessley attributed adrenaline's hypotonic effect on the eye to vasoconstriction leading to decreased aqueous humour production (Wessley, 1900). This theory is still believed by some workers to be the primary mechanism of action of adrenaline on intra-ocular pressure (Thomas and Epstein, 1981).

Darier's original work stimulated much clinical research into the role of adrenaline for the treatment of P.O.A.G. (Darier, 1900; MacCallan, 1903; Knapp, 1921; Thomas et al., 1981; Podos, 1980). Today topical adrenaline alone or in combination with other drugs is considered to be a primary agent for the medical management of primary open angle glaucoma (Weinreb, Ritch et al., 1986; Thomas, 1981). Adrenaline is now known to increase outflow facility and have complex effects on aqueous humour production (See discussion).

In 1967, Philips et al., reported the effects of propranolol a non-specific beta-blocker on intra-ocular pressure after systemic administration. These authors reported that both intravenous and oral administration of propranolol lowered intra-ocular pressure dramatically in patients with POAG (Philips et al., 1967). This report stimulated research work directed towards developing a suitable topical beta-blocker for use in P.O.A.G. (Bonomi et al., 1975; Eliott et al., 1975; Smith et al., 1979; Krieglestein, 1981; Krieglestein et al., 1981). During 1978, topical (-)timolol maleate a non-specific blocker was introduced for the treatment of POAG. This drug is still the most widely used non-specific beta-blocker for the treatment of POAG (Kaufman, 1980; Novack, 1987). It effectively lowers intra-ocular pressure by inhibiting aqueous production and has a b.d. dose schedule. Topical (-) timolol maleate does however have significant local and systemic side-effects. (Kaufman, 1980). Other beta-blockers recently introduced include, levobunolol, carteolol and metipranolol, all non-specific beta-blockers, and betaxolol hydrochloride a beta-1 specific blocker (Duzman et al., 1982; Berrospi and Leibowitz, 1983; Novack, 1987).

Despite over fifteen years of investigation into beta-blockers and their role in intra-ocular pressure control, it is still not known exactly how these agents work. It is believed by some authors that beta-blockers exert their intra-ocular pressure lowering effect by binding to beta-adrenoceptors in the epithelial cells of the ciliary processes (Thomas et al., 1981; Kaufman, 1980; Neufeld, 1979; Woodward et al., 1986). These workers believe that beta-blockers may prevent

active transport, or possibly ultra-filtration of ions and fluid across the epithelial barrier (Kaufman, 1980; Neufeld, 1979) (See discussion). As previously indicated this theory is controversial as it is not known whether beta-adrenoceptors exist in ciliary processes. The next section contains a review of work done to determine whether receptor mechanisms control aqueous production and I.O.P.

VI. Review of the techniques to determine receptor mechanisms controlling aqueous production

The interesting phenomenon that beta-blockers lower intra-ocular pressure by reducing aqueous production has stimulated research workers to examine the ciliary processes to determine whether these agents exert their effects through beta-adrenoceptors located in the epithelium of the ciliary processes. Three major pathways have been investigated to determine whether beta-adrenoceptors exist in the ciliary processes; 1) pharmacological techniques, 2) receptor adenylate cyclase interactions, 3) radio-ligand studies.

A) Pharmacological techniques

Prior to the development of direct ligand binding studies, receptors were defined indirectly by observation and quantitation of certain physiological effects occurring subsequent to hormone or drug receptor interactions. Such studies have provided a great deal of information and have served to define the main patterns of drug specificity which, in turn, define most receptors. Therefore, on the basis of a large body of experimental data, beta-adrenoceptors have been defined as cellular structures which bind agonist catecholamines with a certain order of potency, namely:
Isoprenaline > adrenaline > noradrenaline (Ahlquist, 1948). Similarly antagonists have definable potency series with propranolol being amongst the most potent beta blockers.

Similar techniques have been used on the eye to try to determine the receptors controlling aqueous production and intraocular pressure (Nagasubramanian, 1982; Bonomi et al., 1981; Coakes et al., 1978;

Townsend et al., 1980; Innemee et al., 1982; Sears, 1986). These indirect tests (which include the use of fluophotometry) have been extrapolated by various authors to implicate adrenergic receptors (and in particular beta-adrenoceptors) as being important in aqueous humour production. It is obvious, however, that these pharmacological tests do not allow for exact identification or anatomical location of the receptor sites controlling aqueous humour production. For example (-) timolol maleate, a non-specific beta blocker, may decrease aqueous humour in-flow but one cannot say with certainty that this effect on aqueous production is due to specific beta blockade of the ciliary process epithelial receptor sites as has been claimed by some authors (Alm et al., 1981). The effect on aqueous production could be due to a vascular effect on the choroid or by some other as yet unidentified mechanism (Langham et al., 1979; Smith, 1987; Phillips, 1987).

Extrapolation of these indirect pharmacological tests has resulted in markedly differing opinions in the literature with regard to which receptors in which structures control aqueous humour production and intraocular pressure (Langham et al., 1979; Nagasubramanian, 1982; Coakes et al., 1978; Berrospi et al., 1983; Chiou, 1982; Sears, 1986; Phillips, 1987).

It is clear that if a technique was developed to detect beta-adrenoceptor in the ciliary processes of human or pigmented animal eyes, it would be of great help in resolving this controversy. The development of a direct radioligand binding technique to study ciliary process beta-adrenoceptors was therefore a major aim of this research project. Details of methodology and results are reported in the

appropriate sections of this thesis.

B) Beta-adrenoceptor adenylate - cyclase interactions

As previously indicated Sutherland and Robison were the first to report that the stimulatory effect of catecholamines on adenylate cyclase was initiated by beta-adrenoceptors (Sutherland et al., 1966). Waitzman and Woods were the first authors to report that beta agonists activate adenylate cyclase in the ciliary processes of rabbit eyes (Waitzman et al., 1971). Neufeld has reported changes in aqueous humour cyclic AMP after adrenaline stimulation and sympathectomy (Neufeld et al., 1973).

Recent in vitro work by Nathanson, Cepelik and Elena has revealed that animal and human ciliary process epithelium responds to beta 2 stimulants by producing cyclic AMP. This indirectly suggests that beta 2 receptors exist in the ciliary processes (Cepelik et al., 1981; Elena et al., 1981; Nathanson, 1980). This is an important observation in relation to the anatomical situation and the subtype of beta receptor controlling aqueous humour production.

Despite the advantages of studying beta-adrenoceptors coupled to adenylate cyclase, such experiments are still considered indirect (Lefkowitz, 1976). The characteristics of the receptors being inferred from observed changes in enzyme activity.

C) Radioligand Studies

Until recently there were no methods to directly investigate adrenergic receptors. In 1974 beta adrenergic radioligand techniques were first described (Levitzki et al., 1974; Lefkowitz et al., 1974;

Auerbach et al., 1974). As previously indicated these techniques allow the direct detection, characterization (sub-types) and concentration (i.e. receptor numbers) of beta-adrenoceptors in a tissue sample (Lefkowitz, 1976; Motulusky et al., 1982). Radioligand techniques have been applied to various tissues of the body including lymphocytes, platelets, placenta, lung, brain and other tissues. This work has been reviewed by Motulsky et al and Stiles et al (Motulusky et al., 1982; Stiles et al., 1984).

Very little work has been performed on the eye using radioligand binding studies. Most attempts to detect beta-adrenergic receptors using ligand binding techniques have been performed by Neufeld et al., (Neufeld et al., 1977; Neufeld et al., 1978). This work was performed on iris ciliary body homogenates from albino rabbit eyes. Albino rabbits were used as melanin present in non-albino animals binds ligand and distorts the results (see later). Major deficiencies in these studies are described later (see discussion). In 1980 Bromberg et al., described a novel radioligand technique to try to identify beta-adrenoceptors specifically in the ciliary processes of albino rabbit eyes (Bromberg et al., 1980). This technique has never been verified (see developments section). The successful development of a repeatable radioligand technique, applicable to small amounts of pigmented ocular tissue forms a large portion of this study (Trope et al., 1982).

VII. Review of Animal Models to Study Aqueous Humour Production and Glaucoma

A) Measurement of Aqueous Humour Production

In 1981 Vareilles and Lotti (Vareilles and Lotti, 1981) described a technique to study aqueous humour production in rabbit eyes. This test was based on studies performed by Auricchio and Diotallevi in which I.O.P. recovery rate curves were studied after eye ball compression (Auricchio and Diotallevi, 1965). The technique involves the use of hypertonic saline as an osmotic agent to temporarily lower I.O.P. Briefly, this technique involves initially measuring rabbit intra-ocular pressure. Drug under test is then placed onto one eye of a rabbit and the intra-ocular pressure remeasured. Hypertonic saline is then injected intravenously into the rabbit via a marginal ear vein at a rate of 1 ml/min over 10 minutes. At the end of the infusion I.O.P. rapidly drops and slowly returns to normal. Intra-ocular pressure is then measured at various time intervals throughout the experiment. If a test drug inhibits aqueous humour production, intra-ocular pressure recovery will be delayed in that eye compared with both the untreated eye and a control group of eyes. This technique has been used by a number of investigators to study the effects of a variety of drugs on aqueous humour production (Vareilles and Lotti, 1981 Chiou, 1982 Chang et al, 1985 Conway et al, 1987). It allows one to determine aqueous humour production by measuring the slope of the intra-ocular pressure recovery line. The intra-ocular pressure recovery rate is expressed in millimetres of mercury per minute.[2] This technique was used to determine the effects of beta-2 specific antagonists on aqueous

production.

B) Animal Glaucoma Models

Many workers have tried to induce glaucoma in rabbits and other animal eyes. Techniques that have been used to block outflow channels include injection of air, pigment granules, methylcellulose, red blood cells, ghost cells, cotton and mineral oil into the anterior chamber (Lessel and Kuwabara, 1969; Bartels, 1984; Shields, 1982). Ligation of vortex veins, diathermy and cautery of subconjunctional tissues at the limbus have also been tried (Gelatt, 1975). Other techniques to produce raised I.O.P. include; blunt trauma, water loading, encircling rubber bands, I.V. 5% glucose, and aqueous paracentesis with iris stimulation (Gelatt, 1977) (Bonomi et al, 1979). None of these techniques are reliable models of glaucoma with transient elevations of intra-ocular pressure being the usual response (Gelatt, 1977; Rowland and Potter, 1980; Bartels, 1984). Alpha-chymotrypsin injection into the anterior chamber however can produce long-term elevations in rabbit intra-ocular pressure due to zonular fragments obstructing the outflow channels. This elevation in pressure however is associated with severe intra-ocular inflamation, lens dislocation and vitreous disturbances with loss of the eye as a consequence on occasion (Sears, 1974) (Gelatt, 1977). Subconjunctional injections of 4 mg Betamethasone 3 times per week is claimed to induce ocular hypertension in rabbit eyes after 3-5 weeks. This technique however precludes the use of the second eye as a control and produces undesirable systemic effects (Bonomi et al, 1979).

Experimental glaucoma has been induced in rabbits and rhesus monkeys by repeated circumferential argon laser photo-coagulation of the trabecular meshwork (Gaasterland and Kupfer, 1974) (March, Gherezghiher, and Coss et al., 1984). Due to the difficulties involved in visualizing the rabbit angle gonioscopically plus the expense and other difficulties of using monkeys, these animal models are not commonly used.

Congenital glaucoma occurs in rabbits (Fox et al., 1970) (Kolker et al., 1963) (Gellat, 1977) dogs and in chickens exposed to continuous light (Kinnear, Lauber and Boyd, 1974). The rabbit glaucoma is due to an outflow obstruction and is characterized by large corneal diameter, cloudy cornea and raised intra-ocular pressure (Gelatt, 1977).

Buphthalmic rabbits have been reported as being good animal models for human glaucoma as they respond in a similar fashion to topical antiglaucoma drugs (Vareilles, Conquet, and Lotti, 1985). Breeding and maintenance of these rabbits however can be difficult (See Discussion).

In this study buphthalmic rabbits were used to investigate the effects of $beta_2$ specific drugs on intra-ocular pressure.

VIII. AIMS OF THIS THESIS

1. To develop a radioligand technique to determine whether beta-adrenoceptors exist in the ciliary processes of pigmented eyes.

2. To determine the sub-type of receptors present in the ciliary processes.

3. To apply the results of the ligand binding study to the in-vivo situation:

a) To determine whether the appropriate beta-specific antagonists inhibit rabbit aqueous humour production.

b) To determine whether the appropriate beta-specific antagonists lower I.O.P. in rabbits with glaucoma.

MATERIALS AND METHODS

This section deals with the methods used to detect and characterise beta adrenergic receptors in the ciliary processes (Trope and Clark, 1982). It also contains details of techniques used to study intraocular pressure recovery rate (aqueous humour index) in rabbits and the measurement of intraocular pressure in rabbits with congenital glaucoma.

I) In vitro study of beta receptors.

A) Dissection technique to isolate ciliary processes.

Eight eyes from freshly slaughtered sheep were obtained from an abattoir and immediately enucleated and placed on ice. The ciliary processes were dissected free from the rest of the eye as follows:

1) A posterior sclerotomy was made in the region of the equator to decompress the eye. A small amount of vitreous humour was allowed to prolapse from this incision.

2) An incision $\pm$ 6 mm from and parallel to the limbus was made down to the choroid and extended 360 ^{o} using scissors (Figure 7). Once complete the scissors were introduced into the anterior chamber from the suprachoroidal space through the scleral spur (Figure 8). This procedure enabled the cornea plus a rim of sclera to be peeled off the scleral spur (Figure 9). The iris was then dissected off the underlying iris/ciliary body diaphragm to reveal the ciliary processes (Figure 10). To free the ciliary processes the ciliary body was radially incised and then dissected free from the ciliary processes as indicated in Figure 11. The ciliary processes were then dissected off the lens and underlying vitreous using an operating microscope and microsurgical instruments (Figure 12). Within one hour of death the ciliary processes

were freed and placed into cold saline and transported to the laboratory.

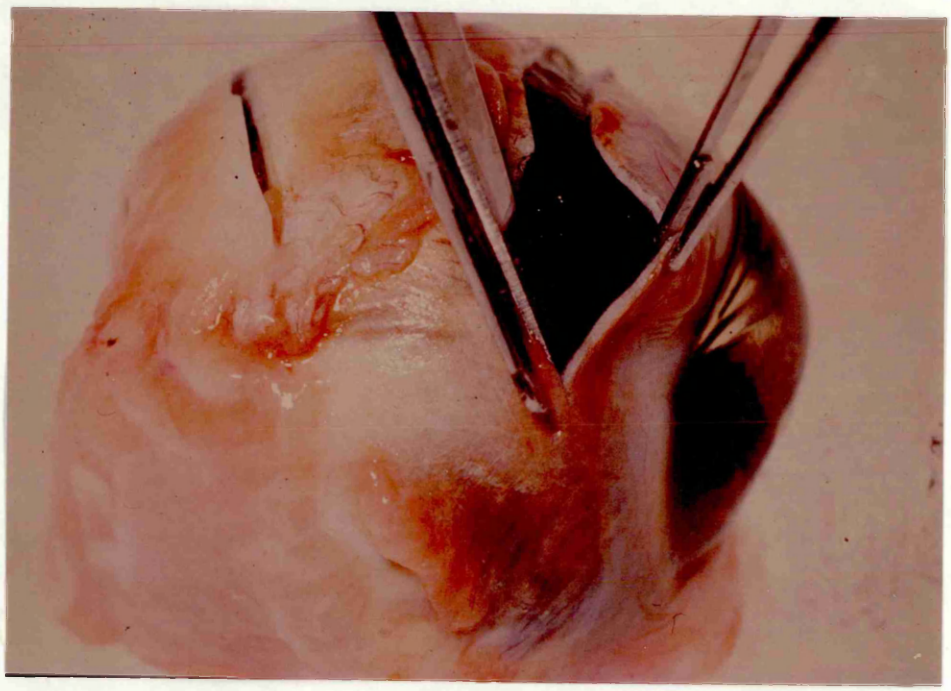


Figure 7: Initial dissection procedure. Note posterior sclerotomy and 6 mm rim of sclera dissected off underlying choroid.

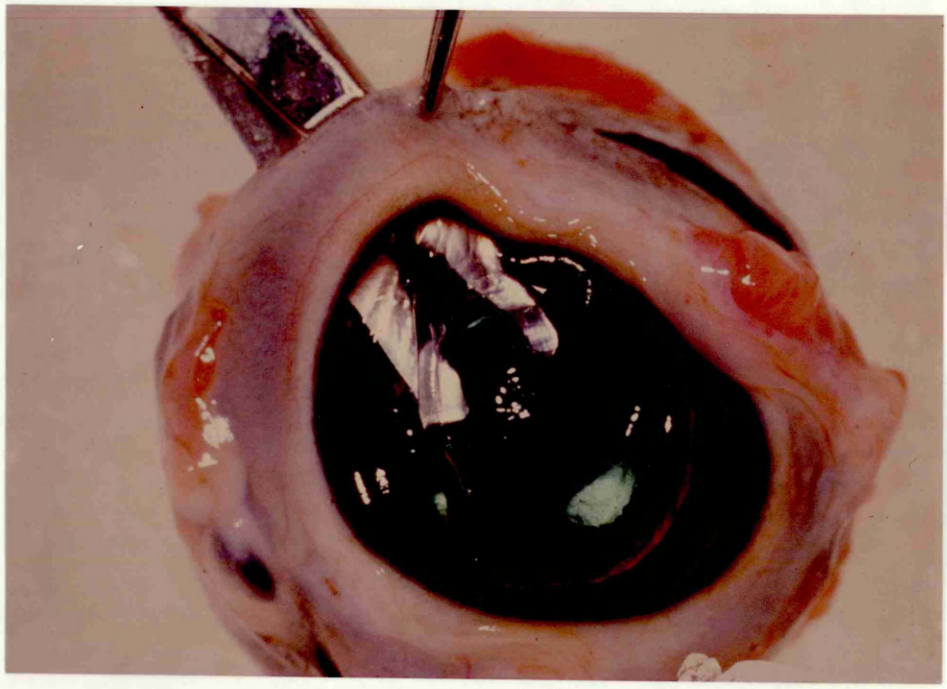


Figure 8: Scissors introduced into anterior chamber through the scleral spur.

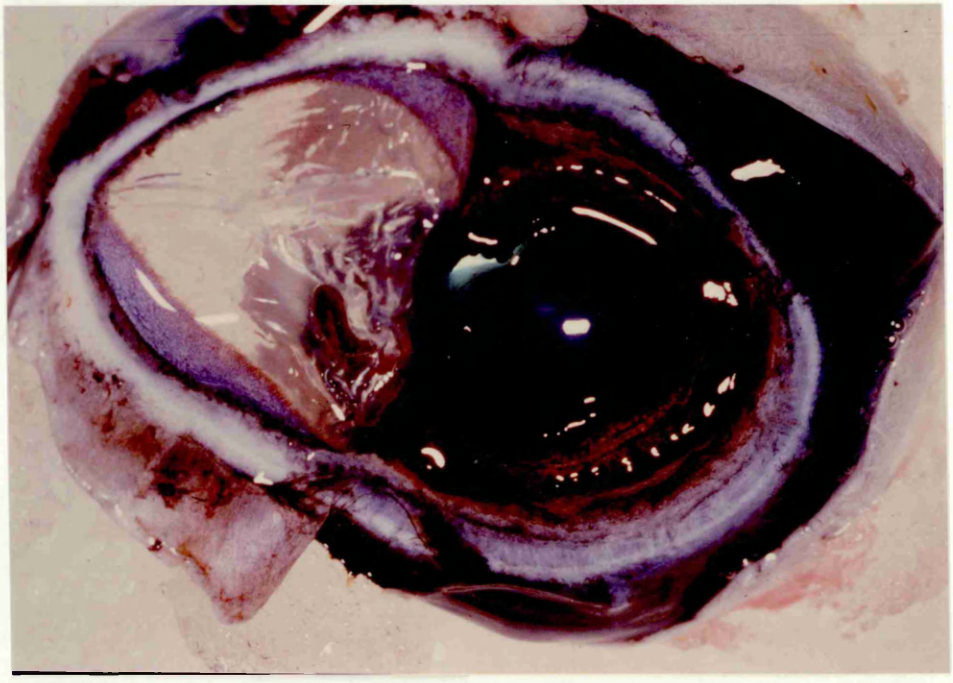


Figure 9: Sclera and cornea peeled off scleral spur to reveal iris and ciliary body.

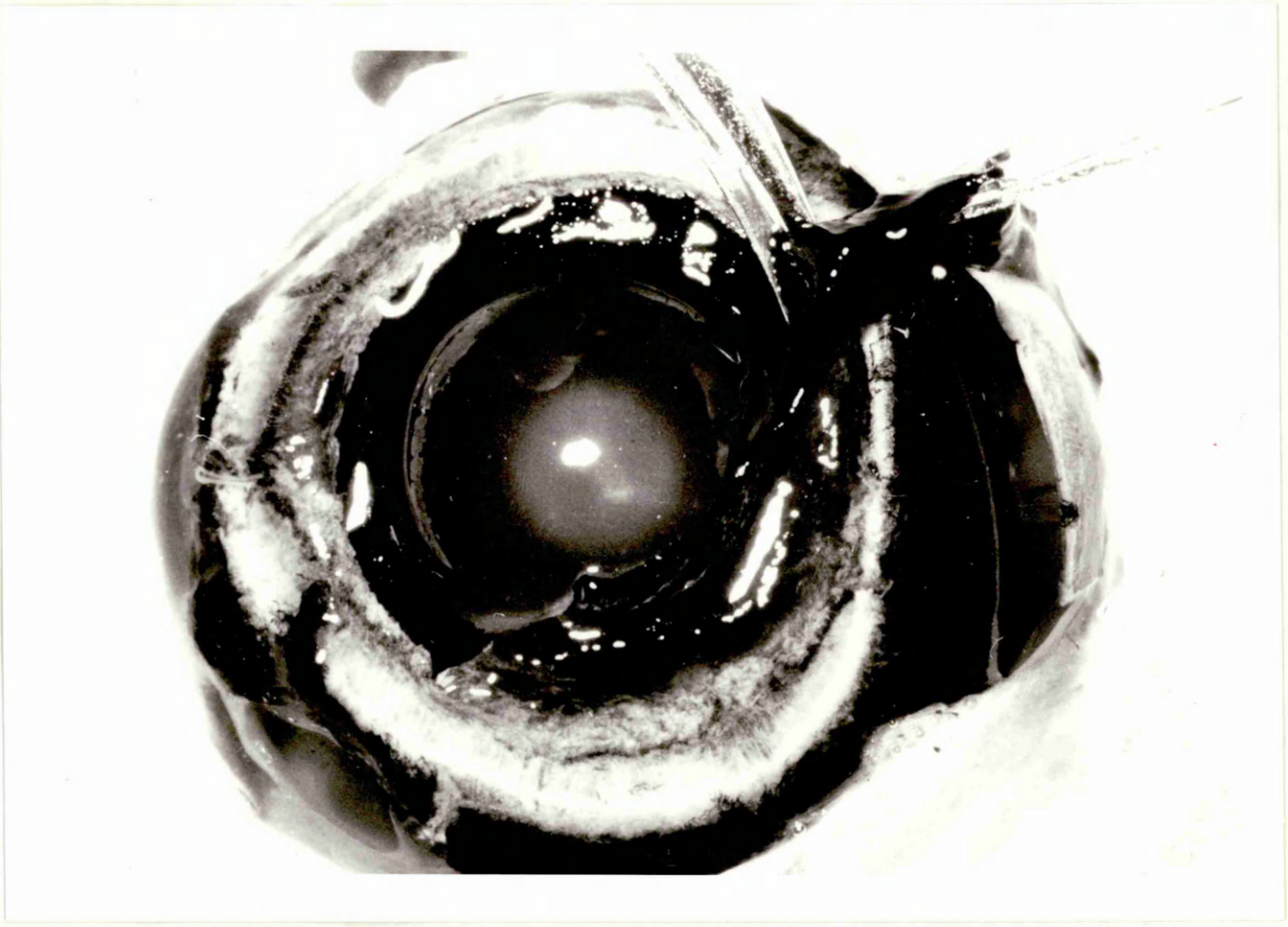


Figure 10: Iris dissected off ciliary body to reveal ciliary processes.

Figure 11: Ciliary body dissected from ciliary processes.

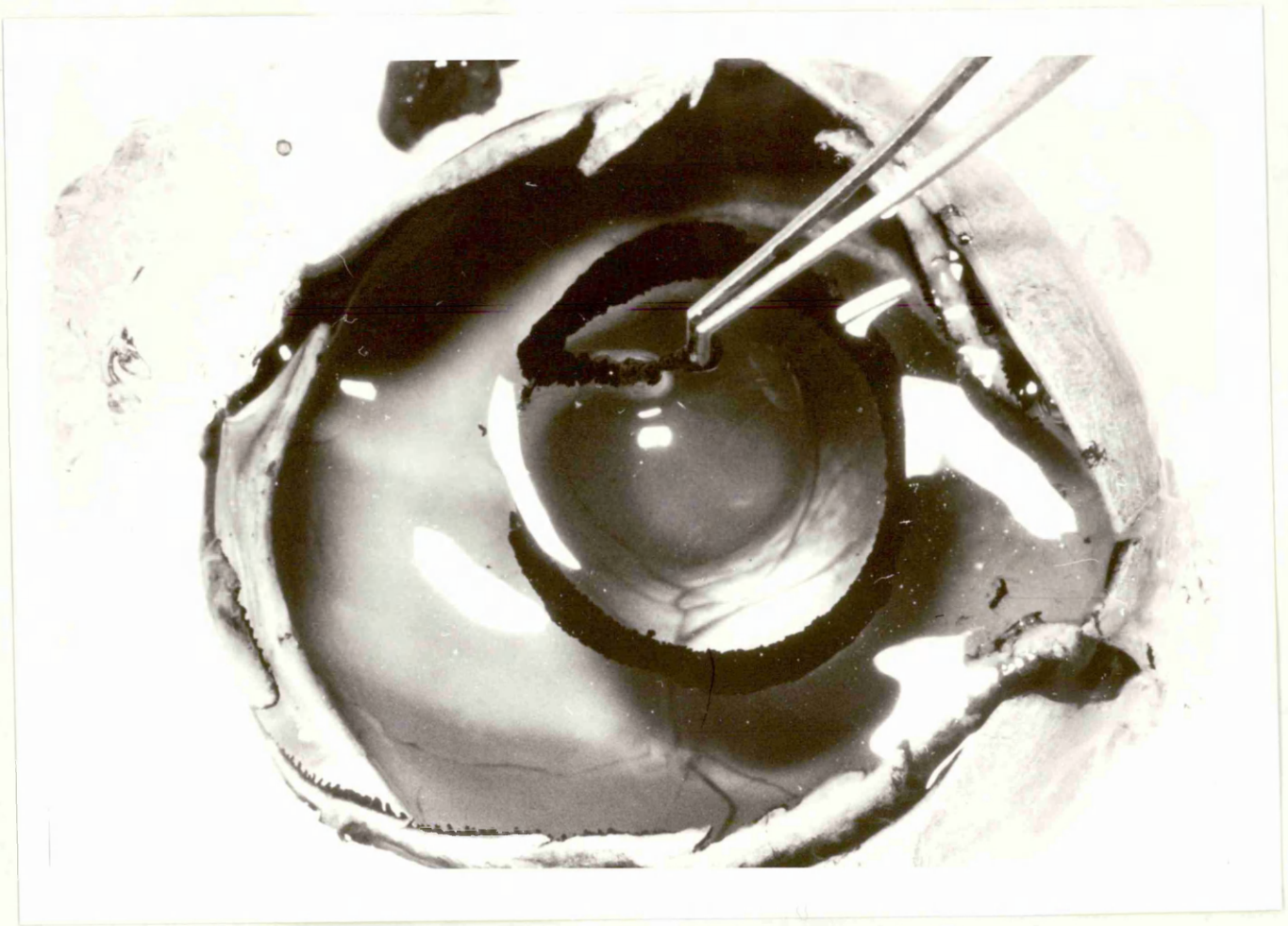


Figure 12: Ciliary processes dissected off the lens and anterior vitreous face using microsurgical techniques.

B) Membrane Preparation

a) The ciliary processes from 8 eyes were pooled in a cellulose nitrate tube containing 3 ml of a buffer solution (pH 7.5) consisting of 50 mmol/l Tris and 1 mmol/l-ascorbic acid (buffer A) at 4°C.

b) The tissue was homogenized with an Ultraturrax (75 % full speed, 5 sec, at 4°C).

c) This homogenate was further homogenized in a ground glass homogenizer using 5 strokes.

d) The homogenate was allowed to stand for one minute in a ice bucket and then centrifuged at 3000 g for 20 minutes at 4°C.

e) The pellet was then resuspended in 3 mls of cold buffer A and further re-homogenzied with the Ultraturrax (25% full speed, 2 x 5 seconds at 4°C).

f) The Ultraturrax was then washed with 1 ml of cold buffer A. This solution was saved and added to the homogenate.

g) The homogenate was then loaded onto a discontinous sucrose gradient consisting of 2 1/2 mls of 50% sucrose and 7 ml of 5% sucrose in buffer A plus 0.1% calf serum at 4°C. The cellulose nitrate tube was topped up with buffer A and placed in a balanced Beckman SW27 rotor and centrifuged at 131,000 g (22,000 rpm) 18 hours at 4°C. This process removed all melanin to the bottom of the tube (Figure 13).

h) Membrane fragments were then removed from the interface (Figure 13) and placed in cold buffer A in a cellulose nitrate tube. The tube was then sealed and re-centrifuged at 300,000 g for 3/4 of an hour at 4°C in a balanced beckman type 65 rotor head.

i) This process produced a depigmented pellet (Figure 14). Table 3 summarizes the membrane preparation technique.

j) The pellet was then re-suspended in the appropriate amount of assay buffer (pH 7.6) containing 20 mmol/l-Hepes, Earles balanced salt solution and 0.3 mmol/l-catechol at 4°C. Once in solution the membrane particles were transfered to a cold ground glass homogenizor where they underwent further homogenization (5 strokes) with a glass hand homogenizer.

Homogenization and Centrifugation Technique

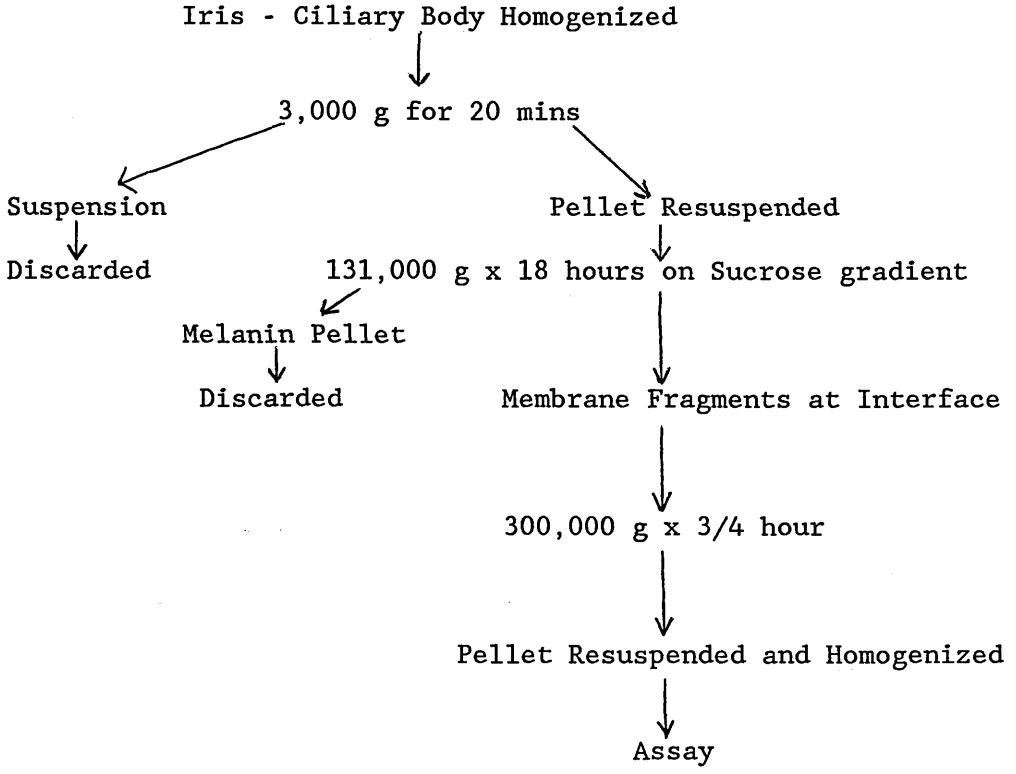


Table 3: Membrane preparation technique for ciliary processes. g = gravitational force.

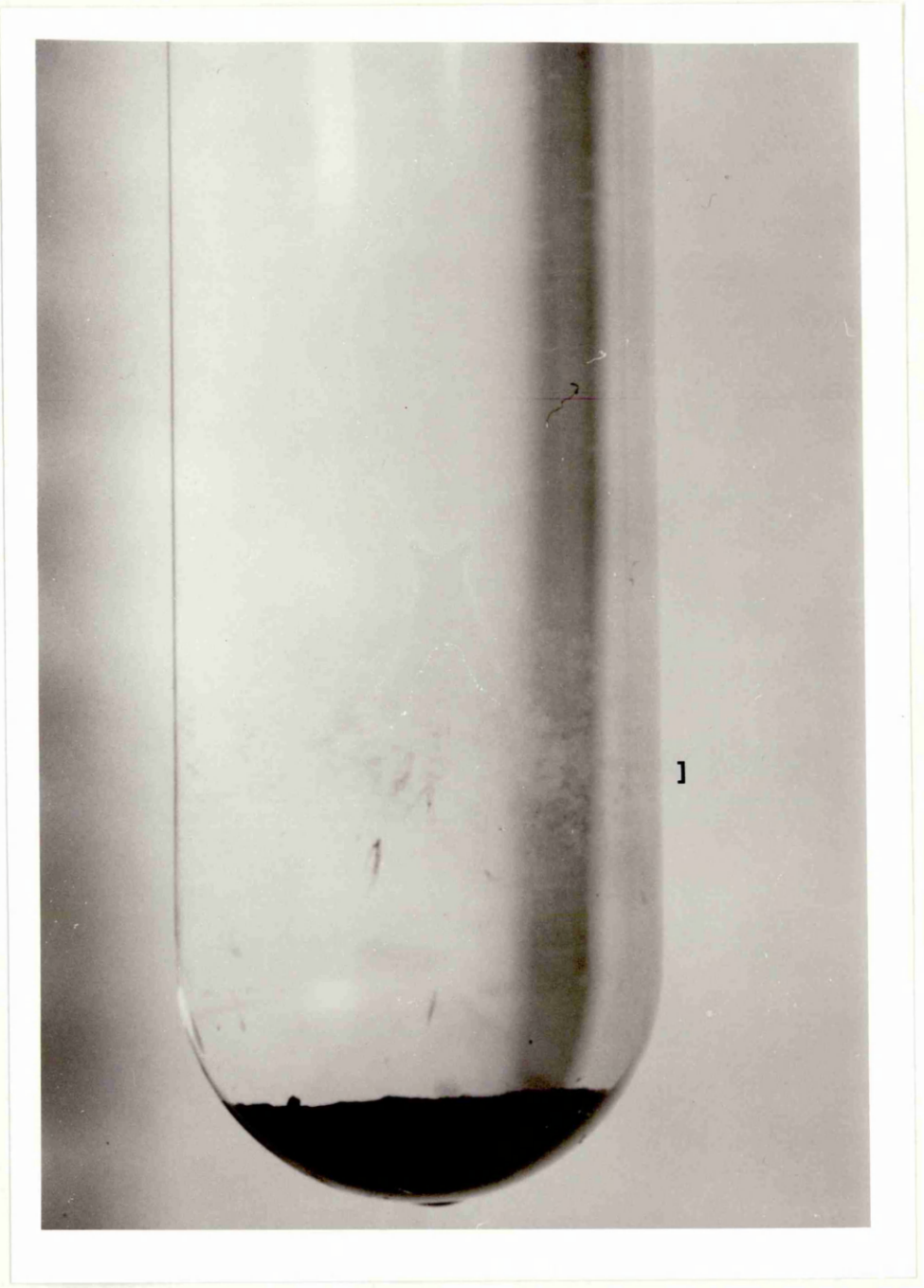


Figure 13: Melanin displaced to bottom of the tube after ultracentrifugation at 300,000 g for 18 hours on a sucrose gradient. Note interface of cell membrane fragments between 5%: 50% sucrose gradient (see bracket).

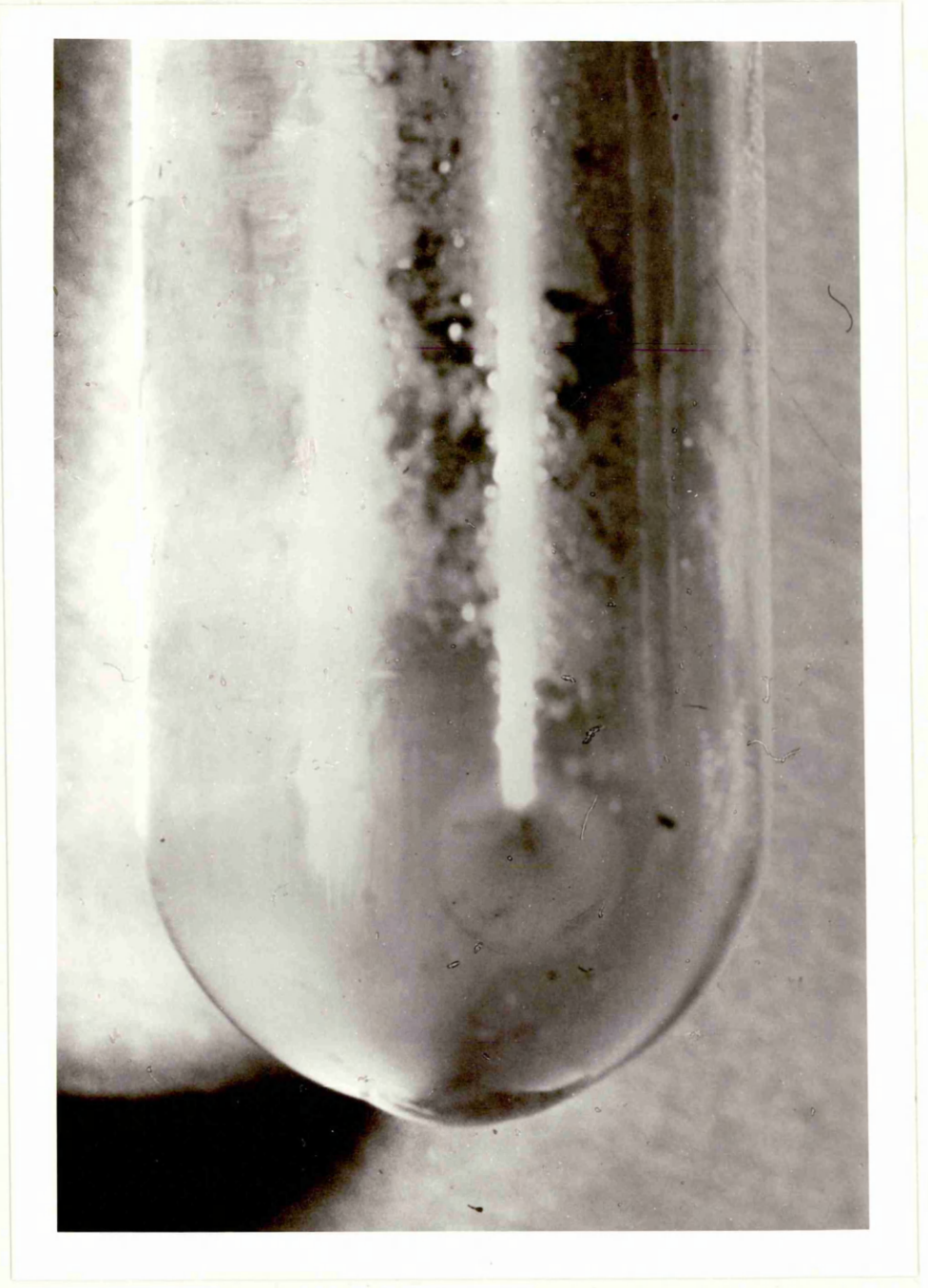


Figure 14: Pellet obtained from sucrose gradient interface. Pellet obtained by ultracentrifugation at 300,000 g for 3/4 of an hour at 4°C.

C) Ligand Binding Techniques

1) Saturation experiments.

Saturation assays were prepared on ice and done in polypropylene tubes in a final volume of 250 ul.

a) For total binding:

1) 100 ul of assay buffer was placed in duplicate test tubes.

2) 100 ul of homogenized cell membrane preparation (5-20 ug protein) was then added to each tube.

3) 50 ul of [^{125}I]-HYP in concentrations ranging from 25 - 500 pM was next added to the appropriate tubes.

b) To determine non-specific binding:

1) 50 ul of assay buffer was initially placed into duplicate tubes.

2) 50 ul of 1 uM (-)propranolol was then added to the tubes.

3) 50 ul of the appropriate concentration of [^{125}I]-HYP was then added to the mixture.

4) finally a 100 ul of cell membrane suspension was placed in appropriate tubes.

Totals and non-specific binding were all estimated in duplicate at all [^{125}I]-HYP concentrations. See subsection 4 for incubation and filtration proc edures.

Radioligand dilutions from 25 - 500 pM were prepared from stock in assay buffer (without catechol) using a dilution program developed by Clark for use on the Apple computer and Wang microprocessor (Clark et al., 1983).

All drugs were freshly prepared in assay buffer (without catechol).

Table 4 reviews details of the amounts and concentrations of cold drug, buffer, radioactive ligand and membrane used in these saturation experiments.

2) Competition Curves/Stereospecificity and Characterization Experiments

All of these experiments were prepared on ice and done in polypropylene tubes in a final volume of 250 ul. Table 5 reviews volumes and concentrations of agents used. For total binding:

a) 100 ul of assay buffer was placed in the tubes.

b) 50 ul of radioligand was then added to the tubes.

c) 100 ul of membrane suspension (5-20 ug protein) was then added to the tubes.

In the competition (test) tubes, the following was inserted:

a) 50 ul of buffer was placed in each of the tubes.

b) 50 ul of the appropriate concentration of cold ligand was then added to the tubes.

c) 50 ul of a fixed concentration of radioligand was then added to the tubes.

d) 100 ul of membrane suspension (5-20 ug protein) was finally placed in the tubes. 450 pM of [^{125}I]-HYP was the concentration used in these experiments. Specific activity was 1,850 Ci/mmol. See section 4 for washing and filtration procedures.

Each experiment was performed from two-four times, using duplicate preparations and on occasion triplicate preparations.

Drugs used in these experiments included (-) and (+) propranolol, practolol, betaxolol hydrochloride, salbutamol, IPS 339, LI - 32-468, ICI 118551 and (±) timolol maleate.

		Total binding	Non-Specific binding
1)	Buffer	100 ul	50 ul
2)	1 uM (-)Propranolol	Nil	50 ul
3)	$[^{125}I]$-HYP*	50 ul	50 ul
4)	Membrane	100 ul	100 ul

*$[^{125}I]$-HYP : 25 - 500 pM

Table 4: Volumes and concentrations of agents used in saturation and kinetic experiments.

		Total	Test
1)	Buffer	100 ul	50 ul
2)	Drug (10^{-11}M - 10^{-3}M)	Nil	50 ul
3)	$[^{125}I]$-HYP*	50 ul	50 ul
4)	Membrane preparations	100 ul	100 ul

*$[^{125}I]$-HYP = 450 pM

Table 5: Volumes and concentrations of agents used in competition stereospecificity and characterization experiments.

3) Kinetic Experiments

A) Time Course of Binding (Association)

For these experiments a final volume of 250 ul was used. Radioligand concentration was 450 pM. Duplicate preparations of total and non-specific binding tubes were prepared on ice in volumes as indicated in table 4 except that membrane preparations were added to the tubes at 5 second time intervals beginning with the last assay tube (i.e. the 90 minute tube first). After membrane preparations had been added to all tubes, the assay mixtures were then incubated for specific time intervals: 1 minute, 2.5, 5, 10, 15, 20, 30, 40 , 60 and 90 minutes. At the end of each of these periods assay tubes were removed from incubation and the reaction stopped with Tris saline, filtered and washed as indicated below (See section 4).

B) Reversal of Binding (Dissociation)

For this experiment duplicate assays consisting of total and non-specific binding tubes were prepared using 450 pM of [^{125}I]-HYP (See Table 4). Total volume in each tube was 50 ul, (i.e. Buffer, colddrug, radioligand and membranes were all made up in one fifth volume). All tubes were incubated for 40 minutes at 35^{o}C.

At the end of 40 minutes all samples were transfered to ice and diluted with 5 mls of cold buffers (100 x dilution). The reaction was then stopped, filtered and washed at times 0, 3, 6, 10, 20, 30, 60 and 120 minute intervals using procedures as detailed below.

Association and dissociation experiments were performed at least three times.

4) Incubation Washing and Filtration Procedures

For all the above experiments the assay mixtures were prepared on ice and incubation was commenced by transfer of the agitated tubes to a 36°C water bath at 30 sec intervals. When transfer was complete, the bath was covered and the samples incubated in the dark (to minimize photolysis of the [^{125}I]-HYP) for 40 minutes.

Each reaction was stopped by adding 2.5 ml of buffer B (pH 7.5) consisting of 50 mmol/l Tris, 0.9% sodium chloride and 0.1 mmol/l ± propranolol. Each sample was then allowed to stand for 45 seconds in this buffer before filtering through Whatman GFC fibreglass filter papers which had been pre-soaked for 90 minutes in buffer B. Filtration occurred by applying a vacuum of 4 pounds per square inch to a series of millipore filtration bowls. The membranes in each chamber were washed using a zipette with 3 x 8 ml of buffer C (pH 7.5) consisting of 50 mmol/l Tris and 0.9% sodium chloride buffer. Filtering and washing was completed within 12 seconds. Table 6 summarizes the constituents of the various buffers and solutions used in all of the above experiments.

Radio-activity (c.p.m.) on the filters was determined using a Packard Auto Gamma Scintillation Spectrometer. For the saturation and kinetic experiments 50 ul of each standard was placed on a GFC filter paper in a test tube and radioactivity counted.

Protein levels were estimated for each experiment using a spectroscopic technique utilizing the commercially available Bio-Rad kit.

Table 7: This table documents chemicals and radioligands used in the experiments.

Table 8: This table documents equipment used in this study.

Table 9: This table details the cold drugs studied and the manufacturers names.

Buffer A - pH 7.5

1. 50 mmol/l Tris
2. 1 mmol/l Ascorbic Acid

Assay Buffer - pH 7.6

1. 20 mmol/l Hepes pH 7.6
2. 2 mls Earles Balanced Salt Solution (without sodium bicarbonate) in 18 mls H_2O
3. 0.3 mmol/l Catechol

Buffer B - pH 7.6

1. 50 mmol/l Tris
2. 0.9 % sodium chloride
3. 0.1 mmol/l (±)propranolol

Buffer C - pH 7.6

1. 50 mmol/l Tris
2. 0.9% sodium chloride

Table 6: Components of buffers

1. Trizma base (2-Amino-2-hydroxymethyl-propane-1,3-dioz) (Tris)-Sigma Chemical Company Ltd., USA and England.
2. Ascorbic Acid - AnalaR-BDH Chemicals, Poole, England.
3. Earles balanced salt solution without Na HCO_3 - Gibco Bio-Cult Ltd. Paisley, Scotland.
4. Hyalase ovine powder - Fisons Loughborough, England.
5. Aseptic calf serum - Gibco Bio-Cult Ltd. Paisley, Scotland.
6. Sodium Chloride-AnalaR-BDH Chemicals Ltd. Poole, England.
7. Hepes buffer Powder (4-(2-Hydroxyethyl)-1-Piperazinethanesulphonic Acid) - Flow Labs Ltd. Ayrshire, Scotland.
8. Catechol (O-dihydroxybenzene)-BDH Chemicals Ltd. Poole, England.
9. Inderal ($\pm$ Propranolol Hydrochloride 1 mg/ml) for injection - ICI labs, England, U.K.
10. Bio-Rad Protein Assay kit Cat./No 500-0001.- Biorad Labs, Watford, England.
11. RADIOLIGANDS
    A. [^{125}I]-iodohydroxybenzylpindolol: Specific radio-activity 2200 Ci/mmol- New England Nuclear, Dreieich, West Germany.

Table 7: Chemicals and Radiochemicals with manufacturers names.

I Vacuum pump - general electric No 5KH33EN251.

II Water bath - Grant x Co. Cambridge, U.K.

III Millipore filtration bowls - No xx270 2550. Millipore (U.K.) Ltd. Middlesex, England.

IV Glass microfibre filter papers GF/C 2.5 mm - Whatman Lab Sales, Maid Stone, England.

V Polyproprolene tubes (12 x 75 mm) Cat. No 55 526 - (Canlab) Sarstedt Ltd. Leicester, England.

VI Beckman centrifuge cellulose nitrate tubes 5/8" x 3" No 302235/344085.

VII Ultraturrax type TP 18110 - Jankel and Kunkel 1KA-Products, Belmont, Surrey.

VIII Beckman ultracentrifuge Type L5-65: Beckman Inst. Inc. California, U.S.A.

Rotors: Type 65 No E4 165

Type SW 24 for CDFG

IX Zipette 10 ml - Jencon Scientific Ltd. U.K.

X Macro Oxford 10 ml pipette.

XI Finnpipettes 50 - 250 ul, 0-50 ul, 250 ul - 1,000 ml.

XII Packard auto gamma scintillation spectrometer.

Table 8: Instruments, Consumables and Disposables used in these experiments.

I Salbutamol. Allen & Handbury Ltd. Middlesex, England.

II (-) and (±) timolol maleate - Merk Frosst (M.S.D.), Quebec, Canada and Hoddesdon, England.

III (-), (±) and (+) propranolol - Imperial Chemical Industries (I.C.I.) Pharmaceuticals Division, Cheshire, England.

IV LI 32468 - Sandoz, Basel, Switzerland.

V IPS 339 - Dr. LeClerk, University Louis Pasteur, Strasbourg, France.

VI ICI 118551 - ICI - Cheshire, England.

VII Practolol - ICI - Cheshire, England.

VIII Atenolol - ICI - Cheshire, England.

IX (-) Metoprolol Hydrochloride- Ciba-Geigy Pharmaceuticals Division, Horsham West Sussex, U.K.

X Betaxolol Hydrochloride - Alcon Labs, Fortworth, Texas, U.S.A.

Table 9: Drugs with manufacturers names.

II. Intraocular Pressure Recovery Method (Aqueous Formation Index).

Six pigmented rabbits were used to investigate each drug. A one week wash out period was used between experiments. All rabbits were accommodated to handling and to the restraint boxes in the laboratory environment prior to the study.

Intraocular pressure readings were obtained using a Digilab pneumatonometer which had been calibrated for rabbit eyes (see developments section). (Moses and Grodezki, 1979). Pressure readings were taken three times and the mean used as final IOP. Topical anaesthesia was placed in the eye of each animal before I.O.P. readings were measured (1/2% Proparacaine Hydrochloride).

Intraocular pressure was measured before instillation of drug or buffer. 100 ul of drug was then instilled into each rabbit lower conjunctival fornix using a micropipette. 100 ul of phosphate buffer solution was placed into the lower fornix of the animals second eye. Drug and buffer administration were performed randomly in a masked fashion. I.O.P. was then re-measured 50 minutes later. A 23.4% solution of sodium chloride was then infused intravenously into each rabbit via a marginal ear vein at a rate of 1 ml/minute over a period of 10 minutes using a Sage pump. At the end of this infusion period intraocular pressures were measured at the following time intervals 0, 5 min, 10 min, 15 min, 20 min, 40 min, 50 min, 60 min, 70 min, 80 min, 120 min, 130 min, and 140 min or until intraocular pressure recovered to preinfusion values in one or both the eyes.

A control experiment was initially performed as described above but buffer alone was placed in one eye. The purpose of having control eyes

was to determine whether systemic absorption of drug influenced I.O.P. recovery rate in the second eye (consensual effect) (See Discussion). The same six rabbits were used throughout the study.

Compounds studied included a non-specific beta blocker (-) timolol maleate (Timoptic (Merk Frosst)), a beta-1 specific blocker betaxolol hydrochloride (Betoptic (Alcon)) and three beta-2 specific blockers namely IPS 339 (Dr. G. LeClerk, Strasborg) LI32-468 (Sandoz), and ICI 118551 (I.C.I.). All compounds used were either commercially prepared (1/2 % Timoptic and 1/2% Betoptic) or were freshly prepared as a 1% solution in phosphate buffer pH 7.4 (IPS 339, LI 32-468, ICI 118551).

Table 10, documents instruments and materials used in sections II and III of this study.

1. Digilab pneuma-tometer: Digilab Model 30D pneuma-tonometer, Cambridge, Mass., U.S.A.
2. Sodium chloride for intravenous injection: Lymphomes Canada Inc., Mississauga, Ontario.
3. Phosphate buffer pH 7.4: Fisher Scientific New Jersey, U.S.A.
4. Sage syringe pump model 351.

Table 10: Instruments and materials used to measure intraocular pressure recovery rate and I.O.P. in normal and buphthalmic rabbit eyes.

III. Method to determine the effects of beta-adrenergic antagonists on intraocular pressure in buphthalmic rabbits

For this series of experiments 5 buphthalmic New Zealand albino rabbits supplied by Maple Lane Farms, Toronto, were studied.

All rabbits were allowed a minimum of three days to become accustomed to animal handling.

Topical anesthesia was placed in the eye of each animal before intraocular pressure readings were measured (1/2% proparacaine hydrochloride).

Each rabbit was initially subjected to a diurnal pressure curve using a digilab pneuma-tonometer over a 12 hour period from 7 am to 7 pm. These pressure readings were used as the experimental control. A mean of three readings was taken to be the actual pressure. Diurnal curves were performed within 1 week of each experiment and were repeated for each drug.

Intraocular pressure was initially measured in both eyes of each animal $\pm$ 10 minutes before drug administration. At 7 a.m. (t = 0 min) 50 ul of a 1/2 - 1% dose of test drug or phosphate buffer was randomly administered in a masked fashion to one eye of each rabbit with congenital glaucoma. The second eye was not treated but its intraocular pressure was measured over the test period. Intraocular pressures were measured every 15 minutes for the first hour, every 30 minutes for the second hour, and then hourly for the duration of the experiment (12 hours). After the first hour of experimentation the animals were returned to their cages. They were subsequently removed for each pressure check and then returned. The experiment was repeated a week

later alternating test drug and buffer.

A wash out period of at least a week was allowed before each rabbit underwent further testing. Compounds studied in this section of the study included IPS 339, ICI 118,551, LI 32-468, 0.5% (-) timolol maleate and 0.5% betaxolol Hydrochloride.

IV. Analysis of Data

A. Saturation binding and competition curves were drawn and analyzed using micro-processor assisted curve fitting programs, as published by Clark et al (Clark et al., 1983; Clark et al., 1984). Kinetics of binding data was analysed and drawn using a computer program developed by Steinberg et al (Steinberg et al., 1985)

B. The least squares method was used to determine intraocular pressure recovery rate (aqueous humour index) over time for each rabbit. Analysis of variance of the change in I.O.P. over time was performed. Comparisons were tested using the F test.

C. Analysis of variance was used to determine Drug effects in buphthalmic Rabbits. The sources of variation considered were:

1. Variation among different rabbits.

2. Differences between the times of measurement.

3. Differences among the treated eye, untreated opposite eye and the same control eye.

4. Random variation.

The test statistic used was the F statistic. The amount of random variation was used to estimate the standard error of the mean intra-ocular pressure for each time and treatment when required.

Three treatment effects were studied:

1) Differences among the three treatments i.e. the treated eye, the corresponding control eye and the untreated opposite eye.

2) Changes occuring over time e.g. 1 and 2 hours after treatment.

3) Interaction between treatment and time i.e. was the difference (if any) between treatments the same at 1 hour after drug instillation as at 2 hours.

When examining short-term effects, the measurements at time 0, before any treatment was given, was used as a co-variable; thus controlling for the starting values of intra-ocular pressure in the analysis for all rabbits eyes. The mean I.O.P. readings at 2 hours was used to express the mean results in percentage terms.

For sections B and C statistics were performed at the University of Toronto, Canada by Dr. M. Chipman, Ph.D., Director, Clinical Research Support Unit.

DEVELOPMENTS

I. Development of Radioligand Technique

The development of radioligand techniques performed on the ciliary processes evolved from a series of experiments performed on sheep iris ciliary body diaphragm. These initial experiments were in many cases not verified in view of the fact that they were performed during an early stage of this project.

A. Dissection of Iris ciliary body diaphragm and ciliary processes.

The technique used to dissect ciliary processes as outlined in the materials and methods section was developed from iris ciliary body diaphragm dissections. After the first few attempted dissections of ciliary body it soon became apparant that a posterior sclerotomy was required to initially decompress the eye. This sclerotomy allowed anterior segment structures to settle back. A second circumferential incision down to the suprachoroidal space enabled removal of the cornea from the scleral spur without distorting anterior segment anatomy. Iris ciliary body was dissected free from choroid using an operating microscope. Finally, iris-ciliary body diaphragm was dissected off the anterior vitreous face and lens.

This technique was modified for ciliary process experiments by freeing the ciliary body from its attachments to the chloroid and then removing the iris (Figure 15). This allowed the ciliary body to be gently elevated off its vitreous attachments and under tension expose the ciliary processes attached to the lens. The ciliary processes were

dissected from the ciliary body and finally dissected off the lens and placed in cold buffer A. Later, as surgical skills improved, a time saving surgical procedure was performed which involved removing iris from the ciliary body to allow direct visualization of the ciliary processes. Ciliary processes were then directly freed from attachement to ciliary body and then dissected off the lens with De Weckers scissors using an operating microscope as indicated in the methods and materials section. All dissections were performed on ice and took twenty to thirty minutes per eye.

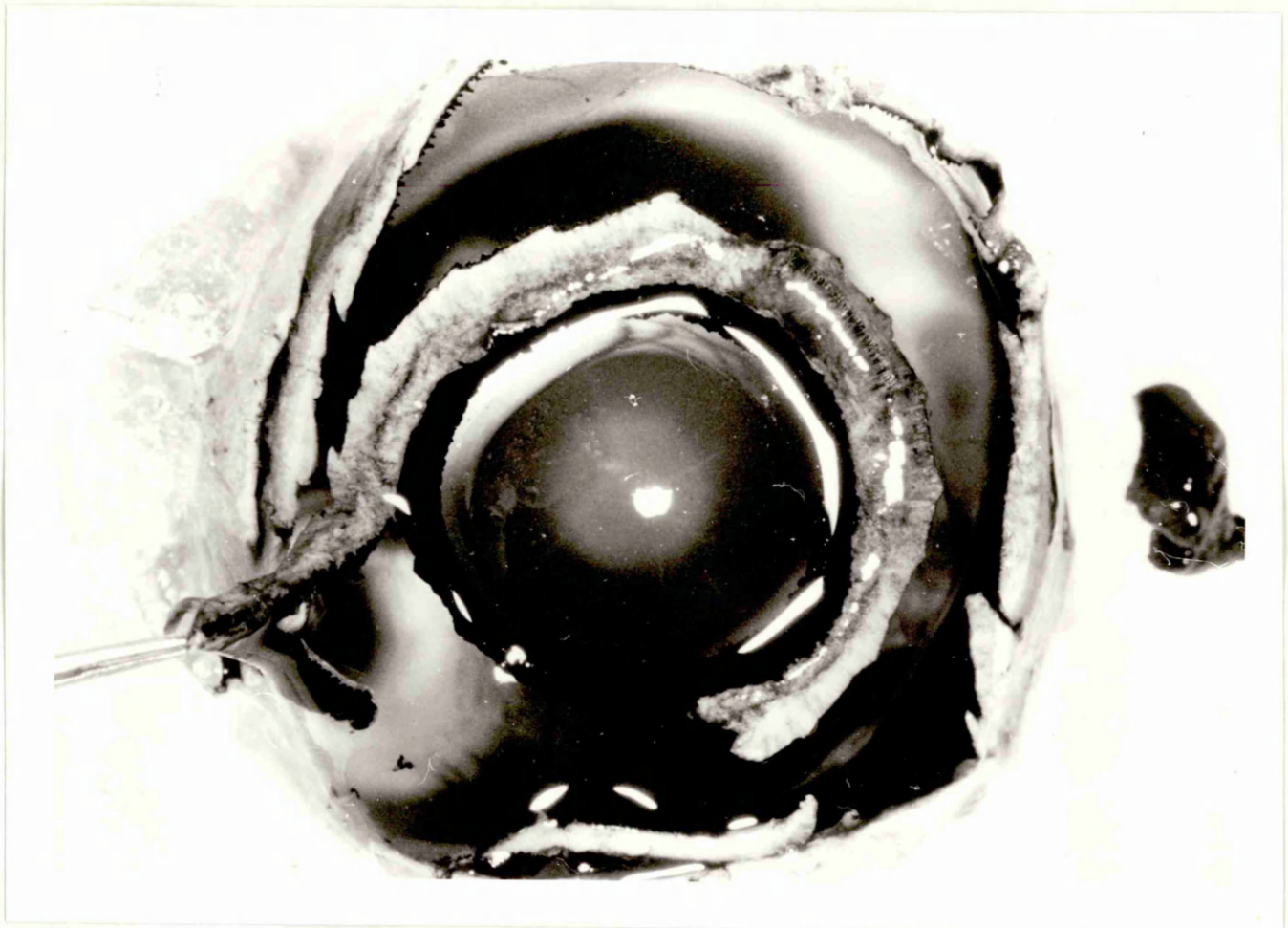


Figure 15: Ciliary body dissected from its attachment to the choroid before ciliary processes dissected off the lens. Later experiments ciliary body was not dissected from choroid.

B. Membrane Preparation and Binding Technique Developments.

Initially an attempt was made to duplicate the membrane preparation and binding technique described by Bromberg et al (Bromberg et al., 1980). It soon became apparent however that this technique was impossible to repeat using eyes containing pigment, as pigment bound virtually all the radioligand making it impossible to determine specific binding. Furthermore, this technique was extremely difficult to perform and unconventional in that it involved puncturing discontinuous sucrose gradients and collecting various fractions including the membrane interface without adequate techniques to separate bound from free ligand.

Bromberg did however report that maximum binding seemed to occur at the 5:50% interfacing of a discontinuous sucrose gradient after a 1 hour spin at 190,000 g. It was therefore decided to continue to use a sucrose gradient technique but to modify the gradient concentration and spin time so as to both collect membrane fragments and get rid of the melanin to the bottom of the tube. Various gradients and spin times were tried. As indicated on table 11, a spin time of 18 hours at 131,000 g on both a 5:75 %, and 5:50 % sucrose gradient in Buffer A produced a melanin free membrane interface with a melanin pellet at the bottom of the tube.

The next stage of development was to ensure that the interface was indeed 1) made up of membrane fragments and 2) melanin free. The tissue derived from the interface was pelleted by spinning at 131,000 g for 3 hours (later, this was modified to 300,000 g for 3/4 hour for ciliary processes experiments (table 3) . The pellet was fixed in tissue

fixative and studied by transmission electron microscopy in the Department of Ophthalmic Pathology and verified by members of the Department of Dermatology, University of Glasgow. Figure 16 is a electron micrograph showing melanin free ciliary epithelial membrane fragments obtained from the 5:75 % sucrose density gradient interface. Similar melanin free membrane fragments were derived from ciliary process tissue preparations at the 5:50 % interface.

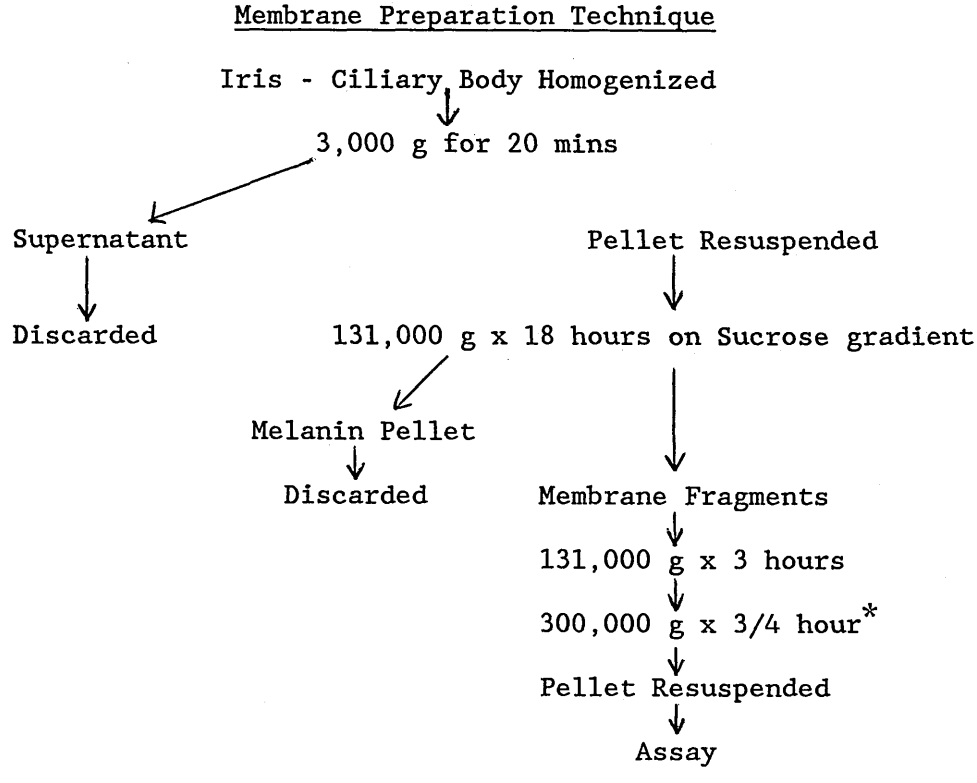


Table 11: Homogenization and spin technique to prepare membrane fragments from iris/ciliary body diaphragm and ciliary processes.

g = gravitational force.

* = ciliary processes.

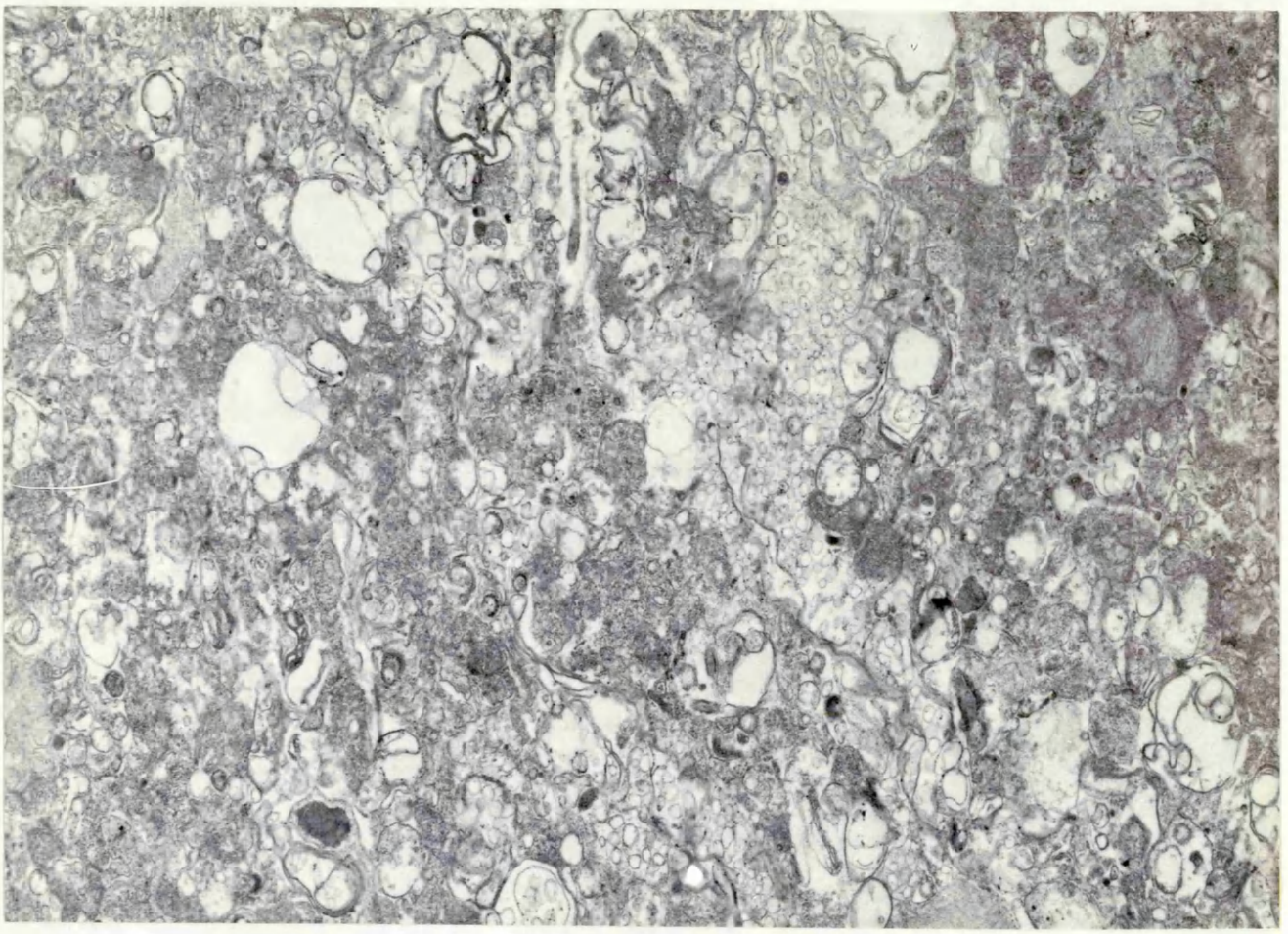


Figure 16: Transmission electron micrograph showing melanin-free membrane fragments isolated from the 5%:75% sucrose density gradient interface. (Magnification x 8000).

At this stage a simple experiment was performed which confirmed that Brombergs technique of adding radioligand to homogenised ocular tissue was inappropriate for pigmented eyes (Bromberg et al., 1980). 50 pM of [^{125}I]-HYP was added to homogenised pigmented iris ciliary body diaphragm and incubated for 30 minutes at 36^{o}C. The suspension was layered on a 5:75% sucrose gradient and spun at 131,000 g for 18 hours. At the end of the spin the melanin pellet and membrane fraction were separated and their radioactivity measured. Ocular melanin bound 98.9% of the counts. The membrane fraction bound 0.58%.

The next stage of the study was spent developing the binding technique.

All buffers and general techniques used to study and characterize beta receptors were derived from standard radioligand binding techniques as described by Yamamura et al., Nahorski et al., and Titinchi et al., (Yamamura, 1975; Nahorski, 1978; Titinchi, 1983).

As indicated in the material and methods section, all preliminary and final experiments were performed in a final volume of 250 ul (Nahorski, 1978). Routine filtration and washing techniques were utilized. One extra procedure however was performed. This involved the use of a "stopping" solution consisting of 50 mmol/l tris (pH 7.5) 0.9 % sodium chloride and 0.1 mmol/l ($\pm$) propranolol at 36^{o}C (Buffer B). This 45 second stopping procedure was performed before filtration and washing. It was performed for a number of reasons. Firstly, it was felt that dilution of the 250 ul sample with 2.5 ml of stopping solution would minimize sample loss on transfer of the tube contents to the millipore filter bowl. Secondly, the [^{125}I]-HYP concentration falls by

a factor of 11 on the addition of 2.5 ml. Since the kinetics of binding of non-specifically bound ligand are more rapid then those of specifically bound ligand, it was felt that this stopping procedure would increase the tendency for non-specifically bound ligand to leave the membrane (Maguire, 1976). Thirdly, this process is assisted by the use of propranolol at 0.1 mmol/l which for similar reasons encourages non-specifically bound [^{125}I]-HYP to leave the membrane while not significantly affecting specifically bound ligand.

Since [^{125}I]-HYP also binds avidly to GFC filters buffer B was used as an anti-adsorbant. Towards this end the filter papers were presoaked in this buffer for 90 minutes. After filtration and washing, all filters were air dried overnight at room temperature and radioactivity measured in an auto-gamma scintillation spectrometer as indicated in the methods and materials section.

Tables 12 and 13 indicate volumes and concentrations of agents used in the initial saturation and competition studies on membrane preparation derived from iris ciliary body diaphragm. As indicated on Table 13 initial concentration of radioligand was 150 pM.

The next section contains the preliminary binding results on ciliary body diaphragm and ciliary processes.

	Total	Non-Specifc Binding
Buffer	100 ul	50 ul
1 uM (-)propranolol	Nil	50 ul
$[^{125}I]$-HYP*	50 ul	50 ul
Membrane Preparation	100 ul	100 ul

*$[^{125}I]$-HYP: 25-500 pM

Table 12: Volumes and concentrations of agents used in saturation binding experiments on iris ciliary body diaphragm.

	Total	Non-Specific Binding
Buffer	100 ul	50 ul
Drug (10^{-11}M -- 10^{-3}M)	Nil	50 ul
$[^{125}I]$-HYP*	50 ul	50 ul
Membrane	100 ul	100 ul

*$[^{125}I]$-HYP = 150 pM

Table 13: Volumes and concentrations of agents used in competition/stereospecificity/characterization experiments.

C) Preliminary Binding Results on Iris Ciliary Body Diaphragm and Ciliary Processes

Figure 17 illustrates a representative example of 1 of the 2 early saturation studies on iris ciliary body diaphragm. As indicated computer analysis of the data revealed Bmax of 72.9 fmol/mg protein. K_D was 140 pM. Correlation coefficient was 94.9%.

A repeat study (graph not shown) revealed a Bmax of 122 fmol/mg protein with a K_D of 120 pM. Correlation coefficient for the 6 points was 91.8%.

The mean Bmax for the 2 experiments was 97.45 fmol/mg protein. The mean K_D was 130 pM.

As this saturation data looked encouraging, a competition curve using (+) and (-) propranolol was performed using 150 pM [^{125}I]-HYP.

Figure 18 indicates the results of the stereospecificity experiment using (+) and (-) propranolol.

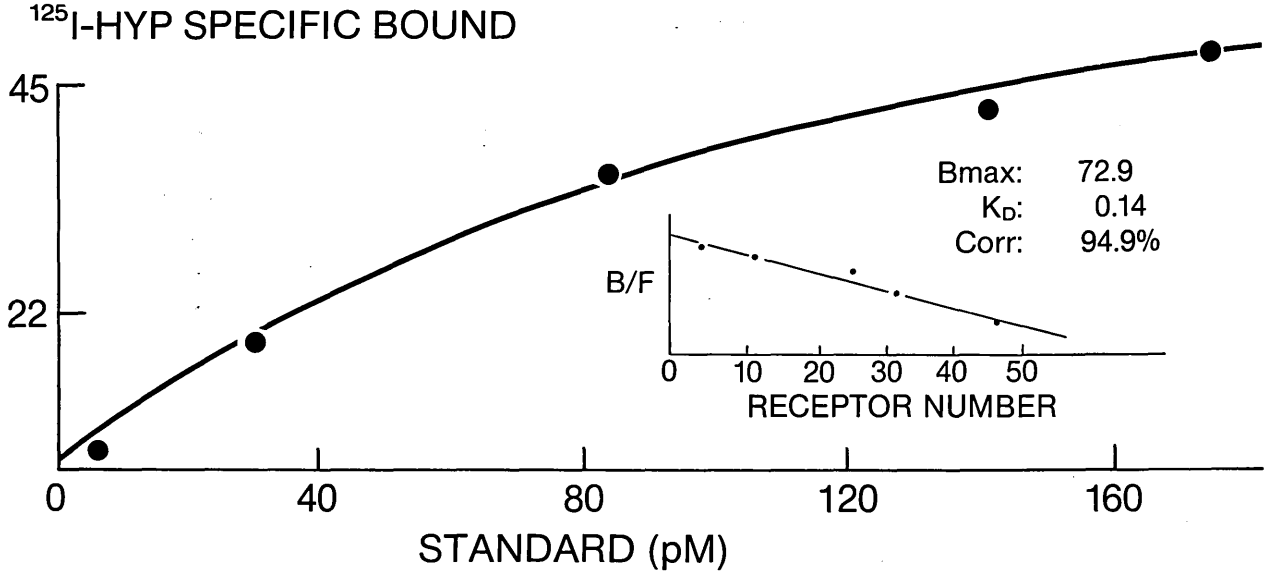


FIG 17 Saturation study (1) on sheep iris ciliary body diaphragm.

Figure 17: Specific binding of [^{125}]I-HYP to sheep iris ciliary body diaphragm receptor sites. Insert, Scatchard plot of [^{125}I]-HYP binding.

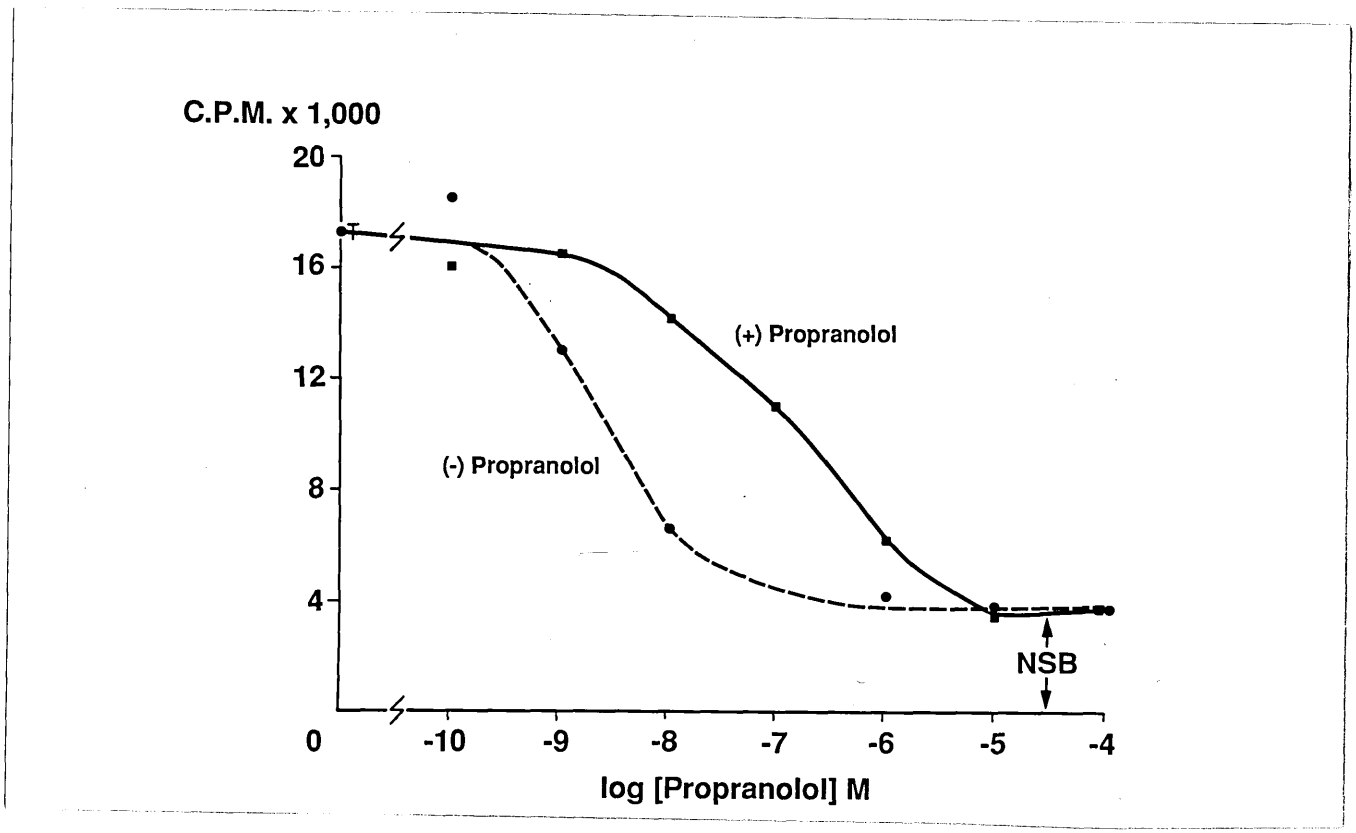


Figure 18: Stereospecificity competition curve performed on membrane preparations from iris ciliary body diaphragm.

[T = Total binding, NSB = Non-specific binding.]

K_i values derived using the formula $K_i = IC_{50}/(1 + \frac{[L]}{K_D})$ were:

(-) propranolol: 4.36×10^{-10} M

(+) propranolol: 3.28×10^{-8} M.

This figure is typical of a stereospecific beta receptor (i.e. there is a difference in potency by a factor of 75).

Figure 19: This first "Recfit" computer generated competition curve shows that the $beta_2$ selective drug, salbutamol, displaced radioligand more potently than the $beta_1$ specific drug practolol, suggesting a preponderance of $beta_2$ receptors in this tissue. Recfit computer analysis of the raw counts revealed a two site fit for each drug, indicating a mixed receptor subpopulation (i.e. $beta_1$ and $beta_2$) in the iris ciliary body diaphragm.

Table 14: This table reviews the K_i's and receptor subtypes derived from computer analysis of the curves drawn in Figure 19. As indicated beta-2 adrenoceptors made up between 75% - 91% of the beta-adrenoceptors in this tissue.

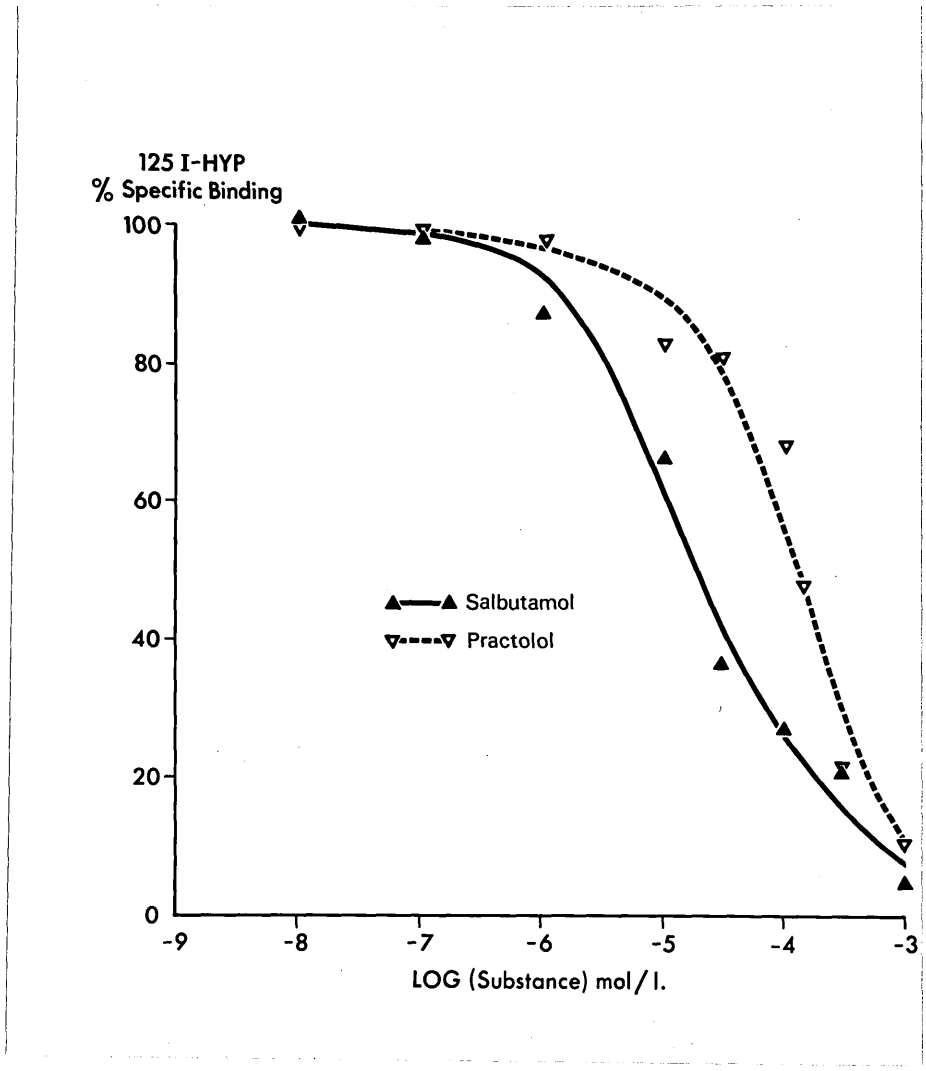


Figure 19: Competition curve comparing the potencies of salbutamol with practolol.

Compound	Affinity Constants K_i (M)		Receptor Subtypes In % Beta-1/Beta-2	Selectivity Ratio
	Beta-1	Beta-2		
Salbutamol (Beta-2 selective)	1.1×10^{-4}	3.0×10^{-6}	25/75	37
Practolol (Beta-1 selective)	3.2×10^{-7}	4.9×10^{-5}	9/91	153

Table 14: The AFFINITY constants K_i (M) and relative concentrations of B_1 and B_2 receptors in iris ciliary body diaphragm derived from Recfit analysis of data.

Once confident that binding could be performed on iris ciliary body diaphragm membrane preparations we then proceeded towards the main objective of the binding study namely to study ciliary processes. Initially a few competition curves were tried on the ciliary process before an attempt was made to verify the results. These preliminary results are documented below.

Figure 20: This graph documents results of the first competition curve attempted on ciliary process membrane fragments. (-) and (+) propranolol were used as cold ligand.

[^{125}I]-HYP used was 420 pM.

Recfit analysis of the data revealed a one site fit:

K_i for (-) Propranolol = 4.6×10^{-9}M

K_i for (+) Propranolol = 3.8×10^{-7}M

Figure 21: This computer drawn displacement curve was generated using (-) metoprolol as cold ligand. Analysis of raw counts revealed a 1 site fit with a K_i of 2.1×10^{-5}M.

Atenolol a B_1 blocker, nadolol (±) propranolol and (-) timolol maleate all non-specific beta blockers (graphs not included) were also studied during this early development stage of the study. K_i for atenolol was 2.2×10^{-5}M. K_i for nadolol was 2.2×10^{-8}M. K_i for (±) propranolol was 7.9×10^{-9}M. K_i for (-) timolol maleate was 6.4×10^{-9}M. Recfit analysis revealed a 1 site fit for all data derived using these drugs.

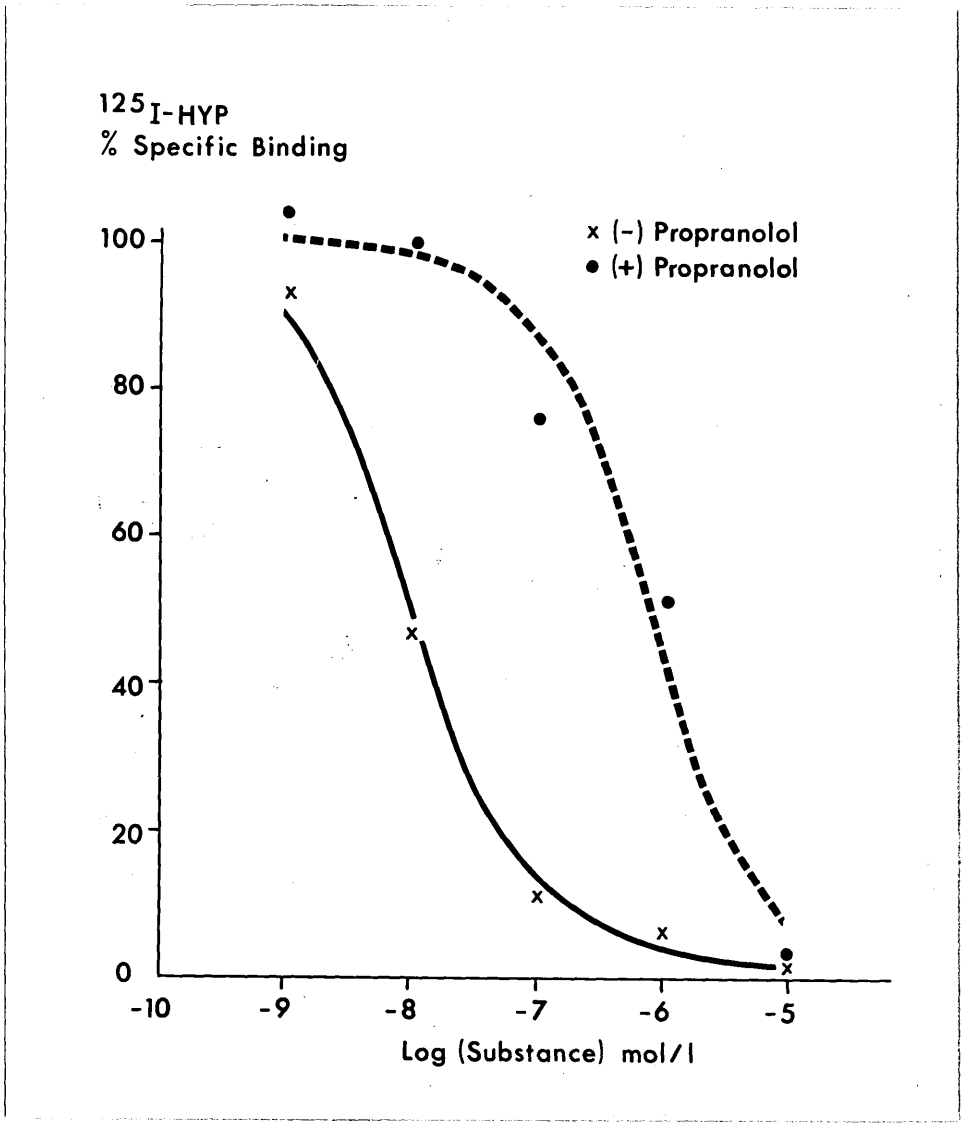


Figure 20: The first competition curve performed comparing the relative potencies of (-) and (+) propranolol to displace [^{125}I]-HYP off ciliary process membrane fragments.

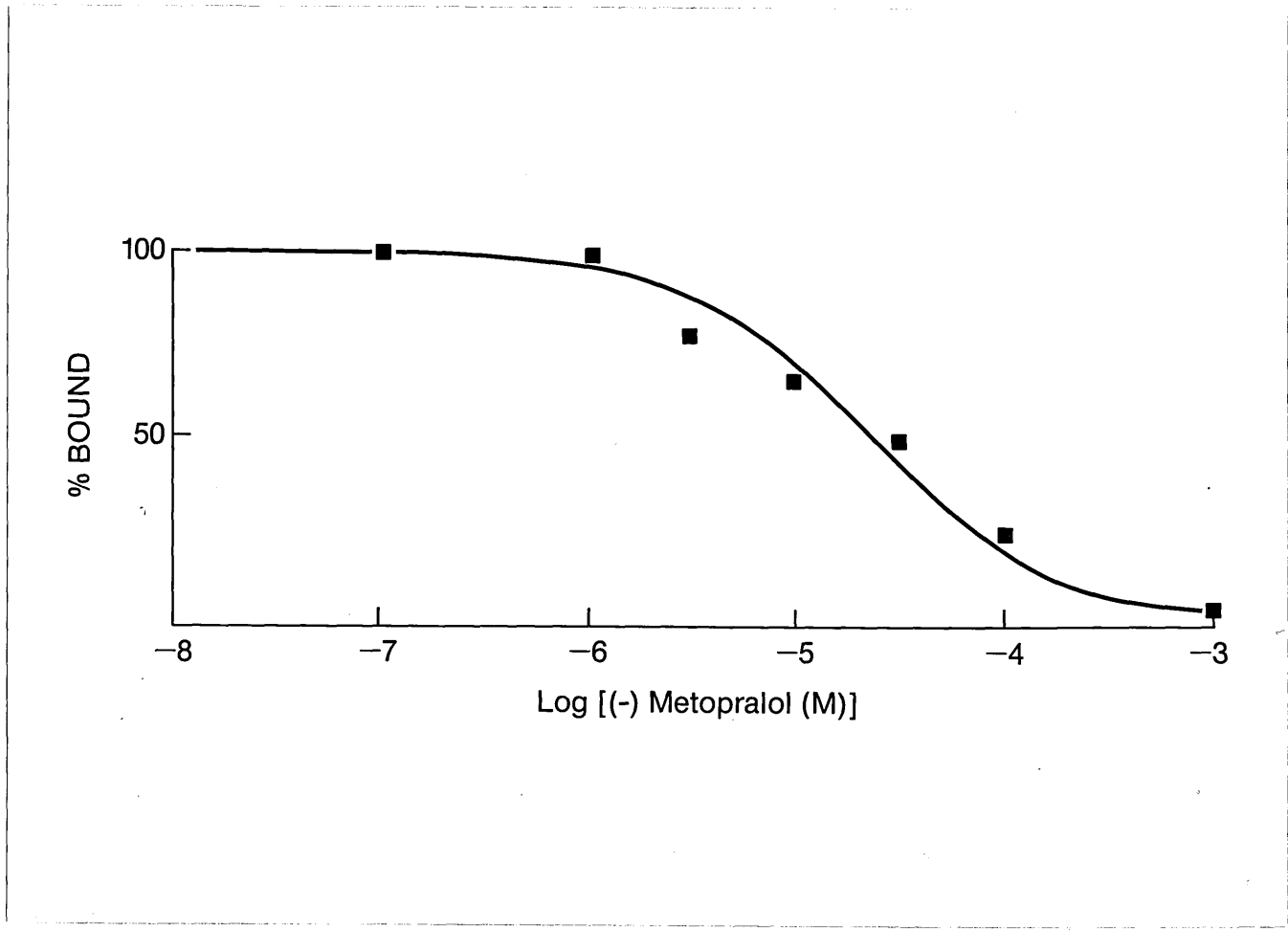


Figure 21: Computer drawn competition curve using (-) metoprolol as cold ligand. The 7 points are mean values of duplicate samples.

D) Results of Ligand Binding Studies on Ciliary Processes

Once satisfied that it was possible to perform radioligand binding on pigment free iris ciliary body and ciliary process membrane preparations, attention was specifically aimed at detecting and characterising beta receptors in the ciliary processes.

Most experiments were performed three times with the final results expressed as mean and standard error.

Figure 22:

This representative computer derived saturation curve and scatchard analysis (insert) is characteristic of a receptor study. As indicated [^{125}I]-HYP bound to ciliary process membrane receptors in a saturable manner.

B_{max} = 98.4 fmol/mg protein

K_D = 0.350 nM

Correlation coefficient = 98.8%

Two further saturation binding studies were performed (graphs not shown). The results were:

I) B_{max} = 110 fmol/mg protein

K_D = 0.350 nM

Correlation Coefficient = 96.9%

II) B_{max} = 85.6 fmoles/mg protein

K_D = 0.34 nM

Correlation Coefficient = 99.6%

The mean (+ S.E.M.) results for the above three experiments were:

B_{max} = 98.0 ($\pm$ 7.30) fmol/mg protein

K_D = 0.363 ($\pm$ 0.0185) nM

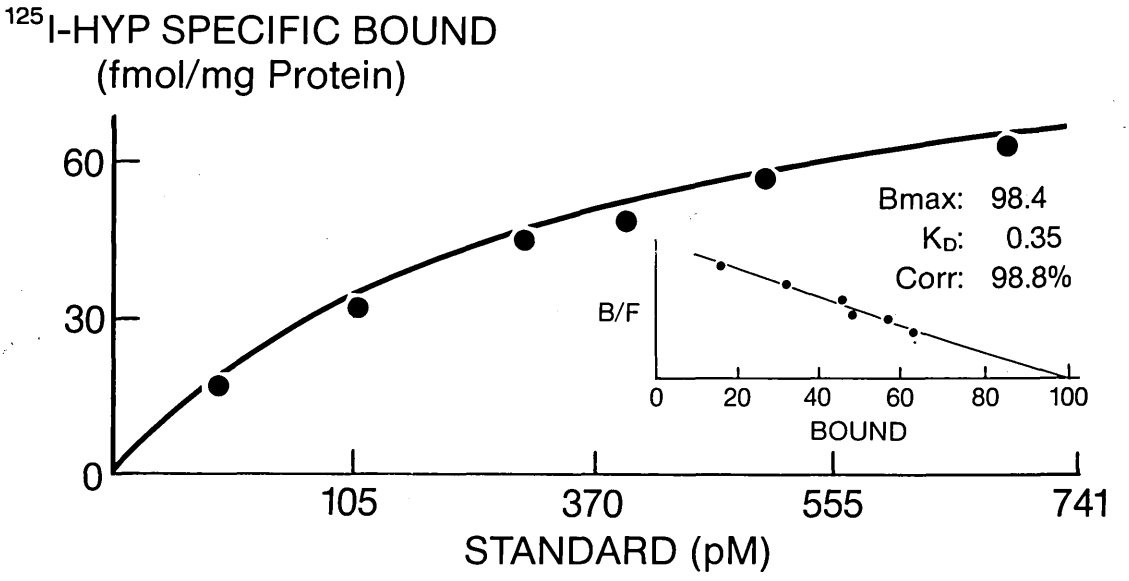


FIG 22 : Saturation curve and Scatchard analysis (insert) of ^{125}I-HYP binding to ciliary process beta receptors. Each point is the mean of duplicate preparations.

Figure 23:

This representative figure is a 1 site fit competition curve using (-) propranolol as cold ligand. This experiment was performed as part of a stereospecificity study using the same membrane preparation as figure 24. Each point is the mean of duplicate prepreparations. This figure indicates that (-) propranolol potently displaced radioligand from ciliary process beta receptors.

Recfit analysis of the data revealed a K_i of 1.44×10^{-8}M.

Two further competition curves (graphs not included) were performed as part of stereospecificity experiments using (-) propranolol as cold ligand.

The K_i's were: 1.16×10^{-8}M.

1.0175×10^{-8}M.

Both were 1 site fit competition curves.

The mean K_i ($\pm$ S.E.M.) results for the three experiments using (-) propranolol = $1.20 (\pm 0.12) \times 10^{-8}$ M.

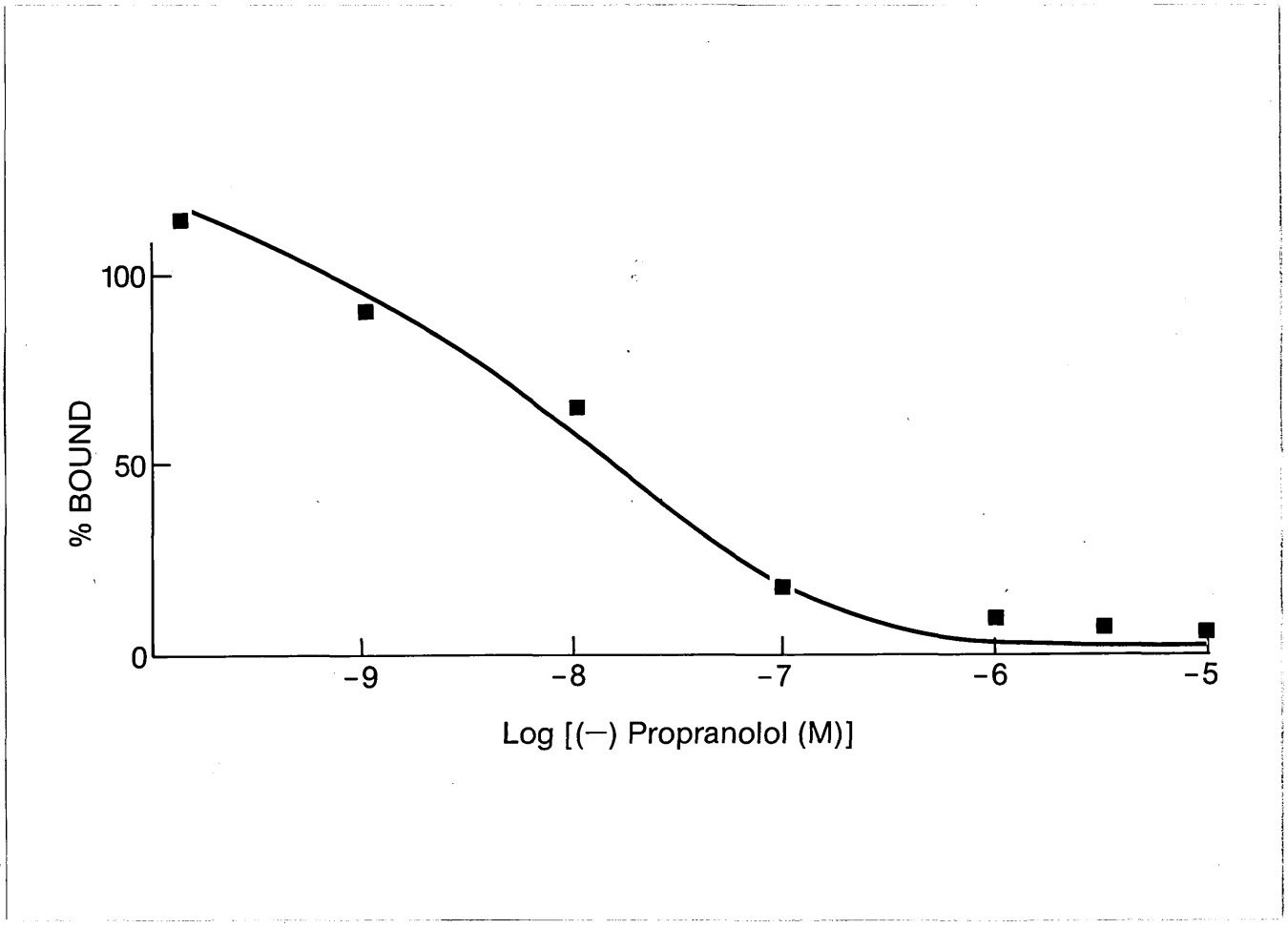


Figure 23: Representative 1 site fit competition curve using (-) propranolol as cold ligand.

Figure 24:

This representative 1 site fit competition curve was generated as part of a stereospecificity experiment using (+) propranolol as competing cold ligand. The same membrane preparation was used as in figure 23. This graph indicates fairly potent displacement of radioligand from ciliary process beta-receptors.

The K_i for this study was 3.76×10^{-7} M.

Two other similar competition experiments using (+) propranolol were also performed (graphs not shown). A 1 site fit competition curve was generated in each case.

K_i for the second (+) propranolol experiment was 2.77×10^{-7}M.

K_i for the third (+) propranolol experiment was 3.67×10^{-7}M.

The mean K_i ($\pm$ S.E.M.) of the three experiments using (+) Propranolol as cold ligand = $3.4 (\pm 0.3) \times 10^{-7}$ M.

As previously indicated the mean of the three experiments using (-) propranolol as cold ligand = 1.20×10^{-8} M. This $\pm$ 30 fold difference between the mean K_i values for (+) and (-) propranolol is consistant with the presence of stereospecific beta-adrenoceptors in ciliary process membrane fragments (See Discussion).

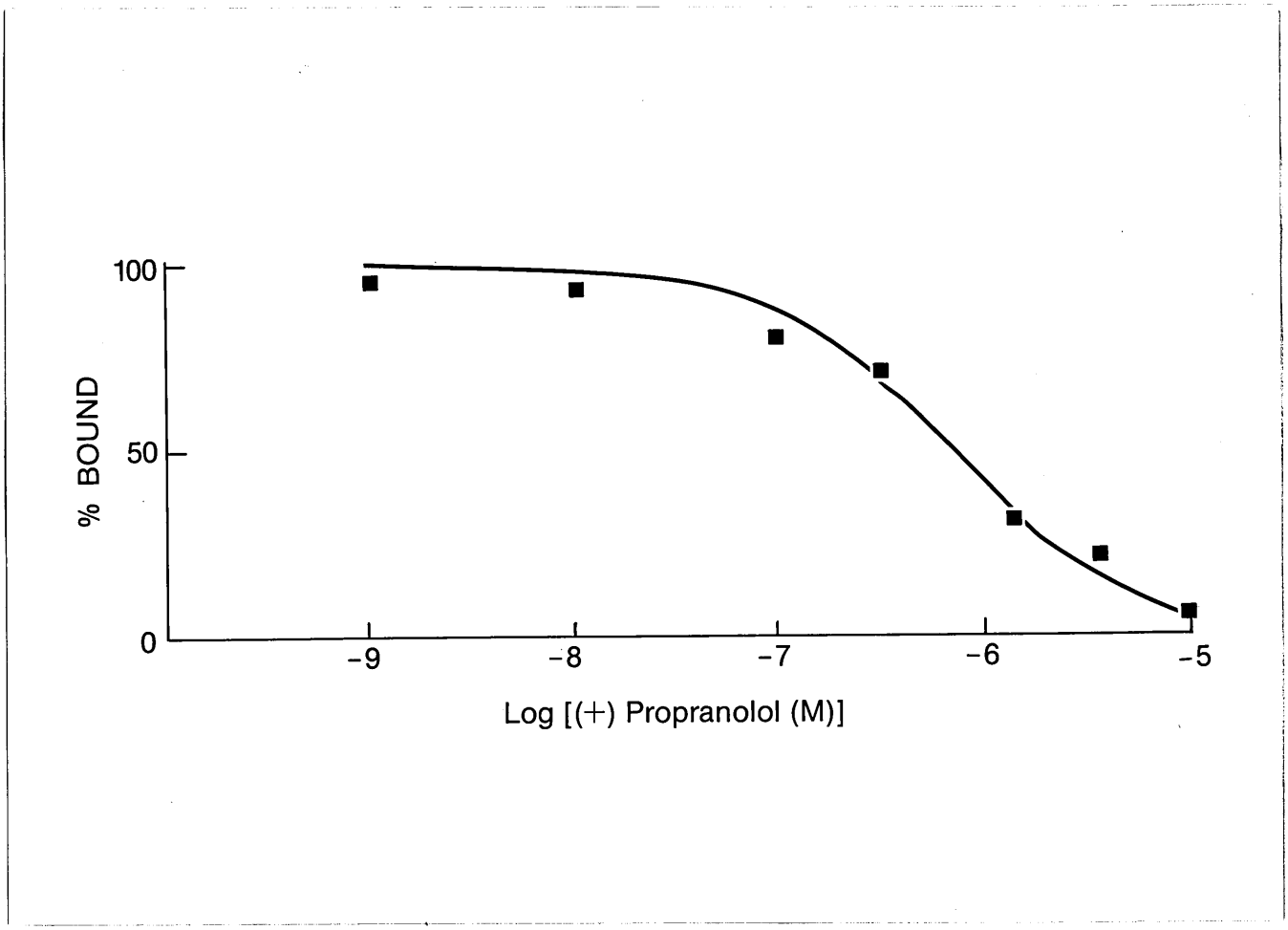


Figure 24: Representative competition curve using (+) Propranolol. Each point is the mean of duplicate samples.

Figure 25:

This representative 1 site fit competition curve was generated using salbutamol as cold ligand. This study was performed on the same membrane preparation as figure 26 as part of a characterization experiment to determine ciliary process receptor subtypes.

The results of this experiment revealed a 1 site fit:

K_i for salbutamol = 1.96×10^{-5} M.

Two other characterization/competition experiments were performed using salbutamol. Recfit analysis revealed a 1 site fit graph in both cases. The result of the two other experiments were;

$K_i = 1.46 \times 10^{-5}$ M.

and $K_i = 1.524 \times 10^{-5}$ M.

Mean K_i for the three salbutamol experiments = $1.99 (\pm 0.50) \times 10^{-5}$M.

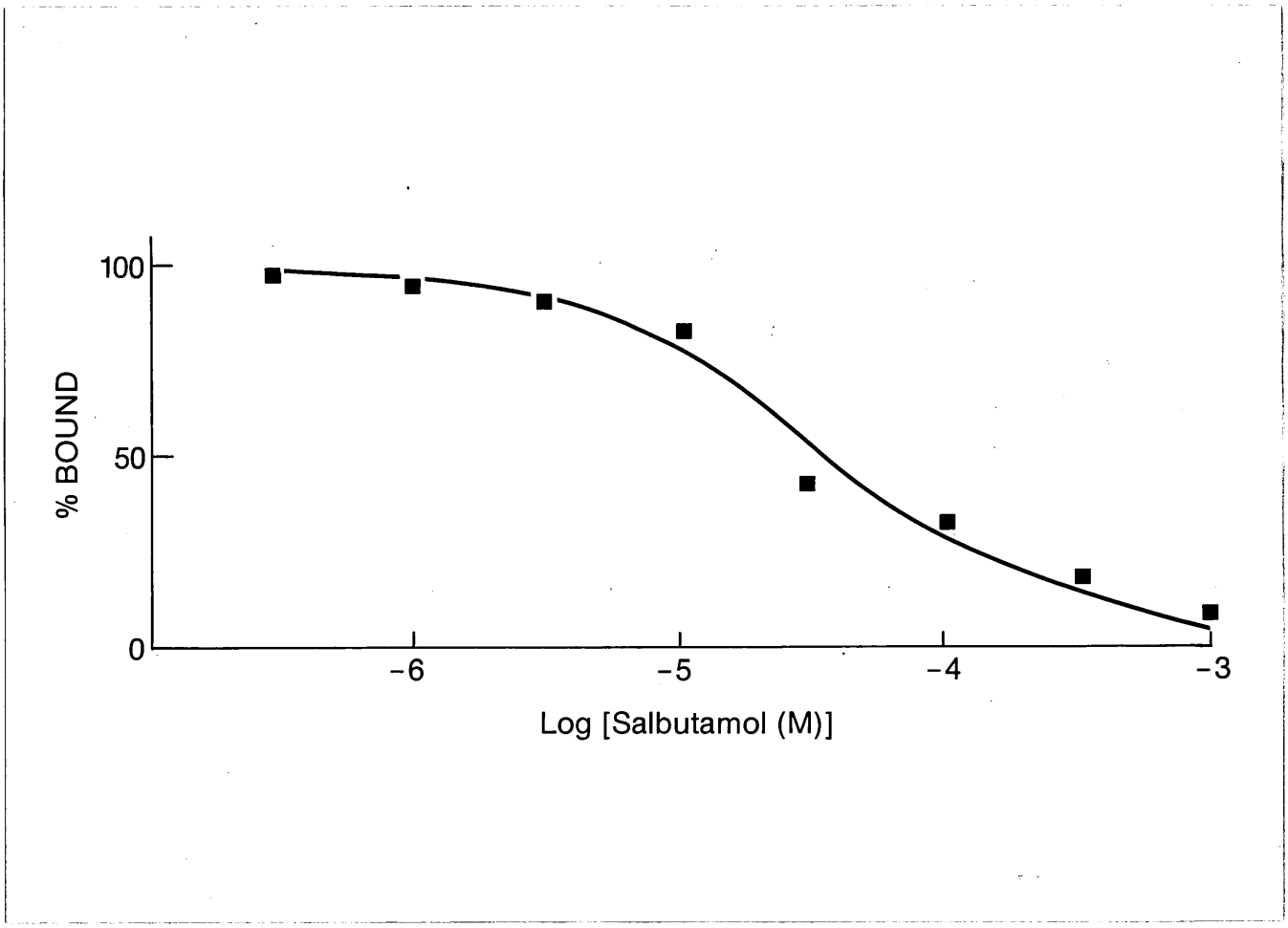


Figure 25: Representative 1 site fit competition curve generated with salbutamol as cold ligand. Each point is the mean of duplicate preparations.

Figure 26:

This experiment was performed on the same membrane sample as experiment 25 as part of a characterization experiment to determine receptor sub-types. In this case practolol ($beta_1$ blocker) was used as competing cold ligand. This graph shows weak displacement of [^{125}I]-HYP from ciliary process beta receptors.

The K_i result derived from this 1 site fit competition curve = 1.10×10^{-4} M.

Two further similar experiments were performed.

K_i for the second experiment = 0.82×10^{-4} M.

K_i for the third experiment = 1.013×10^{-4} M.

Mean K_i ($\pm$ S.E.M.) for the three practolol experiments = $0.97 (\pm 0.08) \times 10^{-4}$ M.

As indicated (Table 15) Salbutamol was noted to be more potent than practolol in displacing radioligand off receptor sites implicating beta-2 receptors as the major receptor subtype in this tissue (see later).

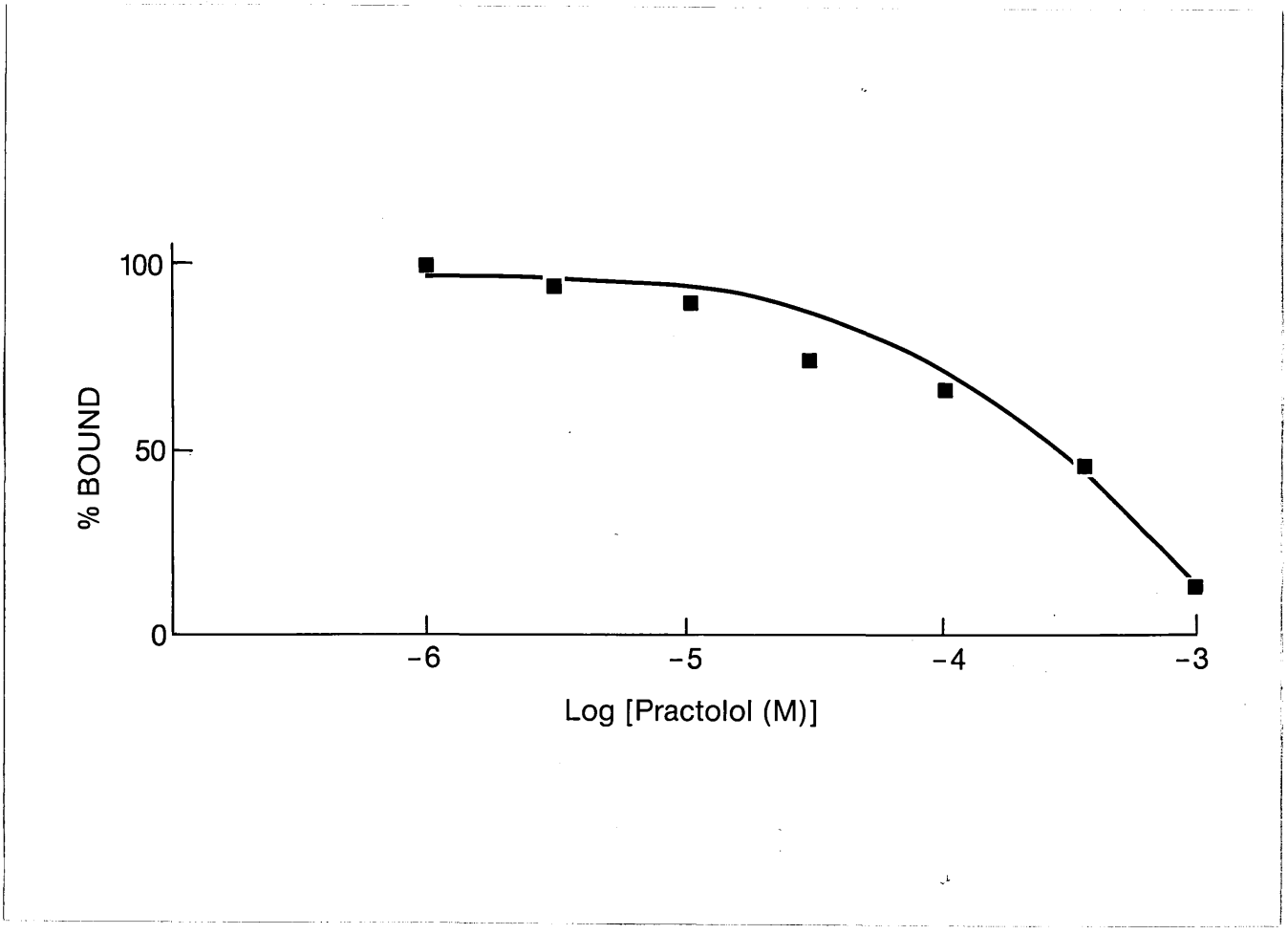


Figure 26: Representative competition curve using Practolol as cold ligand.

Figure 27:

This representative 1 site fit competition curve was generated using (±) timolol maleate as competing cold ligand.

The K_i for this experiment = 2.00×10^{-5} M.

Two other competition experiments were performed using (±) timolol maleate as cold ligand.

K_i for the second experiment = 1.03×10^{-5} M.

K_i for the third experiment = 1.16×10^{-5} M.

The mean K_i value for the three (±) timolol experiments were:

$1.40 \ (\pm 0.30) \times 10^{-5}$ M.

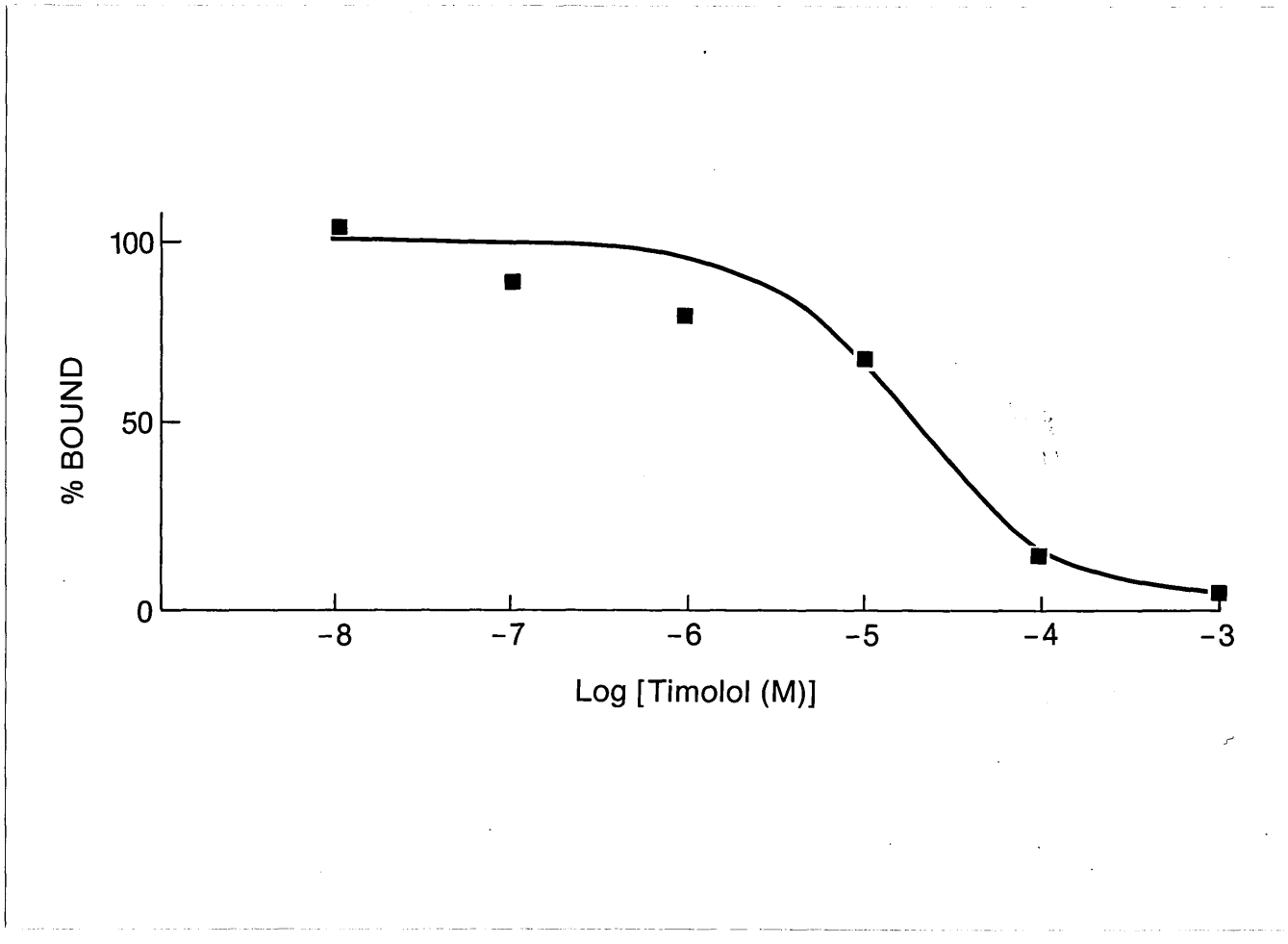


Figure 27: Representative 1 site fit competition curve using (±) timolol maleate as competing cold ligand.

Figure 28:

This representative 1 site fit competition curve was generated using betaxolol hydrochloride, a $beta_1$ blocker as cold ligand. This study was performed on the same membrane preparation as figure 29.

The K_i for this displacement curve = 6.87×10^{-6} M.

A repeat competition study revealed a 1 site fit for betaxolol hydrochloride = 5.89×10^{-6} M.

Mean K_i for the two betaxolol hydrochloride studies =

$6.39 (\pm 0.50) \times 10^{-6}$ M.

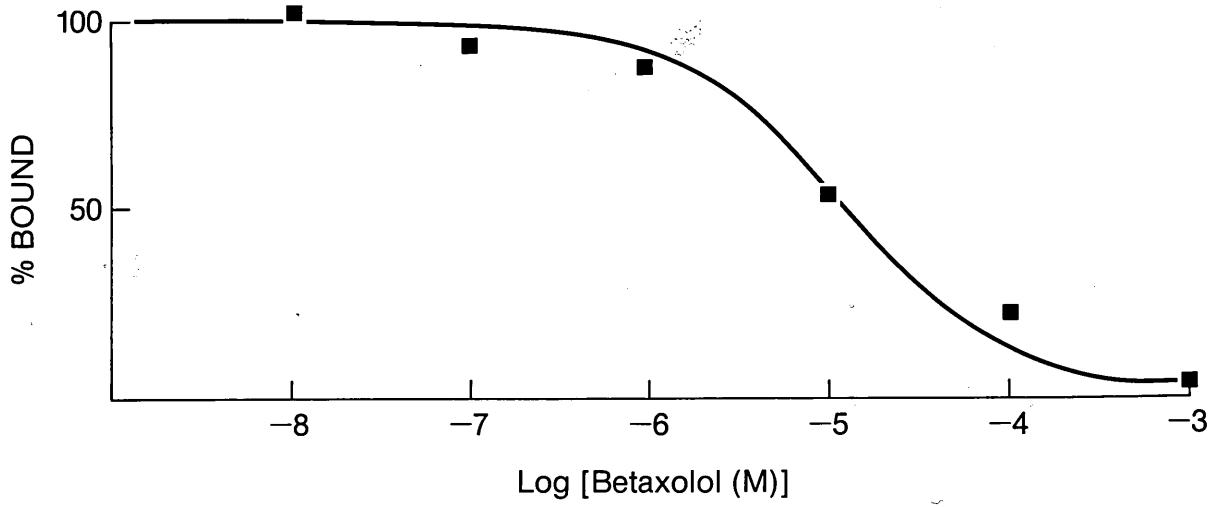


Figure 28 : Representative competition curve for betaxolol hydrochloride. Each point is the mean of duplicate preparations.

Figure 29:

This representative 1 site fit competition curve was generated using ICI 118551 (a beta-2 blocker) as cold ligand. This experiment was performed on the same membrane preparation as figure 28. ICI 118551 displaced [^{125}I]-HYP potently off ciliary process membrane receptors. Recfit analysis of the data revealed a K_i for this drug = 2.78×10^{-8} M.

A repeat experiment using ICI 118551 revealed another 1 site fit with a K_i of 5.53×10^{-8}M.

Mean K_i for the 2 experiments = $4.65 (\pm 0.81) \times 10^{-8}$ M.

As indicated on table 15, the beta-2 blocker ICI 118551 displaced radioligand much more potently than betaxolol hydrochloride (beta-1 blocker) off the same receptor preparations. This result indicates that beta-2 receptors are the major receptor subtype in this tissue.

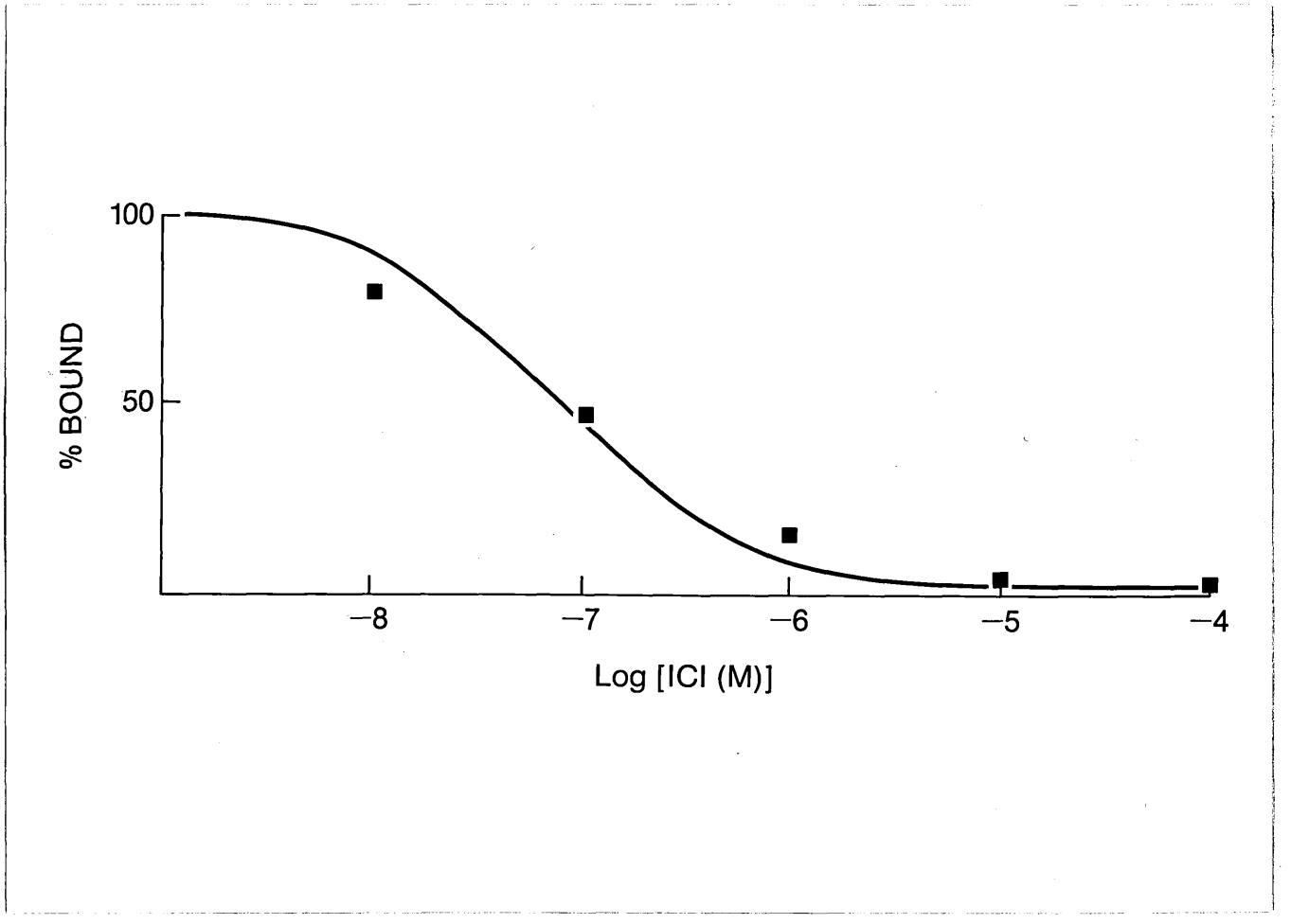


Figure 29: Representative 1 site fit competition curve using ICI 118551 as cold ligand. Each point is the mean of duplicate preparations.

Figure 30:

This representative 1 site fit competition curve was generated using (-) metoprolol (a beta-1 blocker) as cold ligand.

The K_i for this (-) metoprolol experiment = 2.1×10^{-5} M.

Two other similar experiments were performed. Both were 1 site fits.

K_i from the second experiment = 1.03×10^{-5}M.

K_i from the third experiment = 1.06×10^{-5}M.

Mean K_i for (-) metoprolol ($\pm$ S.E.M.) = $1.39 (\pm 0.3) \times 10^{-5}$ M.

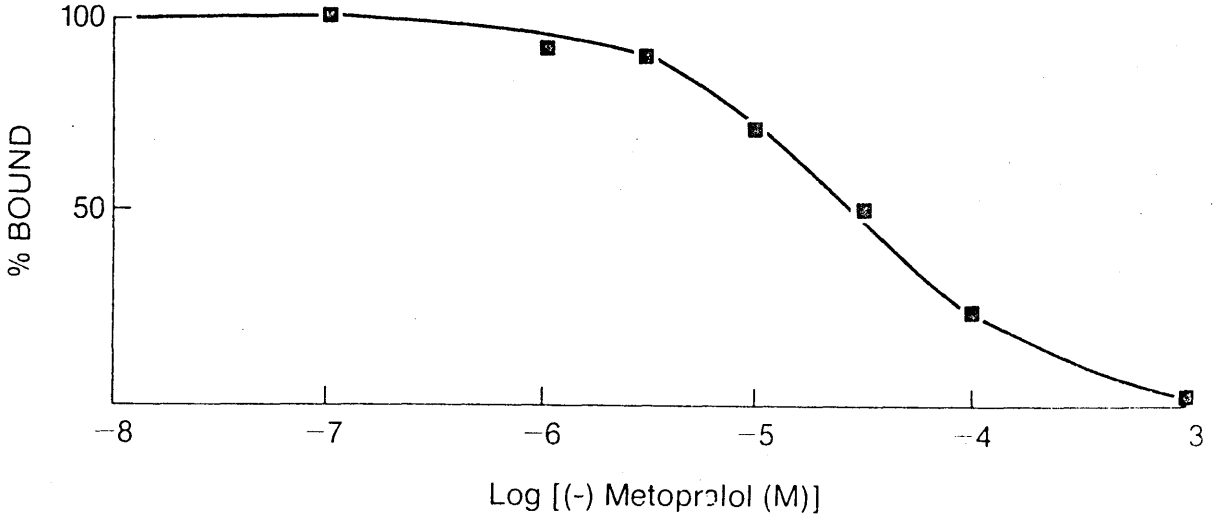


Figure 30: Representative competition curve using (-) metoprolol as competing ligand. Each point is the mean of duplicate preparations.

Figure 31:

This representative 1 site fit competition curve was generated using IPS 339 (beta-2 blocker) as cold ligand. As indicated IPS 339 potently displaced [^{125}]I-HYP off cilliary process receptor sites.

K_i for IPS 339 was 1.03×10^{-7} M.

Two further experiments were performed with IPS 339. Both produced 1 site fit competition curves with K_i values of:

8.31×10^{-8} M and

5.9×10^{-8} M.

Mean results for the three experiments using IPS 339 ($\pm$ S.E.M.) = $8.16 (\pm 1.27) \times 10^{-8}$ M.

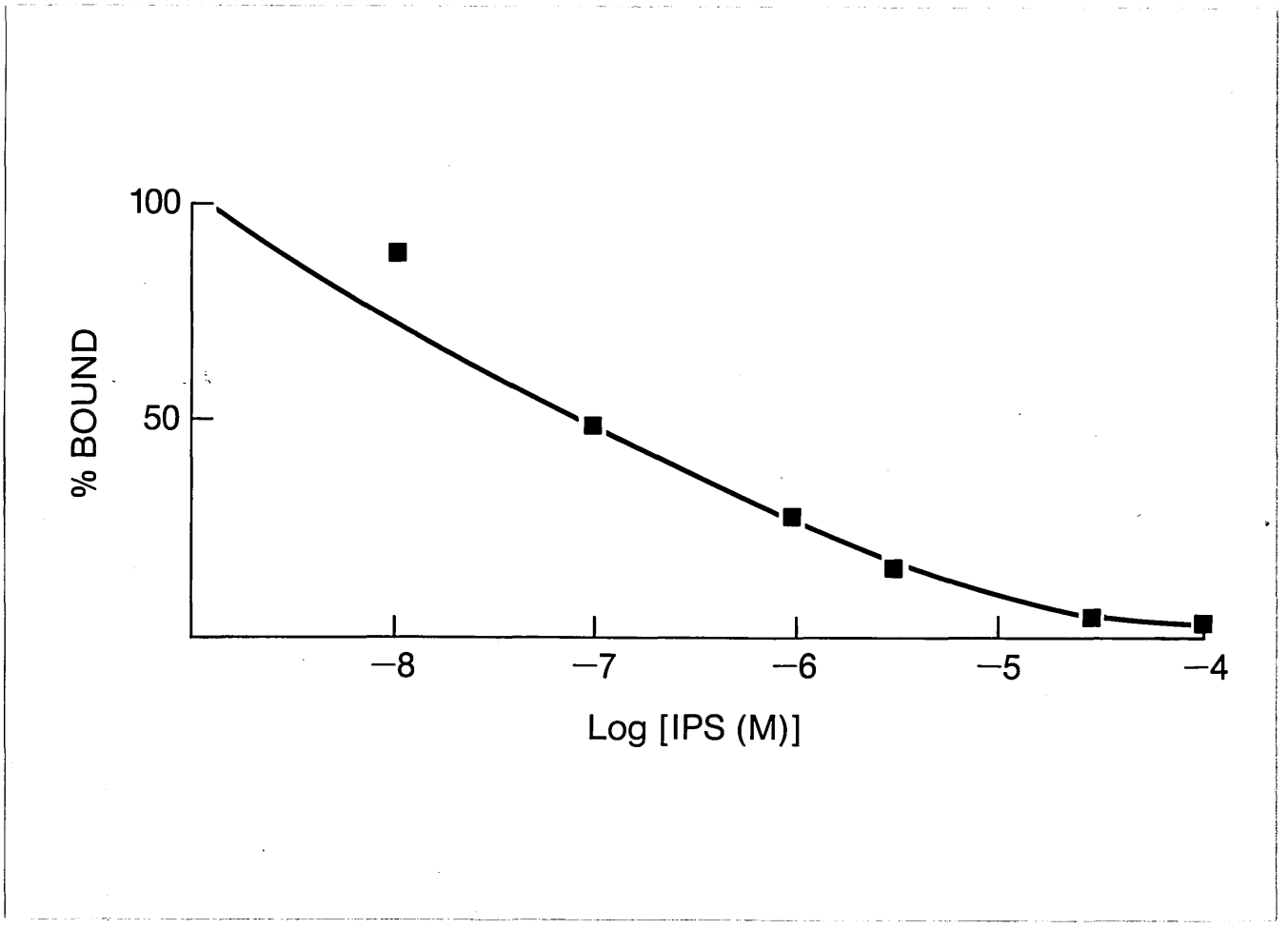


Figure 31: Representative 1 site fit competition curve for IPS 339.

Figure 32:

This representative 1 site fit competition curve was generated using the beta-2 blocker LI 32-468.

This drug potently displaced hot ligand off ciliary process membrane receptors.

Recfit analysis revealed a K_i = 3.112 x 10^{-8} M.

Two repeat experiments were performed with LI 32-468. Both produced 1 site curves with K_i values of: 2.876 x 10^{-8} M and

2.75 x 10^{-8} M.

Mean K_i ($\pm$ S.E.M.) for the three experiments using LI 32-468 = 2.91 ($\pm$ 0.10) x 10^{-8} M.

Non-specific binding for all previous experiments ranged between 25 - 45%.

Table 15: This table reviews all the competition data with cold ligands in rank order of potency. As indicated (-) propranolol was the most potent drug. The three beta-2 blockers all bound potently to ciliary process beta receptors while the beta-1 blockers bound less potently to ciliary process beta receptors. Practolol (a beta-1 blocker) was the least potent of the 10 drugs studied.

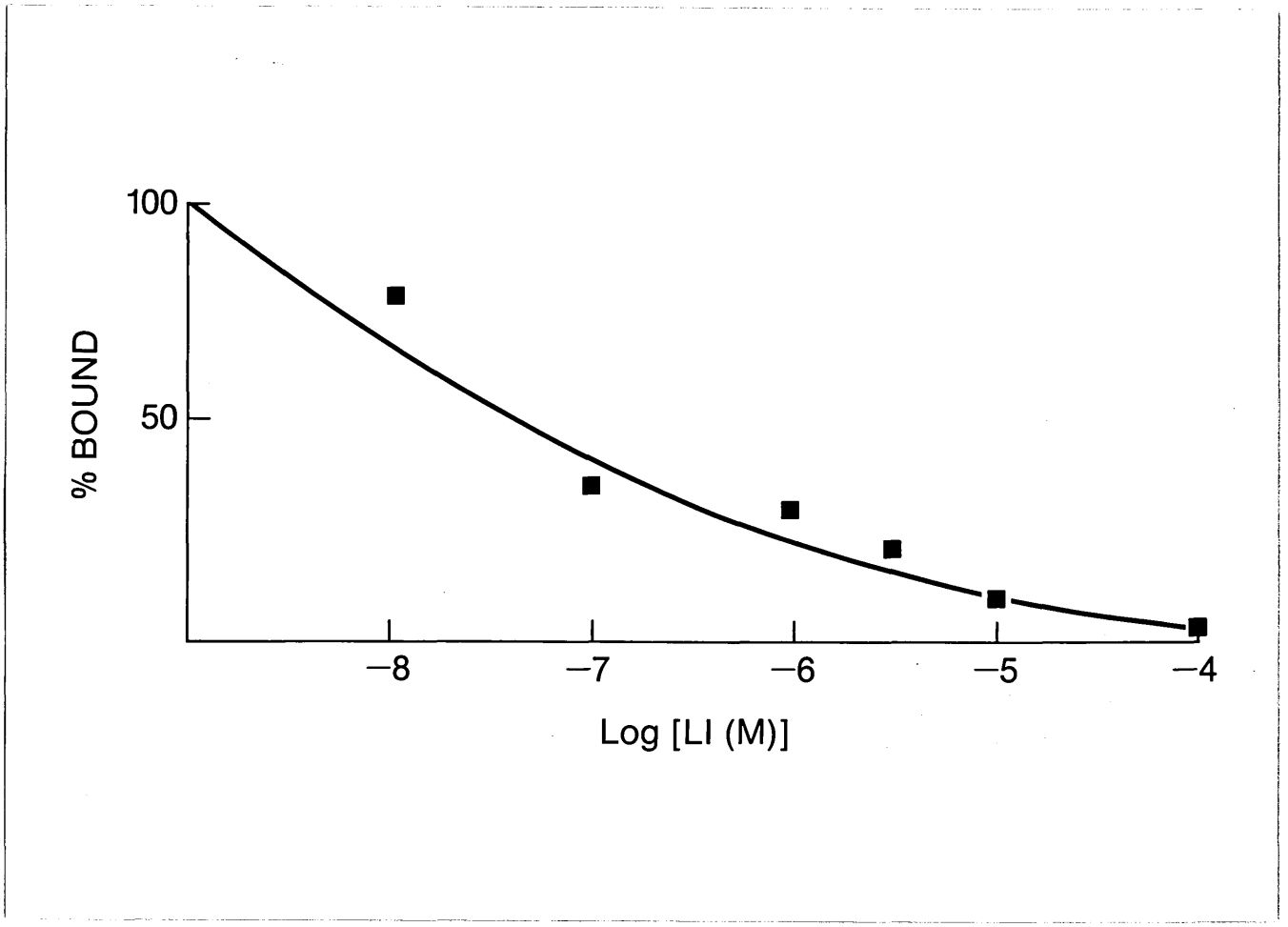


Figure 32: Representative 1 site fit competition curve using LI 32-468 as cold ligand.

Drug		No. of Experiments	Type of Adrenergic Drug	K_i (M) value (± S.E.M.)
1)	(-) Propranolol	3	N.S.B.	1.20×10^{-8} (± 0.12)
2)	LI 32-468	3	B_2	2.91×10^{-8} (± 0.10)
3)	ICI 118551	2	B_2	4.65×10^{-8} (± 0.87)
4)	IPS 339	3	B_2	8.16×10^{-8} (± 1.27)
5)	(+) Propranolol	3	N.S.B.	3.4×10^{-7} (± 0.31)
6)	Betaxolol Hydrochloride	2	B_1	6.39×10^{-6} (± 0.50)
7)	(-) Metoprolol	3	B_1	1.39×10^{-5} (± 0.3)
8)	(±) Timolol maleate	3	N.S.B.	1.40×10^{-5} (± 0.30)
9)	Salbutamolol	3	B.A.	1.99×10^{-5} (± 0.50)
10)	Practolol	3	B_1	0.97×10^{-4} (± 0.08)

Table 15: Review of the Competition data in rank order of potency;

N.S.B. = Non-specific blocker

B_1 = Beta-1 blocker

B_2 = Beta-2 blocker

B.A. = Beta-2 agonist

Kinetics binding experiments

I) On rates:

Figure 33: Is a representative K_{on} computer generated graph. As indicated, equilibrium was reached within 30 minutes.

K_{on} for this experiment was:

$0.3818 \times 10^{9}\ M^{-1}\ min^{-1}$

Two further K_{on} experiments were performed.

The K_{on} results were:

2) $0.4658 \times 10^{9}\ M^{-1}\ min^{-1}$

3) $0.5115 \times 10^{9}\ M^{-1}\ min^{-1}$

The mean ($\pm$ S.E.M.) K_{on} for the 3 experiments was

$.4530\ (\pm\ 0.037) \times 10^{9}\ M^{-1}\ min^{-1}$

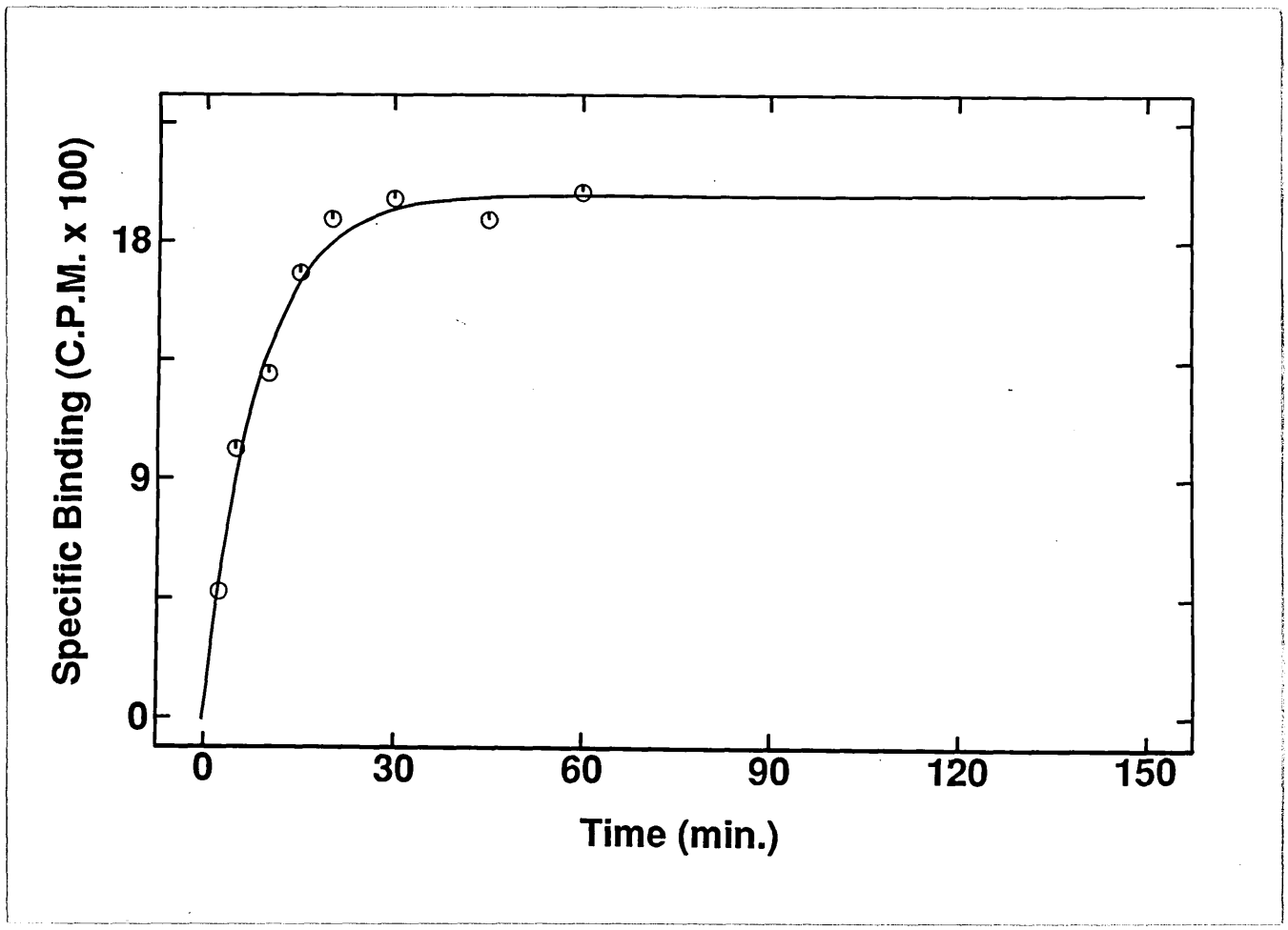


Figure 33: Time course of association (K_{on}) of [^{125}I]-HYP binding to beta adrenoceptors on cell membranes derived from sheep ciliary processes.

Figure 34:

This computer generated graph shows the time course of dissociation for 3 separate experiments. The computer derived K_{off} rates were;

I) $1.124 \times 10^{-1}\ min^{-1}$

II) $9.515 \times 10^{-2}\ min^{-1}$

III) $1.003 \times 10^{-1}\ min^{-1}$

Mean $K_{off} = \underline{1.026\ (\pm\ .051) \times 10^{-1}\ min^{-1}}$

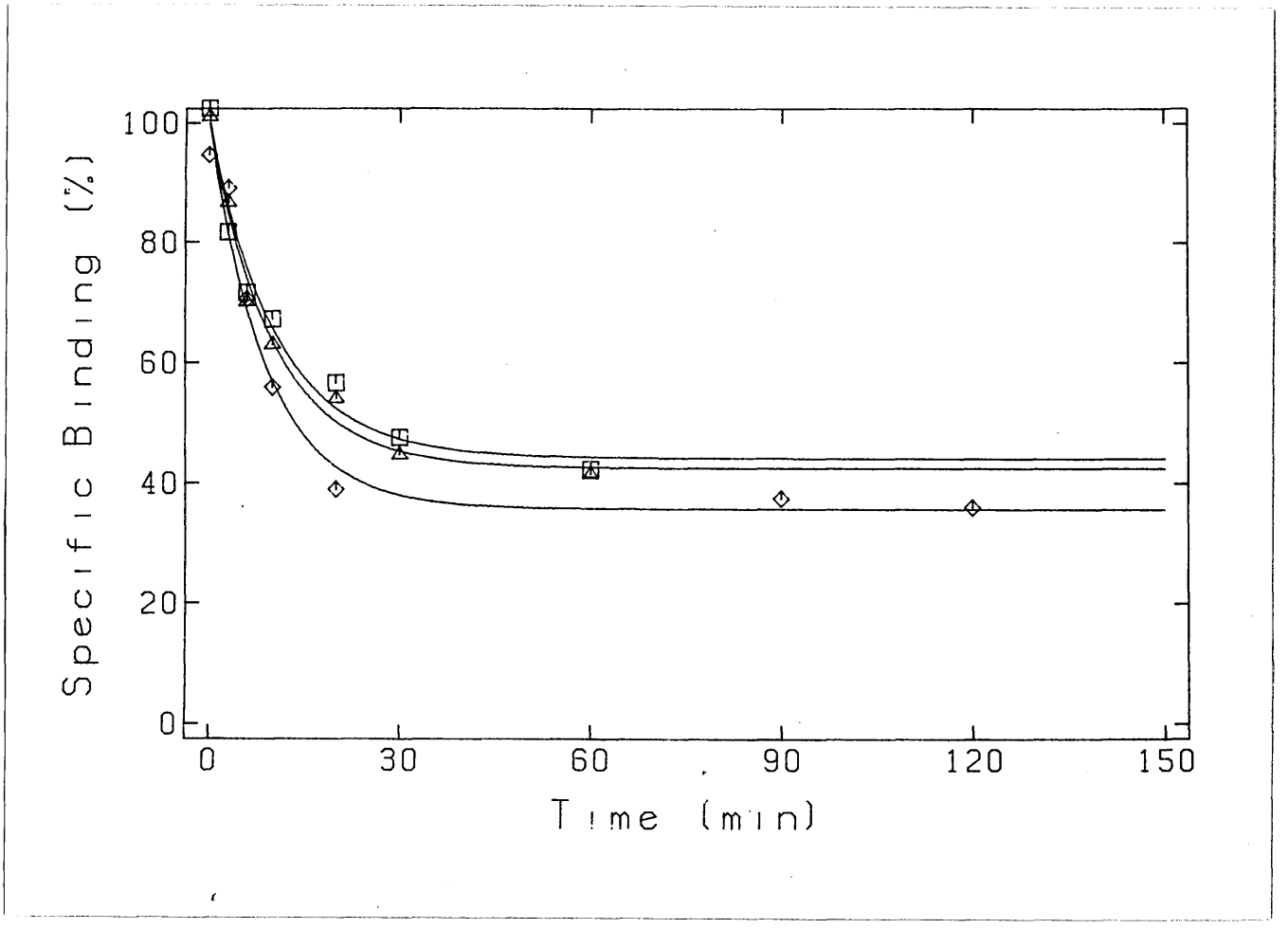


Figure 34: Three (K_{off}) time course of [^{125}I]-HYP dissociation graphs from three separate experiments. Each point is the mean of triplicate preparations.

K_{off} = Ln 2/t (Levitzki, 1984)

L_{n2} = 0.693

$t_{1/2}$ = Ln $2/K_{off}$

$$= \frac{0.693}{1.026 \times 10^{-1} \text{ min}^{-1}}$$

= 6.75 minutes

$$K_D = \frac{K_{off}}{K_{on}}$$

$$= \frac{1.026 \times 10^{-1} \text{ min}^{-1}}{0.453 \times 10^{9} \text{ M}^{-1} \text{ min}^{-1}}$$

K_D = 226 pM

The dissociation constant (K_D) determined from the kinetic studies is in reasonable agreement with the K_D derived from equalibrium saturation binding (K_D = 363 pM).

E) Discussion of Radio-ligand binding results

A major aim of this thesis was to develop a readily repeatable radioligand method to detect and characterize beta-adrenoceptors derived from pigmented iris-ciliary body diaphragm and ciliary processes. The technique described in the methods and material section has allowed this to be accomplished. There are however aspects which require clarification.

1) General discussion on melanin and membrane preparation.

The first major obstacle to overcome was the presence of large amounts of melanin derived from the pigmented tissue homogenates. Melanin binds radioligand thereby increasing non-specific binding (Wax and Molinoff, 1987). The melanin problem was eventually overcome by removing it from the tissue homogenate by a process of ultracentrifugation using a discontinuous sucrose gradient. This process removed the melanin to the bottom of the tube enabling membrane fragments to be collected from the interface.

Membrane fragments were prepared for ligand binding studies using the described method. Bromberg et al used homogenised membrane fragments and sucrose gradients in their study on the ciliary processes (Bromberg et al., 1980). Despite an inadequate washing technique Bromberg et al reported maximum ligand binding to cellular fragments at the 5:50% interface and presumed the presence of membrane fragments. These workers did not study the tissue they were working with by either light microscopy or electron microscopy. Our transmission electron micrographs confirmed that membrane fragments do indeed collect at this 5:50% interface.

It is important to determine the source of these membrane fragments. In the preliminary iris-ciliary body experiments they came from a combination of ciliary epithelium, iris epithelium, iris and ciliary body stroma, neuronal tissue and muscle fibres. However, for the ciliary process study the site of origin can be more accurately localised. The ciliary processes consist of two layers of epithelium; a stroma of collagen fibres, fibroblasts, nerves and a central vascular channel (Streeten, 1984). Transmission electron microscopic examination of the fragments revealed that they were essentially epithelial in origin and as a result the majority came from ciliary epithelium. Clearly however, exact localization of beta receptors to the non-pigmented epithelium was not proven and has yet to be achieved. Other workers have attempted to directly localize beta receptors to the non-pigmented epithelial cell walls. In 1979 Dafna et al used a fluorescent analogue of propranolol to identify binding sites to anterior segment receptors. Most of the binding in this study occurred in the basal layer of the non-pigmented epithelium. Some binding to both the pigmented epithelium and episcleral vessels however also occured (Dafna et al., 1979). There was no mention regarding binding to receptor sites in the lateral intercellular spaces. A major problem with Dafna's study is the fact that the authors did not consider the problem of non-specific binding by propranolol. Despite this, their paper still has some relevance as a broad indicator of potential beta receptor sites in the ciliary processes. Other workers using different techniques have tried to localise beta receptors to the non-pigmented ciliary epithelium. Recently, an autoradiographic technique was developed to try

to localize these receptor sites, but these results have not been verified (Burnstein et al., 1983). Polansky et al have reported a method to separate non-pigmented cells from pigmented cells in bovine eyes (Polansky et al., 1985). This technique involves dissection of ciliary processes, 1 hour incubation in a variety of chemicals and gentle vortexing. Apparently non-pigmented ciliary epithelial cells eventually separate from the ciliary processes. Perceptable membrane damage to cells does however occur. Despite the authors claims that there is greater than 95% purity, pigment contamination was significant enough for the authors to employ both a centrifugation technique and a 10/20% ficoll gradient to get rid of pigment to concentrate membrane fragments. Clearly, this technique is very complex with little advantage over the method described in this thesis to prepare melanin free membrane fragments. Cell cultures of non-pigmented epithelium would allow accurate receptor identification. A novel technique has recently been described by Coca-Prados et al to culture intact non-pigmented epithelial cells from bovine eyes (Coca-prados et al., 1986). This technique is a complicated one requiring special plastic substrates and hormones and the support of a cell culture laboratory. Carefully monitored incubation times are required to weaken the junctional complexes (Desmosomes, gap junctions, puncta adherence) that bind the two epithelial layers together (Streeten, B.W. 1982, Raviola G. and Raviola, E. 1978). Clearly, if this cell culture techniques works and proves to be repeatable radioligand studies on pure non-pigmented ciliary epithelium will allow for accurate anatomical localization of beta adrenoceptors.

Once melanin contamination was eliminated from the sample and T.E.M. of the interface of the sucrose gradient confirmed the presence of membrane fragments the problem then arose as to which radio-ligand method to use to investigate beta-receptors in anterior segment tissues. The technique described by Bromberg et al using specific numbers of droplets from punctured sucrose gradient tubes without a filtration or washing step proved unrepeatable on pigmented tissue in our hands. As a result a more conventional binding method was developed using a homogenization technique, density gradient ultra-centrifugation and the accurate transfer of membrane fragments to sample tubes as described in the methods and materials section of this thesis. Standard filtration and washing techniques were used in this study (Burt 1985, Titinchi 1984, Nahorski et al., 1978). Temperature control of water baths, tissue preparations and incubation times were systematically optimized as the experiments progressed.

2) Discussion of Preliminary Results on Iris Ciliary Body Diaphragm

Mean B_{max} for the preliminary iris ciliary body experiments was 97.45 fmol/mg with a K_D of 130 pM. The stereospecificity competition curve performed on iris-ciliary body diaphragm, (Fig. 18) revealed Ki values for the two stereoisomers of Propranolol that differed by a factor of 75, which is typical of functional beta-receptors. As indicated in the characterization experiment a predominance of beta-2 receptors ($\pm$ 80%) were detected on computer analysis of the data. This result suggests that beta-1 receptors must have arisen from the iris

and non-epithelial parts of the ciliary body, as later experiments revealed all beta receptors in the ciliary processes to be of the beta-2 sub-type.

A number of different workers have attempted to study beta receptors in iris ciliary body diaphragm. Page and Neufeld reported a Bmax of 330 fmol/mg protein and a K_D of 2.4 nM from albino rabbit iris ciliary body tissue homogenates using ^{3}H-dihydroalprenolol as radioligand (Page and Neufeld, 1978, Neufeld et al., 1978, Neufeld and Page, 1977). It is important to note however, that these authors did not show graphic proof of saturation of beta receptors and scatchard analysis or stereospecificity graphs of their data. Bhargava et al, reported a Bmax of 960 fmoles/mg protein and a K_D of 700 nM in pigmented monkey iris ciliary body homogenates (Bhargava, 1980). No attempt was made to remove melanin from the tissue homogenates by these authors. As a result further discussion of their work is not helpful.

Schmitt et al., recently reported data on Albino rabbit iris-ciliary body diaphragm (Schmitt et al., 1984). These authors reported a Bmax of 272 fmol/mg protein, and a K_D of 1.2 nM. These authors presented data supporting the view that beta-2 receptors are the major receptor sub-type in this tissue. As indicated by Clark et al., Schmitt's paper suffers from a variety of weaknesses (Clark et al., 1985). The major concern relates to Scatchard analysis of the binding data. No saturation curves and scatchard graphs were presented and no stereospecificity or kinetic data were reported. A variety of other concerns exist with regard to this paper, all of which have been summarized by Clark et al., (Clark et al., 1985). Mittag and Tormay

recently published data on New Zealand rabbit iris ciliary body diaphragm. These authors reported a Bmax of 132 fmol/mg protein with a K_D of 0.6 nM. Stereospecificity and kinetic studies however, were not performed by these authors. They conclude however, (from two competition curves using epinephrine and norepinephrine) that beta-2 receptors are the major receptor sub-types in rabbit iris ciliary body (Mittag and Tormay, 1985).

Wax and Molinoff recently published binding data on human iris ciliary body diaphragm. These authors confirmed the importance of removing melanin from tissue homogenates and make reference to our sucrose gradient technique. Wax and Molinoff, used a 20% percoll gradient for this purpose and reported a Bmax of 134 fmol/mg and a K_D of 106 pM. They also detected beta-2 receptors in iris ciliary body diaphragm and reported that 10% of beta receptors were of the beta-1 subtype. (Wax and Molinoff, 1987). These results are very similar to our preliminary data derived from sheep iris ciliary body diaphragm.

3) Discussion of results on ciliary processes

The major purpose of this section of the study was to determine whether beta-adrenoceptors exist in ciliary process and to determine their subtype.

Our experiments revealed a mean Bmax of 98 fmol/mg protein with a mean K_D of 363 pM. Our data was the first radioligand data published on ciliary processes (Trope and Clark, 1982). Three studies have subsequently reported saturation binding data on ciliary processess. Elena et al., published their binding data in 1984. These authors, used homogenized bovine tissue as their source of membranes (Elena et al.,

1984). The major difference in technique was the use of a 45,000 g spin for 30 minutes using a 40% percoll density gradient to separate melanin from membrane fragments. These authors confirmed the presence of saturable beta-adrenoceptors in bovine ciliary processes. Bmax was 1.28 pmol/mg protein with a K_D of 320 pM. The K_D's between the two studies are very similar, i.e., 320 pM versus 363 pM in our study. A problem with Elena study is the reported Bmax. These authors indicated a Bmax of 1.28 pmol/mg protein which is significantly different from that found in our sheep eyes (Bmax = 98 fmol/mg protein). Their Bmax level is also inconsistant with levels reported in other tissues (Mimmeman et al., 1979). Elena et al., have attributed the Bmax difference mainly to species differences (Elena et al., 1984).

Polansky et al., reported their results in 1985. Using tissue preparative technique described earlier, their reported saturation results are similar to ours namely Bmax of 43 fmol/mg protein and a K_D of 216 pM (Polansky et al., 1985). These authors however did not indicate the concentration of radio-ligand used in their binding experiments, nor did they graphically document their saturation data.

Wax and Molinoff have recently reported saturation binding data derived from human ciliary process membrane preparations (Wax and Molinoff, 1987). Their paper reports a Bmax of 180 fmol/mg protein and a K_D of 92 pM. Differences in technique include the use of a percoll density gradient and the use of [^{125}I]-iodopindolol. ([^{125}I]-IPIN). Furthermore they used 200 nM (-) timolol maleate to determine non-specific binding. Despite the species and technical differences, the data derived from both our studies are similar (See later).

The kinetic results reported in this thesis support the view that beta-adrenoceptors exist in ciliary processes of sheep eyes. The K_D derived from kinetic studies was similar to the K_D derived from direct saturation binding studies on ciliary process membrane fragments. Kinetic studies have been reported by Elena et al and Wax and Molinoff (Elena et al, 1982; Wax and Molinoff, 1987). Elena et al reported that equilibrium was reached in 60-70 minutes. Wax and Molinoff however, reported that in human tissue equilibrium was reached around 20 minutes; similar to our study. The K_{on} and K_{off} results reported by Wax and Molinoff are similar to our results. These authors reported K_{on} data of 2.7×10^9 M^{-1} min^{-1} (0.45×10^9 M^{-1} min^{-1}) and K_{off} values of 1.8×10^{-1} min^{-1} with a $t_{1/2}$ of 4.6 min. (1.026×10^{-1} min^{-1}, $t_{1/2} = 6.75$). Our results are in parenthesis.

Stereospecificity data has been reported on bovine ciliary processes by both Elena et al., and Polansky et al., (Elena et al., 1984, Polansky et al., 1985). Polansky reported an IC_{50} for (-) and (+) propranolol of 2×10^{-9} M and 1×10^{-6} M respectively. Elena reported Ki values of 3.4×10^{-9} M for (-) propranolol and 6.275×10^{-7} M for (+) propranolol. In our series of ciliary processes competition experiments (-) propranolol was the most potent of all beta-blockers ($K_i = 1.2 \times 10^{-8}$ M) (Table 15). Our K_i value for (-) propranolol was an order of magnitude larger than that reported by Elena and Polansky. The Ki value for (+) Propranolol was similar to Elena's reported value but one order of magnitude smaller than Polanskys value.

It is interesting to note that two years after we published our preliminary competition data on sheep ciliary processes, (Trope et al.,

1982) Elena et al., published their competition curves derived from bovine eye experiments confirming our data that beta-2 receptors are the major receptor subtype in the ciliary processes. Elena et al., reported a Ki value for IPS 339 of 8.16×10^{-8} M which is similar to our Ki value (2.8×10^{-8} M). Elena et al., however did not study any other beta-2 blockers (Elena et al, 1984). 5 years after our report Wax and Molinoff confirmed our results in human eyes. These authors reported that the beta-2 blocker ICI 118551 inhibited radioligand binding 56 times more potently than the beta-1 blocker ICI 89406. As indicated on table 15, ICI 118551 displaced [^{125}I]-HYP 137 times more potently than the beta-1 blocker betaxolol hydrochloride. Wax and Molinoff also reported that their competition curves fit best to a 1 site fit. Probably due to the tedious nature of preparing large enough quantities of ciliary process membrane preparations, they did not report IC_{50} or K_i values for any other beta-2 or beta-1 blockers (Wax and Molinoff, 1987).

There is other data to support our findings that beta-2 receptors exist in the ciliary processes. In 1980 Nathanson reported for the first time that beta-2 agonists maximally stimulated the production of cyclic AMP in animal and human ciliary processes (Nathanson, 1980). This has been confirmed by Cepelik et al. and Elena et al., (Cepelik et al., 1981; Elena et al., 1984).

In conclusion, I report that beta adrenoceptors exist in sheep ciliary processes and that they are of the beta-2 subtype. Our binding results have very recently been confirmed by studies on human ciliary processes (Wax and Molinoff, 1987).

The next goal of this study was to apply these in vitro results to the in vivo situation. Specifically, we wished to determine whether beta-2 antagonists inhibit aqueous production and lower I.O.P. However, a number of developmental experiments were first performed (as indicated in the next subsection) before this major aim was undertaken.

II) Development of techniques used to study intra-ocular pressure recovery rate and intra-ocular pressure in rabbits with glaucoma.

A) Pneuma-tonometer Calibration

I) For the in vivo animal studies a Digilab pneuma-tonometer was used to measure rabbit intra-ocular pressure. As this instrument is calibrated for use in humans (Langham and McCarthy, 1968) (Quigley and Langham, 1975) it was decided to perform calibration studies on rabbit eyes before using this instrument to determine intra-ocular pressure recovery rate and intra-ocular pressure measurements in buphthalmic rabbits.

The pneuma-tonometer is an applanation tonometer developed by Langham et al. in 1968 (Langham and McCarthy, 1968). It incorporates a tonometric probe, a pressure transducer and a digital readout of intra-ocular pressure. The probe contains a hollow plunger that rides on a porous sleeve. A gas (Dichlorofluomethane (CCl_2F_2)) flows through the plunger to the specialized tip and through the wall of the porous sleeve to form an "air bearing". The gas pressure pushes the plunger outward. When the tip is applied to the cornea, gas escape is impeded from the specialized tip causing the gas pressure to rise. This pressure rise

causes the plunger and tonometer tip to propel forward with greater force until flow of gas resumes. The force required to resume gas flow is measured and according to instrument design is converted to mmHg (Moses and Grodzki, 1979). Moses and Grodzki have suggested that the instrument does indent the cornea somewhat so it should be considered to be both an applanation and identation tonometer (Moses and Grodzki, 1979).

As previously indicated, the pneuma-tonometer is calibrated for human use. To ensure that the instrument reflected accurate readings on rabbit eyes we calibrated the Digilab pneuma-tonometer by using a computerized miniturized universal pressure meter as our standard.

1) Methods and Materials:

1) To ensure that our standard universal pressure meter was accurate, it was initially tested against a mercury manometer. Pressures from 10 mm of mercury to 100 mm of mercury were tested. A constant error of -2 mm of mercury was detected. This constant error was eliminated by the addition of 2 mm of mercury to all measurements.

2) A 25 gage needle was connected to PE 160 polyethylene tubing to one of the stopcock valves attached to the universal pressure meter.

3) A 20 cc syringe was connected to the other stopcock valve.

4) The system was filled with 0.9% saline, all air bubbles were carefully removed and the stop cock closed.

5) A brown pigmented rabbit was anesthetized with Sodium Pentibarbital (35 mg/Kg) intravenously.

6) The rabbits right eye was positioned under the surgical microscope and a lid speculum used to retract the lids. Topical anesthetic was administered to the eye.

7) The anterior chamber was canulated with the 25 gauge needle throught the limbus along the iris plane.

8) Measurements with the pressure meter were then made with the stopcock closed (Varelles et al., 1977). Simultaneous measurements were made with the pneuma-tonometer applied to rabbit cornea. Four more readings were taken in the same fashion with the mean of the results taken as the final IOP.

9) The intra-ocular pressure in the rabbit eye was then artificially raised between 10 to 45 mm of mercury. This was accomplished by opening the stopcock valve to which the saline filled

syringe was connected. Saline was then injected directly into the eye under direct visualization throught the microscope. When the anterior chamber was noted to be deep, the stopcock was closed and the pressure measured simultaneously with the universal pressure meter and pneuma-tonometer. As the I.O.P. declined, approximately 10 intra-ocular pressure readings were taken with both the universal pressure meter and pneuma-tonometer. This procedure was then repeated on the second eye of the rabbit and again repeated on the two eyes of a second rabbit. A total of 35 readings were taken. The results were plotted graphically.

After the experiment was completed the rabbits were given an overdose of anesthetic.

Table 16 documents materials used in the above experiments.

Results:

Figure 35 shows the computer generated correlation graph of the pneuma-tonometer versus the universal pressure meter under closed stopcock conditions. Pressures were measured within the range of 10 - 45 mm of mercury. The result was a straight line with a correlation coefficient of 0.97. The equation of the regression line was $y = 0.821 + 0.859X$.

1) Universal pressure meter - Bio Tech (DPMII) Instruments, U.S.A.

2) Pneuma-tonometer - Digilab Model 301, Cambridge, MA., U.S.A.

3) Surgical Microscope - Zeiss, West Germany.

4) 3 way stopcock

5) Polythyene tubing (PE 160).

6) Sodium pentibarbital - "Somnotol" - MTC pharmaceuticals, Mississauga, Canada.

7) Proparacane Hcl (0.5%) topical eye drops - Allergan Inc., Quebec, Canada.

8) 2 pigmented Rabbits (3.2 and 3.6 Kg).

Table 16: Materials used to calibrate pneuma-tonometer

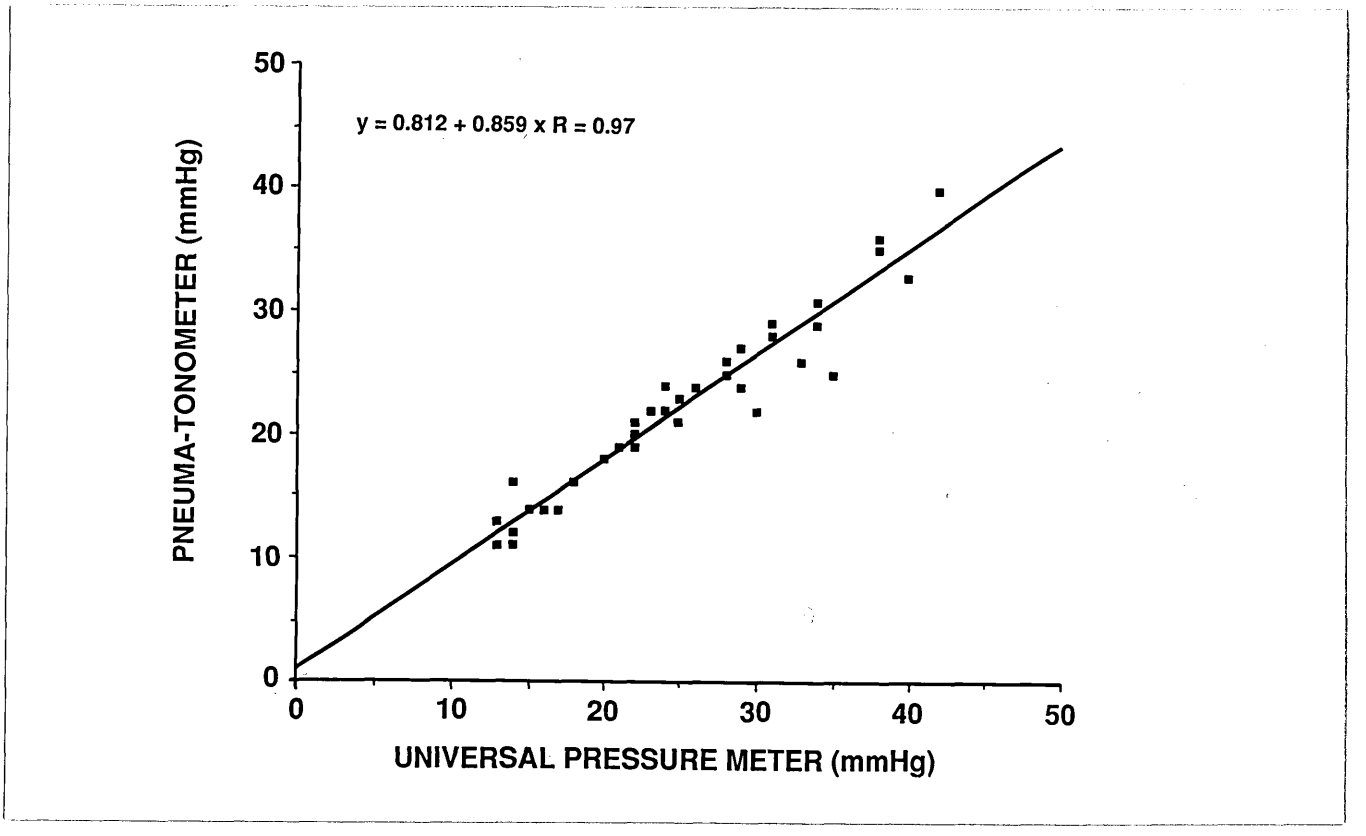


Figure 35: Comparative tonometric measurements of a digilab pneuma-tonometer vs universal pressure meter. Data eerived from 4 experiments.

2) Discussion:

The purpose of this experiment was to calibrate the Digilab pneuma-tonometer for use on rabbit eyes.

For intra-ocular pressures ranging between 10 to 45 mm of mercury a tight linear relationship was found between tonometric measurements (y) and the standard universal pressure meter (x) as reflected by the high correlation coefficient ($R = 0.97$).

In conclusion the pneuma-tonometer appears to be a good instrument for measuring intra-ocular pressure in rabbit eyes. In rabbits with normal IOP's (as used in our intra-ocular pressure recovery experiments) the pneuma-tonometer will produce very accurate I.O.P. measurements. The slightly greater scatter at higher I.O.P.'s indicates the instrument is slightly less accurate for use in rabbits with raised I.O.P. but as most rabbits with glaucoma do not have pressures above 35 mm of mercury, the instrument is both reliable and precise for use in such animals.

B) I.O.P. Recovery Technique Development and Discussion

Once the pneuma-tonometer was calibrated we performed a control experiment to determine intra-ocular pressure recovery in 6 rabbits.

Methods & Materials:

6 Pigmented rabbits underwent the I.O.P. recovery test as outlined in the Materials and Methods section, except that phosphate Buffer (pH 7.4) was randomly placed on 1 eye of each rabbit and nil in the second eye of each rabbit.

Results:

I.O.P. recovery rate for the

1) Untreated eyes was: $0.13346 \pm .01588$ mmHg/minute^2

2) Buffer treated eyes: 0.14121 ± 0.006541 mmHg/minute^2

There was no statistical difference in the slopes of these 2 lines ($p < 0.3$). The buffer values were used as the controls in later experiments.

Figure 36: This figure reveals the slopes of the 2 lines. It indicates that intra-ocular pressure recovered to its original I.O.P. within 140 minutes. Each reading is the mean ($\pm$ S.D.) I.O.P.

Discussion: This first recovery rate experiment provided control values and confirmed the feasibility of using the intra-ocular pressure recovery rate experiments as an indirect method of measuring aqueous production. The results of this control experiment were similar to published data (Varielles and Lotti, 1981). These same 6 rabbits were later used for the I.O.P. recovery rate experiments.

The next section of the thesis contains the results of the in vivo studies.

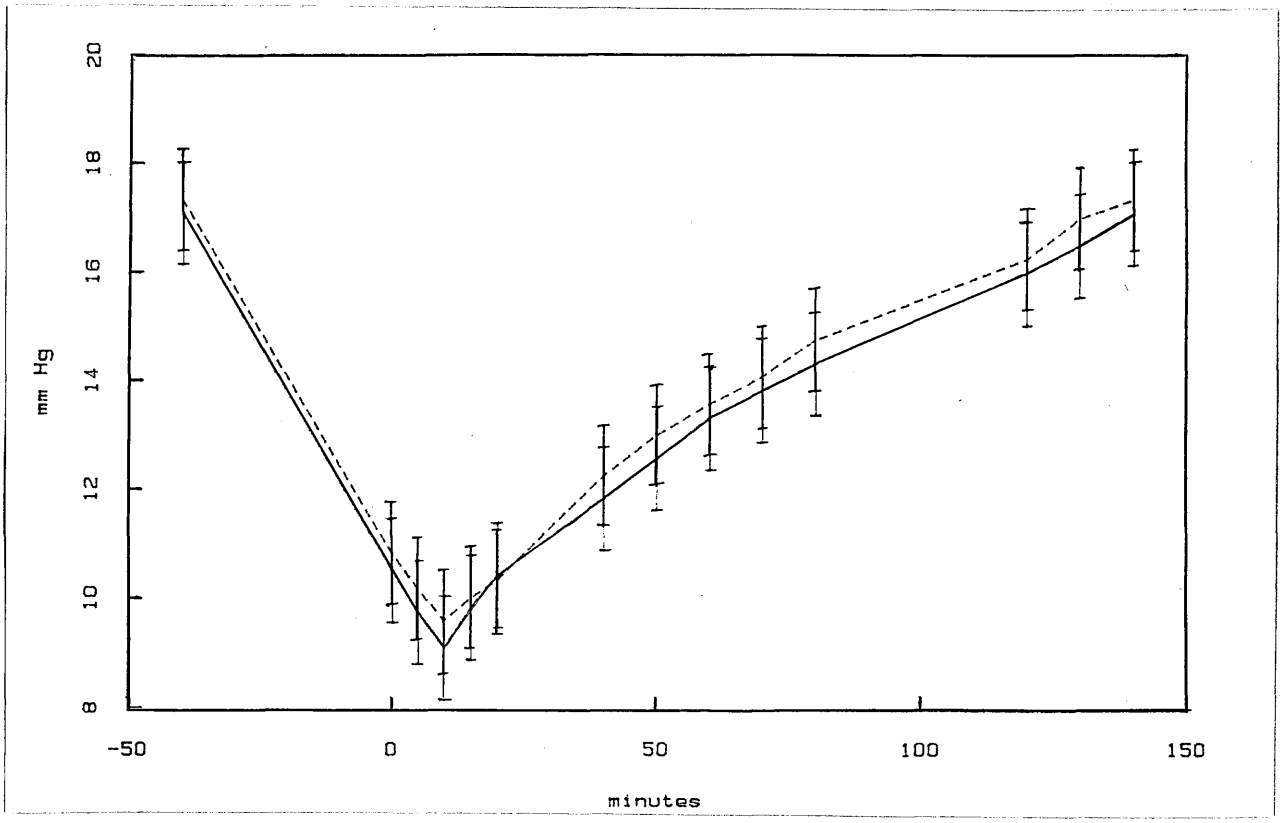


Figure 36: Effect of buffer vs nil treatment on I.O.P. recovery rate (aqueous humour index) in 6 unanaesthetised normal rabbits.

(Dotted Line = Buffer treated eyes).

(Solid Line = Nil treated eyes).

RESULTS

The results section of this thesis contains the results of studies to determine; 1) the effects of beta-2 blockers on aqueous production and 2) the effects of beta-2 blockers on I.O.P. in rabbits with glaucoma.

I) Results of the Intra-ocular Pressure Recovery Rate Experiments (Aqueous Humour Index)

Figure 37: This figure compares the mean (± S.D.) effect of the beta-2 blocker IPS 339 vs buffer on intra-ocular pressure recovery rate.

IPS 339 significantly inhibited intra-ocular pressure recovery rate compared to the buffer treated group of eyes ($P < 0.0042$). IPS 339 produced a 44.54% mean inhibition of intra-ocular recovery rate in the drug vs. buffer treated eyes.

The intra-ocular pressure recovery rate was inhibited by 66.15% in the treated vs the control group of rabbit eyes ($P < 0.0001$) indicating that IPS 339 inhibited I.O.P. recovery in the buffer treated group of second eyes (consensual effect) (Table 17) (See discussion).

IPS 339 had minimal effect (over 50 minutes) on normal rabbit I.O.P. as evidenced by the ± 1 mm Hg drop in mean I.O.P.

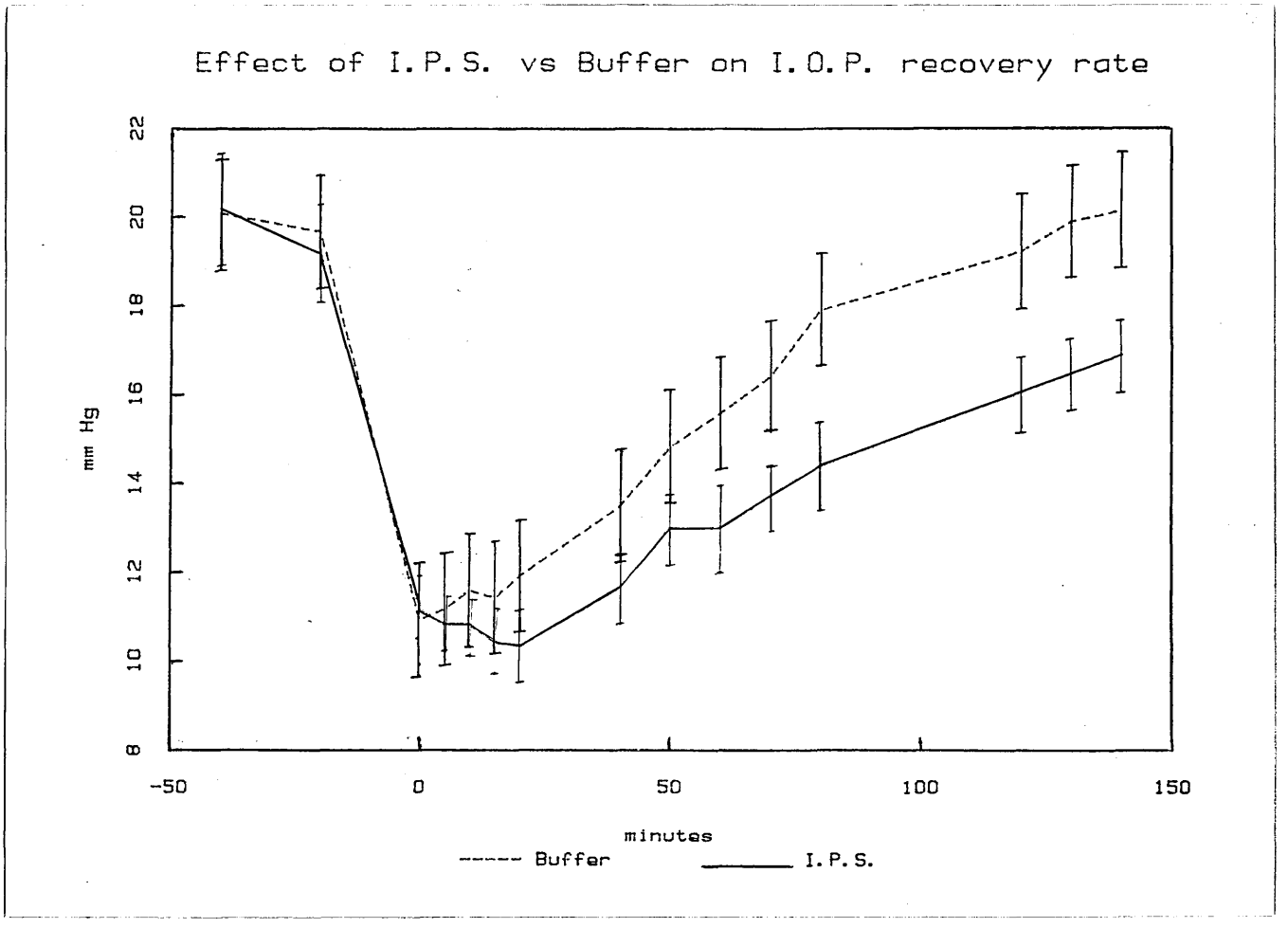


Figure 37: Effect of IPS 339 vs. buffer on intra-ocular pressure recovery rate.

Figure 38:

This figure compares the mean (± S.D.) effect of LI 32-468, another Beta-2 blocker, with buffer on intra-ocular pressure recovery rate.

LI 32-468 significantly inhibited intra-ocular pressure recovery rate compared to the buffer treated group of eyes ($P < 0.0042$). There was a 41.68% mean inhibition of intra-ocular pressure recovery in the drug vs. the buffer treated eyes (Table 17).

The intra-ocular pressure recovery rate was inhibited by 61.10% in drug treated vs the control group of rabbit eyes ($P < 0.0001$) (Table 17) indicating a consensual effect of the drug on the buffer treated second eyes (see discussion).

LI 32-462 also produced minimal effect on normal rabbit I.O.P. There was less than a 1 mm Hg mean drop in I.O.P. over the 50 minute time period before infusion of hypertonic saline.

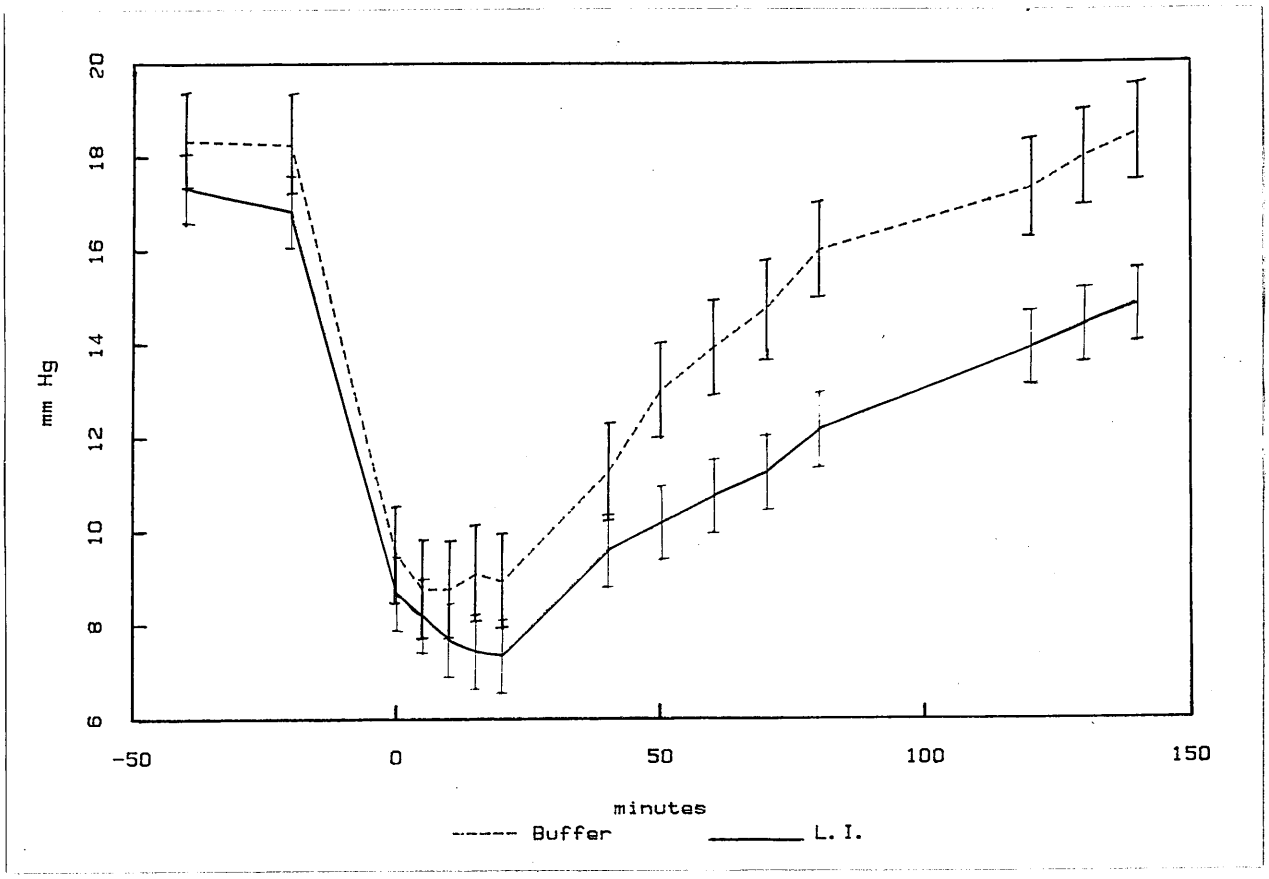


Figure 38: Effect of LI 32-468 vs. buffer on intra-ocular pressure recovery rate.

Figure 39:

This figure compares the effect of ICI 118551, with buffer on intra-ocular pressure recovery rate.

As indicated, ICI 118551 inhibited mean intra-ocular pressure recovery rate compared to the buffer treated group of eyes. There was a mean inhibition rate of 21.61% in the drug vs. buffer treated eyes. This difference however, was not statistically significant ($P < 0.178$).

ICI 118551 however had a marked consensual effect on I.O.P. recovery as evidenced by a 58.99% inhibition of intra-ocular pressure recovery rate in the drug treated vs. the control group of eyes. ($P < 0.0001$) (Table 17). Furthermore, the group of buffer treated second eyes had the slowest intra-ocular pressure recovery rate of all the buffer groups treated ($0.07404\ mmHg/min^{-2} \pm 0.04359$) (Table 17) (See discussion).

As with the previous two beta-2 antagonists drugs, ICI 118551 had minimal effect on normal rabbit mean I.O.P. over the 50 minute period before administration of the hypertonic saline.

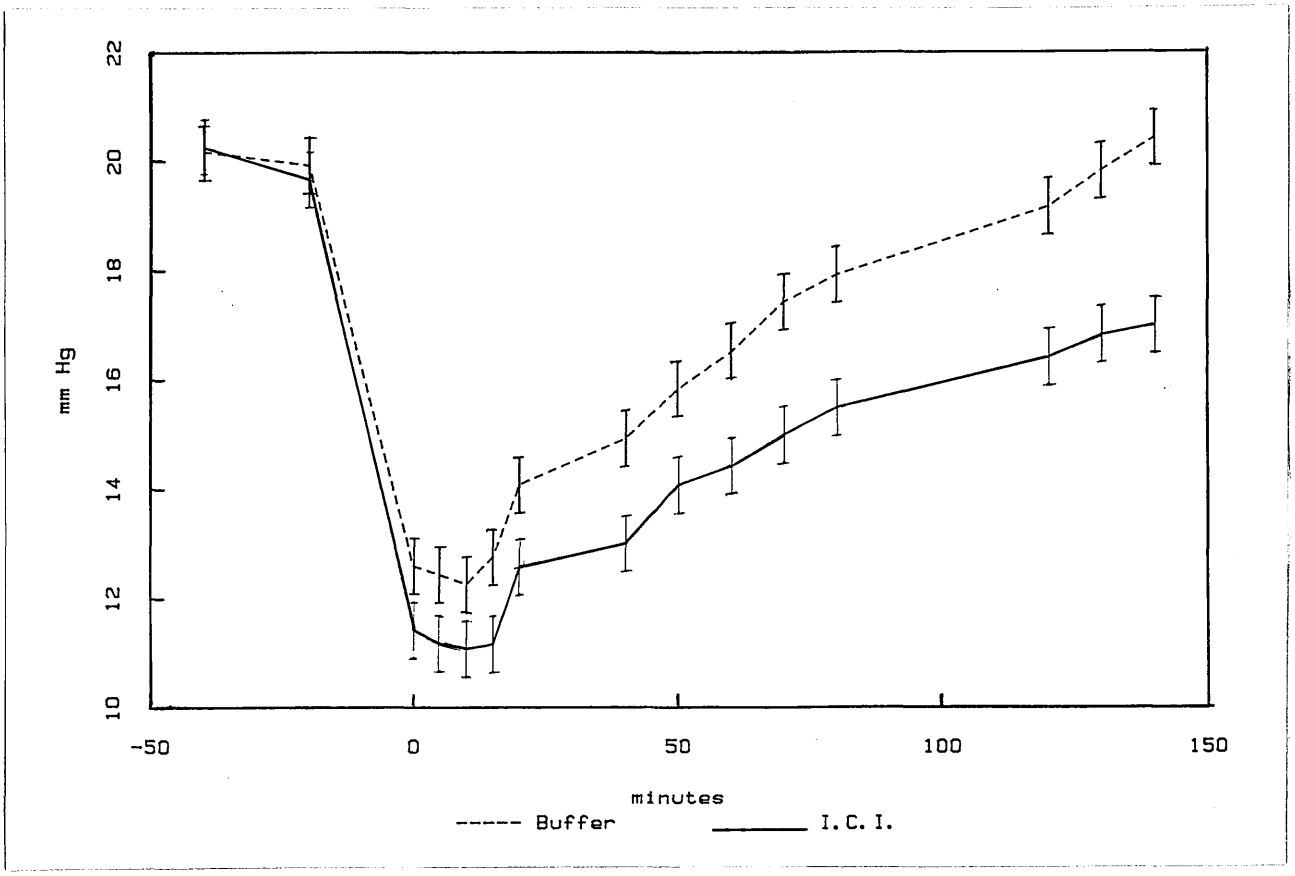


Figure 39: Effect of ICI 118551 vs. buffer on Intra-ocular pressure recovery rate.

Figure 40:

This figure compares the effect of commercially available (-) timolol maleate (Timoptic 1/2%) with buffer on intra-ocular pressure recovery rate in normal rabbit eyes.

(-) timolol maleate significantly inhibited intra-ocular pressure recovery rate compared to the buffer treated group of second eyes ($P < 0.05$). There was a 26.86% mean inhibition of intra-ocular pressure recovery rate in the drug vs. buffer treated eyes.

Like the other drugs (-) timolol maleate also appeared to have a significant consensual effect. There was a 49.97% inhibition of rabbit intra-ocular pressure recovery rate in the drug vs the control group of rabbit eyes. ($P < 0.0001$) Like the other drugs (-) timolol maleate had minimal effect on normal rabbit I.O.P. before administration of the I.V. hypertonic saline.

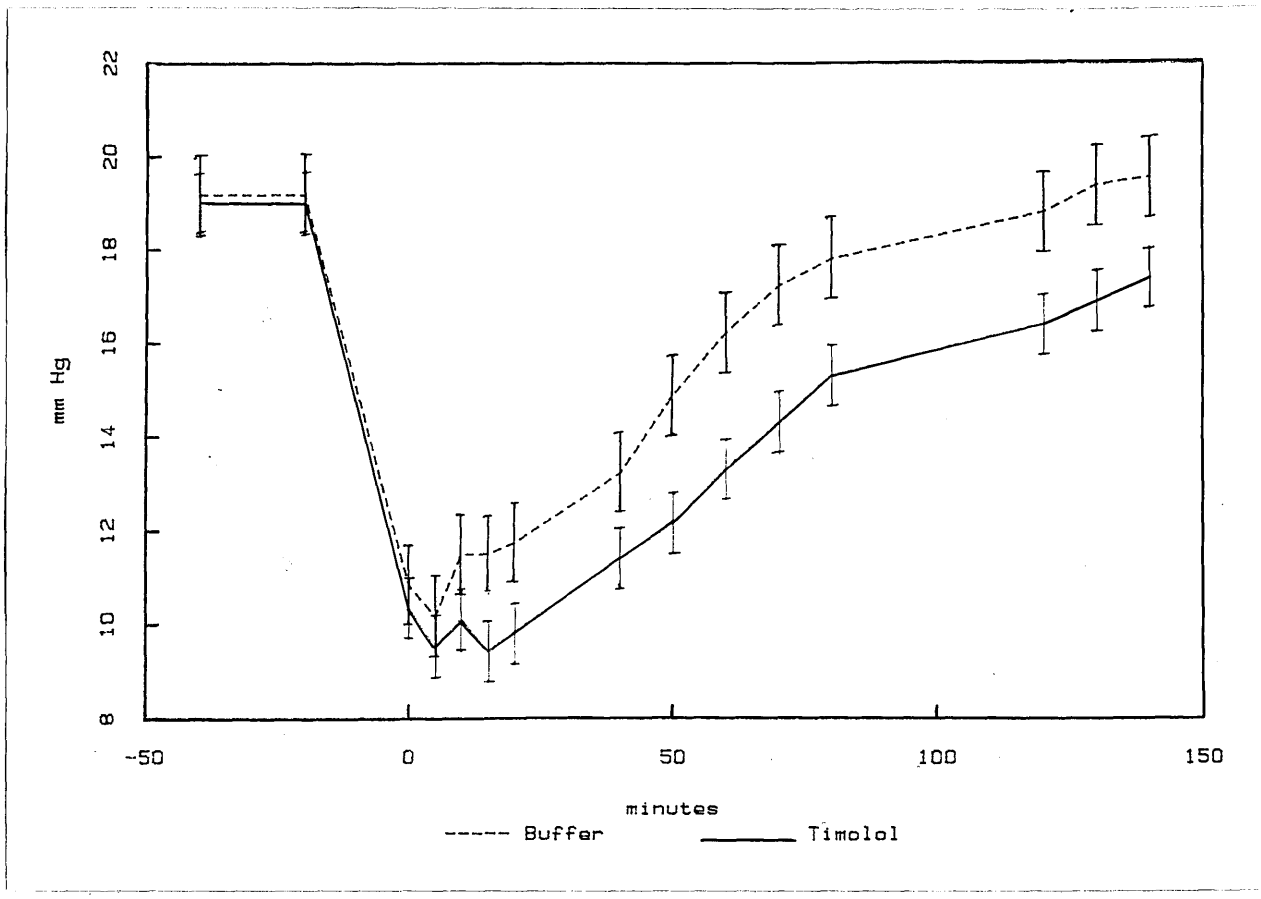


Figure 40: Effect of timolol maleate vs. buffer on rabbit intraocular recovery rate.

Figure 41:

This figure compares the effects of commercially available 0.5% betaxolol hydrochloride (Betoptic) a beta-1 specific blocker, with buffer on rabbit intra-ocular pressure recovery rate.

Betaxolol had a small but not statistically significant effect on the drug vs. buffer treated group of eyes. (7.81% inhibition) ($P < 0.5656$).

Betaxolol hydrochloride did however, have a consensual effect as evidenced by a 41.74% inhibition in intra-ocular pressure recovery rate in the drug vs. control group of eyes (Table 17) ($P < 0.0002$).

Table 17 indicates that betaxolol hydrochloride significantly inhibited aqueous production but had the weakest effect on intra-ocular pressure recovery rate of the five drugs tested (See discussion). As with the other drugs betaxolol hydrochloride had minimal effect on mean I.O.P. before infusion of the hypertonic saline.

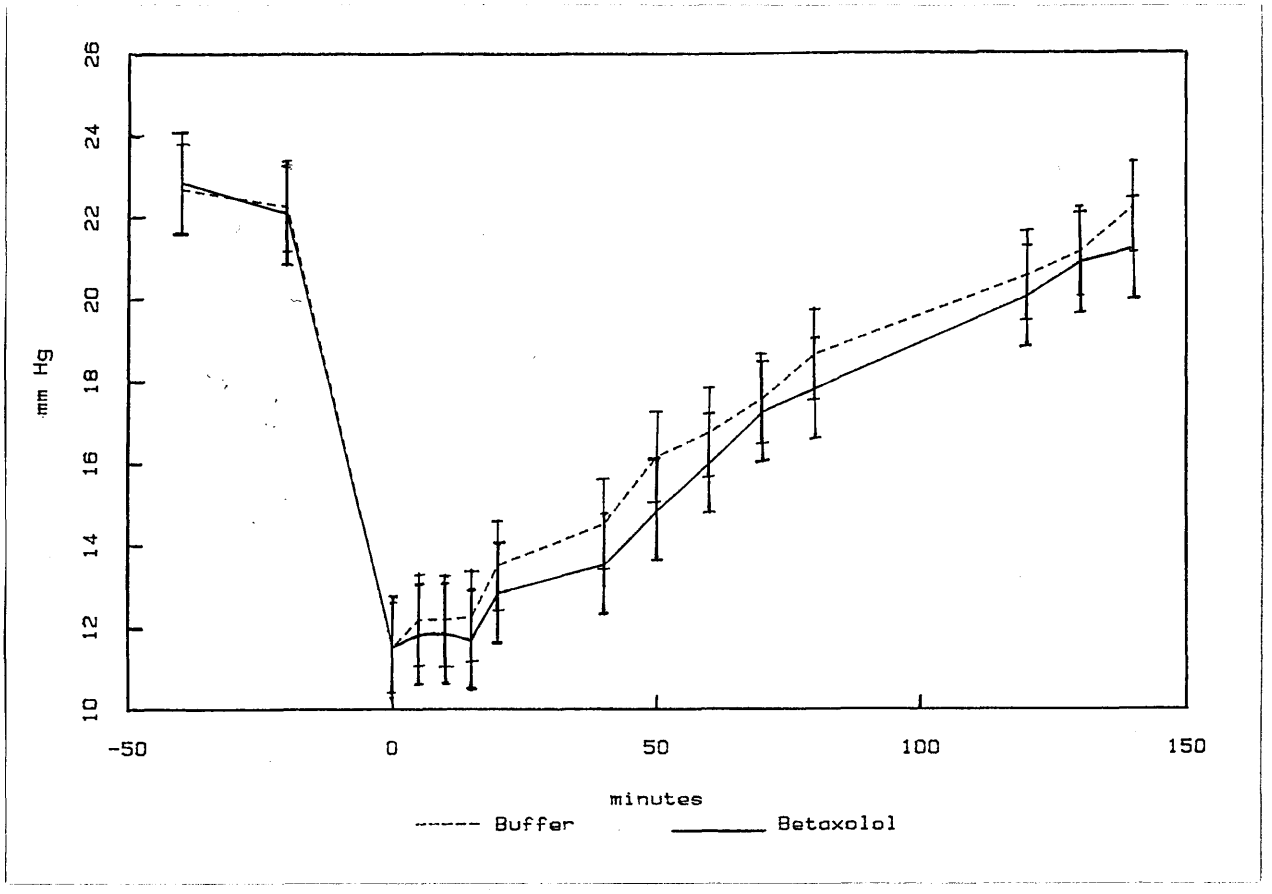


Figure 41: Effect of betaxolol hydrochloride vs. buffer on intraocular pressure recovery rate.

Table 17:

This table details the results (mean $\pm$ standard deviation) and statistical significant of the intra-ocular pressure recovery rate experiments in order of potency. As indicated the Beta-2 blockers IPS 339 LI 32-468 and ICI 118551 were the most potent of the five drugs tested. These three Beta-2 blockers inhibited I.O.P. recovery by 66.15%, 61.10% and 58.99% respectively. (-) timolol maleate inhibited I.O.P. recovery by 49.97% while the B_1 blocker betaxolol HCl (Betoptic) inhibited I.O.P. recovery by 41.74%.

Treatment	Slope of I.O.P. recovery rate ($mm\ Hg/min^2$)	% Difference between drug vs. buffer	p Value	% Difference between drug vs. control	p Value
1) IPS 339	0.0478 ± 0.003457	-44.54%	0.0042	-66.15%	0.0001
Buffer	0.0862 ± 0.005457				
Control	0.14121 ± 0.006541				
2) LI	0.05493 ± .008201	-41.68%	0.0042	-61.10%	0.0001
Buffer	0.09418 ± 0.008201				
Control	0.14121 ± 0.006541				
3) ICI	0.05804 ± 0.00435	-21.61%	0.1782	-58.99	0.0001
Buffer	0.07404 ± 0.004359				
Control	0.14121 ± .006541				
4)(-)Timolol Maleate	0.07065 ± .006601	-26.86%	0.0535	-49.97	0.0001
Buffer	0.0966 ± .006601				
Control	0.14121 ± .006541				
5)Betaxolol HCl	0.08227 ± 0.004638	-7.81%	0.5656	-41.74	0.0002
Buffer	0.0893 ± 0.004638				
Control	0.14121 ± 0.006541				

Table 17: Results (mean ± standard deviation) of I.O.P. recovery rate (aqueous humour index) of drug treated, buffer treated second eyes and control group of rabbit eyes.

$$\% \text{ difference} = \frac{\text{slope of drug - slope of buffer or control}}{\text{slope of buffer or control}} \times \frac{100}{1}$$

II. Effect of Beta-Blockers on Intra-Ocular Pressure in Buphthalmic Rabbits

Figure 42: This figure documents the effects of 50 ul of 1% IPS 339 on glaucoma rabbit intra-ocular pressure.

As indicated mean intra-ocular pressure dropped from 26 mm Hg to 20 mm Hg (-6 mm Hg) (23%) within one hour of topical application of 50 ul 1% IPS 339. The initial change in intra-ocular pressure over the first 2 hours was statistically significant when compared to the Diurnal control group of glaucoma eyes ($P < 0.02$).

This drop in intra-ocular pressure was sustained over the 2 - 12 hour period of the study with a final intra-ocular pressure reading 16.5 mm Hg. The contrast between the treated and control group of eyes was statistically significant over the 2-12 period ($p < 0.0001$).

Table 18: This table reviews the mean I.O.P. ($\pm$ S.D.) readings for the treated and diurnal control eyes.

Figure 43: This figure documents the consensual effect of 1% IPS 339. As indicated IPS 339 was systemically absorbed and dropped mean I.O.P. from 31.5 mm Hg to 24.5 mm Hg (22.2%). Statistical analysis of the data revealed that there was no statistically significant difference between the IPS treated and the untreated fellow eyes over the 0-12 hr period of the study ($p < 0.75$).

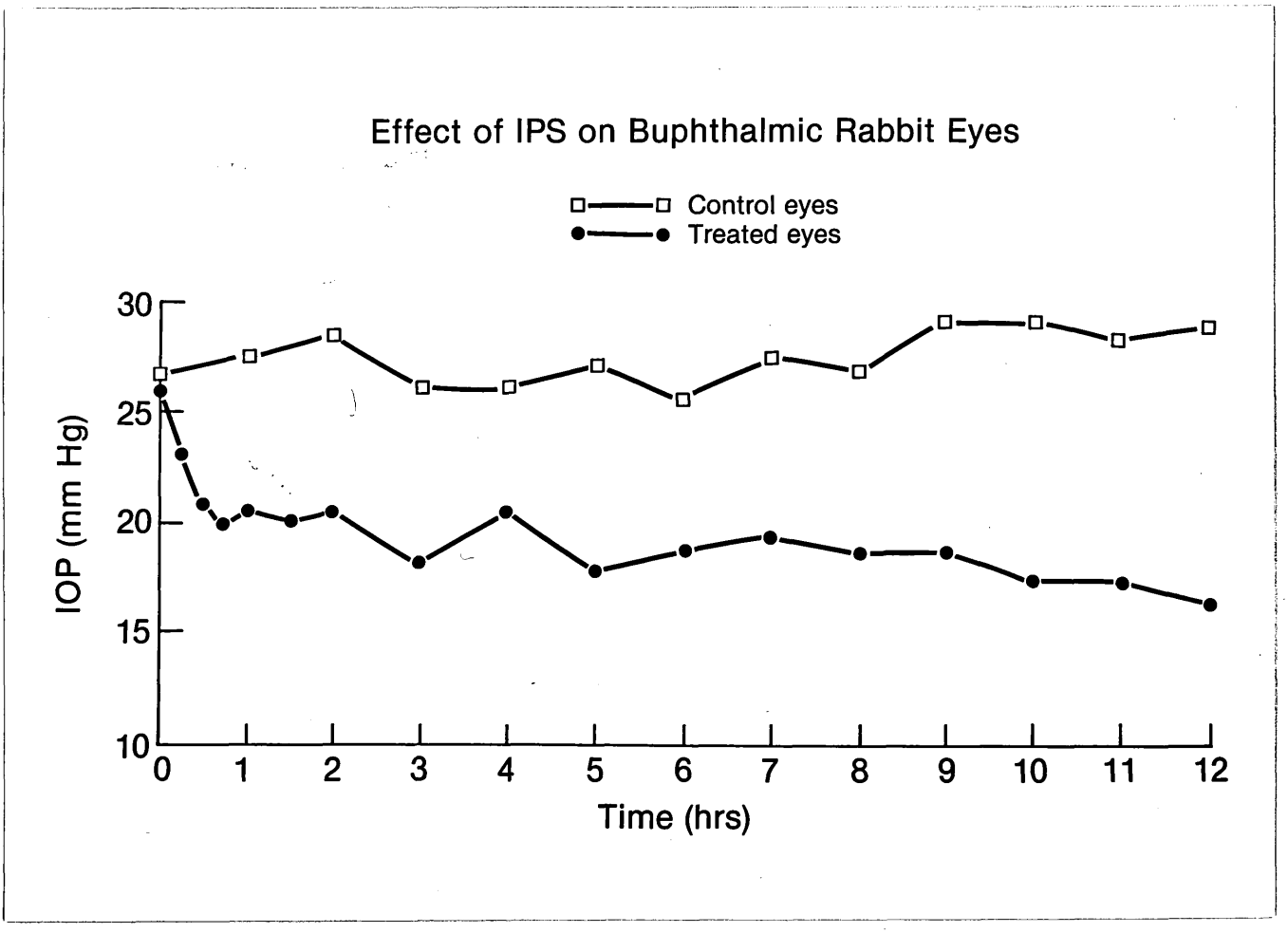


Figure 42: Effect of 50 uls of 1% IPS 339 on Buphthalmic treated eyes. Each point represents the mean I.O.P. of 5 rabbit eyes.

Time	IPS 339 Treated IOP (mm Hg)	S.D. (mm Hg)	Control Group (mm Hg)	S.D. (mm Hg)
0	26	6.3	26.8	4.8
15 mins	23.2	8.2	N/T	
30 mins	20.9	5.4	N/T	
45 mins	20.0	5.6	N/T	
60 mins	20.5	7.1	27.5	4.1
1 1/2 hrs	20.1	6.4	N/T	
2 hrs	20.7	6.2	28.5	3.1
3 hrs	18.1	6.0	26.2	1.5
4 hrs	20.5	6.7	26.2	3.3
5 hrs	17.8	6.7	27.2	4.3
6 hrs	18.8	5.4	25.6	5.6
7 hrs	19.4	4.4	27.6	4.2
8 hrs	18.7	5.7	27.0	5.0
9 hrs	18.8	6.7	29.2	4.5
10 hrs	17.5	5.7	29.1	3.8
11 hrs	17.4	6.0	28.4	3.7
12 hrs	16.5	5.1	28.8	3.9

Table 18: Mean intra-ocular pressure ($\pm$) S.D.) for I.P.S. 339 treated eyes and diurnal control eyes over the 12 hour period of the study.

N/T = Not tested.

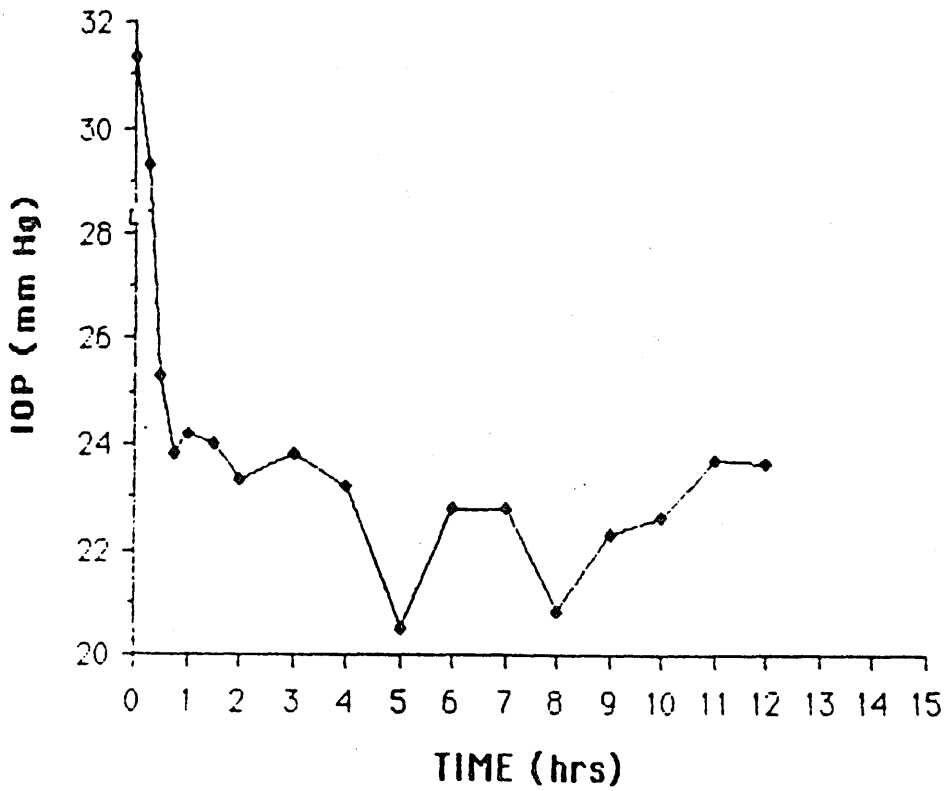


Figure 43: Consensual effect of a 50 ul drop of 1% IPS 339. Each point is the mean I.O.P. of 5 rabbits eyes.

Figure 44:

This figure documents the effect of 50 ul of 1% LI 32-462 on glaucoma rabbit intra-ocular pressure.

As indicated mean intra-ocular pressure dropped 21.8% in the glaucoma eyes from 31.6 mm Hg to 24.7 mm Hg (-6.9 mm Hg) with in the first hour post treatment. The initial change in intra-ocular pressure over the first 2 hours was statistically significant when compared to the diurnal control group of eyes ($p < 0.02$).

This decrease in intra-ocular pressure was sustained over the 2 - 12 hour period of the study. The final intra-ocular pressure reading was 19.8 mm Hg at 12 hours. The contrast between the L132-468 treated and the control group of eyes was statistically significant over this 2 - 12 hour period of the study ($p < 0.0001$).

Table 19: This table Documents the actual (mean $\pm$ S.D.) intra-ocular pressures in the LI 32-468 treated and the diurnal control treated eyes over 12 hours.

Figure 45: This graph illustrates the consensual effect of LI 32-468. As indicated one hour post treatment the mean intra-ocular pressure dropped from 33 mm Hg to 28 mm Hg (15%) in the untreated second eyes. Over the first 2 hours there was no statistically significant difference between the intra-ocular pressure in the treated and untreated eye ($p < 0.06$). Over 2 - 12 hours however, LI did not lower intra-ocular pressure in the untreated eye as significantly as in the treated eye. The contrast between the 2 eyes at this time was significantly different ($P < 0.0001$).

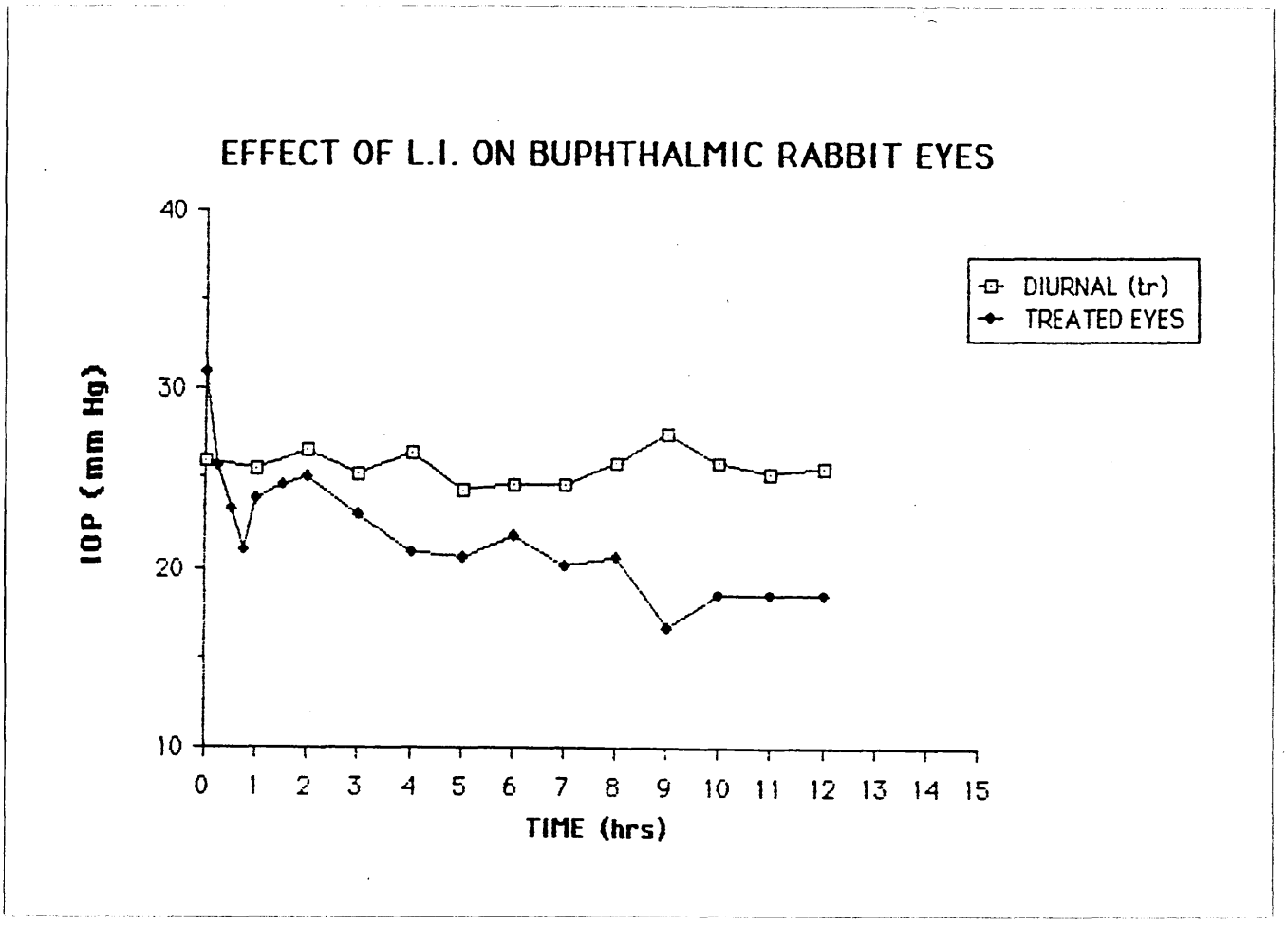


Figure 44: Effect of a 50 ul drop of 1% LI 32-68 on buphthalmic rabbit intra-ocular pressure. Each point is the mean I.O.P. reading taken from 5 rabbit eyes.

Time	LI 32-468			
	Treated eyes (mm Hg)	S.D. (mm Hg)	Control eyes (mm Hg)	S.D. (mm Hg)
0	31.6	6.2	26.0	6.3
15 mins	26.2	6.0	N/T	
30 mins	24.1	6.0	N/T	
45 mins	22.0	6.0	N/T	
60 mins	24.7	5.0	25.5	6.7
1 1/2 hrs	24.7	3.0	N/T	
2 hrs	25.6	1.8	26.6	7.3
3 hrs	24.5	5.2	25.3	6.5
4 hrs	18.6	8.4	26.4	6.5
5 hrs	21.9	4.4	24.3	9.8
6 hrs	22.9	4.6	24.6	6.0
7 hrs	21.2	4.3	24.6	6.8
8 hrs	21.1	4.0	25.9	7.1
9 hrs	18.2	5.3	27.5	5.1
10 hrs	19.8	5.0	25.9	3.6
11 hrs	19.8	5.6	25.2	3.2
12 hrs	19.8	5.4	25.6	6.2

Table 19: Mean (± S.D.) intra-ocular pressures for LI 32-468 treated eyes and diurnal control eyes over 12 hours.

N/T = Not tested.

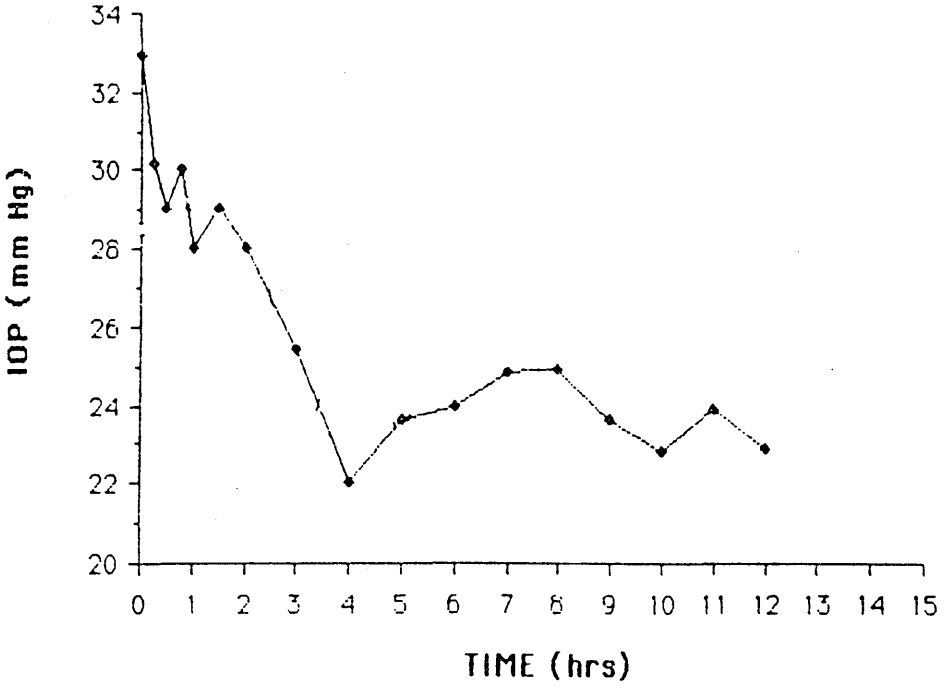


Figure 45: Consensual effect on mean I.O.P. of 50 ul of LI 32-468.

Figure 46:

This figure documents the mean intra-ocular pressure drop in the ICI 118551 treated eyes, untreated second eyes and diurnal control eyes. As indicated mean intra-ocular pressure dropped 16.6% in the drug treated eyes from 24 mm Hg to 20 mm Hg (4 mm Hg) over the first hour of the study. The contrast between the drug treated and control eyes over the first 2 hours of the study was statistically significant ($P < 0.01$). This effect was sustained over the 12 hour period of the study ($P < 0.001$). This figure also indicates that ICI 118551 was systemically absorbed and had a consensual effect. There was no statistical differences between the treated and the untreated eyes over both the 0 - 2 and 2 - 12 hr period of the study ($P < 0.06$) indicating both a short and long term systemic effect.

Table 20: This table documents the actual intra-ocular pressures in the drug and diurnal control group of eyes.

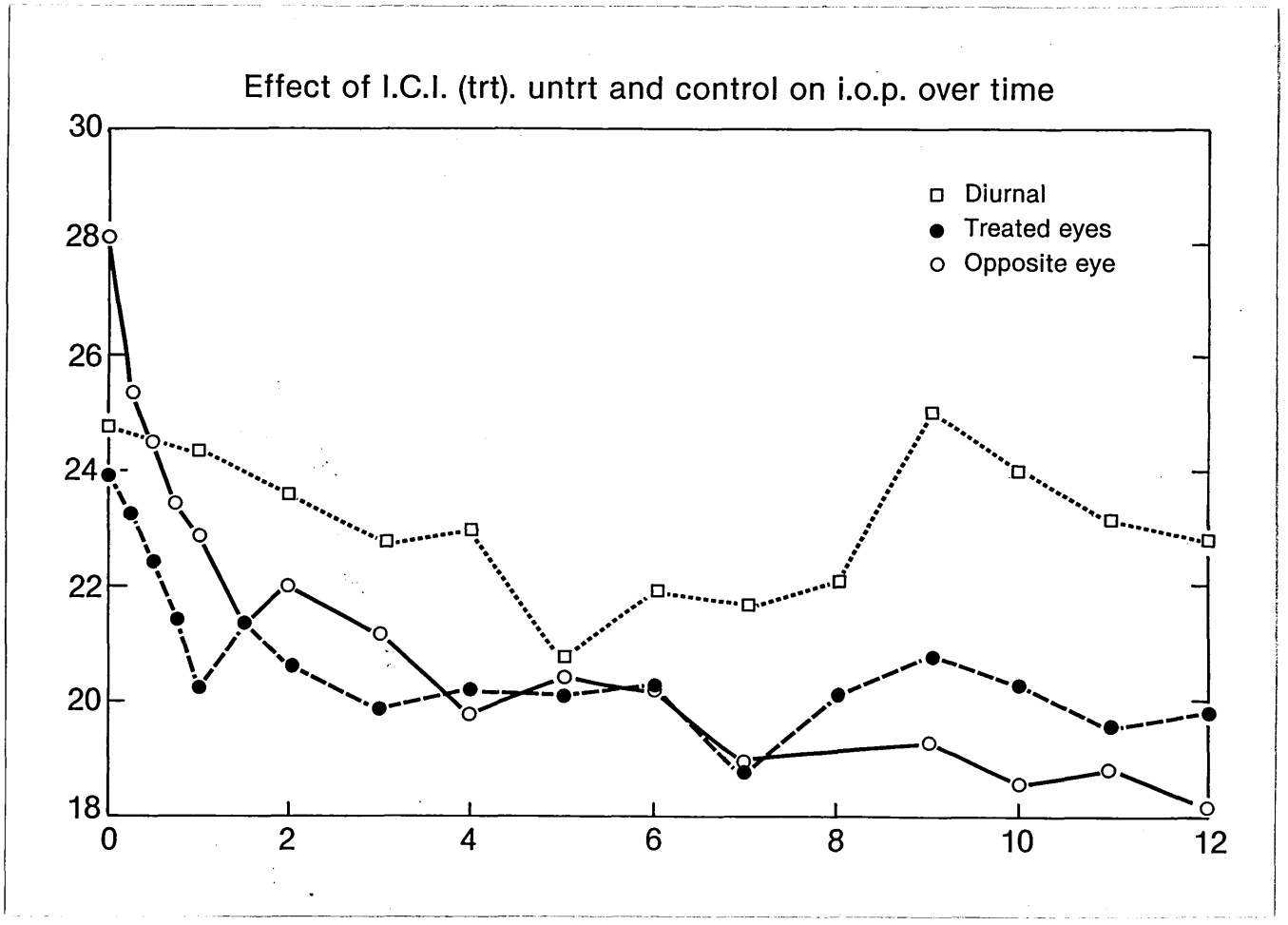


Figure 46: Direct and consensual effect of a 50 ul drop of 1% ICI 118551 on buphthalmic rabbit intra-ocular pressure. Each point represents the mean I.O.P. of 5 rabbit eyes.

Time	ICI 118551 Treated eyes (mm Hg)	S.D. (mm Hg)	Control eyes (mm Hg)	S.D. (mm Hg)
0	23.917 ±	3.8	24.83	5.22
15	23.167 ±	4.622	NT	
30	22.417 ±	3.666	NT	
45	21.333 ±	4.309	NT	
1 hr	20.167 ±	4.803	NT	
1 1/2 hrs	21.333 ±	4.082	24.42	5.60
2 hrs	20.583 ±	4.03	23.67	5.09
3 hrs	19.833 ±	3.869	22.83	4.89
4 hrs	20.167 ±	6.274	23.00	4.43
5 hrs	20.083 ±	4.652	20.67	6.68
6 hrs	20.167 ±	3.642	21.92	4.31
7 hrs	18.667 ±	3.817	21.67	4.27
8 hrs	20.083 ±	3.121	22.08	4.63
9 hrs	20.750 ±	3.029	25.08	4.72
10 hrs	20.250 ±	2.752	24.00	2.19
11 hrs	19.500 ±	2.280	23.17	2.11
12 hrs	19.750 ±	2.162	22.83	2.72

Table 20: Effects of 50 ul of 1% ICI 118551 on glaucoma rabbit intra-ocular pressure.

NT = not tested.

Figure 47:

This figure documents the effects of 50 ul of 1/2% (-) timolol maleate (Timoptic) on glaucoma rabbit intra-ocular pressure.

As indicated mean intra-ocular pressure dropped 23.3% from 34.3 mm Hg to 25.7 mm Hg over the first hour of the treatment (-8 mm Hg). The change in intra-ocular pressure over the first two hours was statistically significant compared to the control group of eyes. ($p < 0.001$).

This decrease in intra-ocular pressure was sustained over the 2 - 12 hour period of the study with a final intra-ocular pressure reading of 24.1 mm Hg. The contrast between the treated and the control group of eyes was statistically significant over the 2 - 12 hour periods of the study ($p < 0.0001$).

Table 21: This table documents the mean ($\pm$ S.D.) intra-ocular pressure change in the (-) timolol maleate treated and control eyes over the 12 hr period of the study.

Time	Timolol Maleate Treated eyes (mm Hg)	S.D. (mm Hg)	Diurnal Control eyes (mm Hg)	S.D. (mm Hg)
0	34.3	6.9	27.6	2.9
15 mins	29.7	4.4	N/T	
30 mins	27.2	7.6	N/T	
45 mins	26.7	6.1	N/T	
60 mins	25.7	7.1	27.3	5.4
1 1/2 hrs	25.4	7.3	N/T	
2 hrs	24.2	8.0	28.8	6.1
3 hrs	23.8	6.0	27.0	6.2
4 hrs	23.4	8.6	25.4	3.6
5 hrs	23.6	7.1	24.9	7.7
6 hrs	23.5	7.5	25.9	7.8
7 hrs	21.6	5.1	27.8	6.4
8 hrs	19.4	8.4	28.2	6.5
9 hrs	22.6	9.5	28.1	3.9
10 hrs	21.9	8.2	29.7	5.2
11 hrs	24.0	8.4	30.2	5.3
12 hrs	24.1	6.3	29.9	4.3

Table 21: Mean intra-ocular pressures (± S.D.) of the (-) timolol maleate treated and control eyes over 12 hours.
N/T = Not treated.

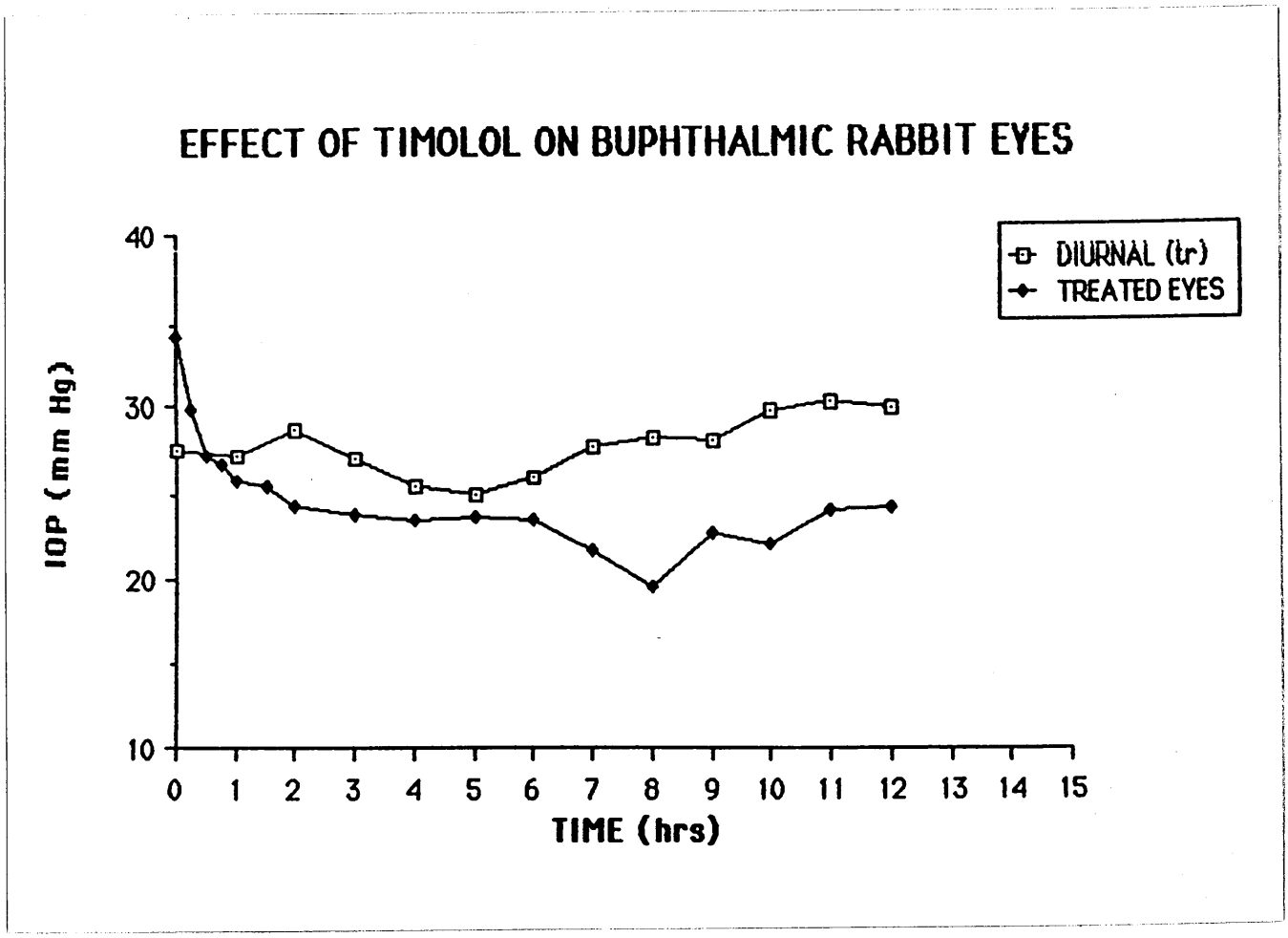


Figure 47: Effect of 1 drop of 0.5% (-) timolol maleate (Timoptic) on intra-ocular pressure in buphthalmic eyes. Each point represents the mean I.O.P. of 5 rabbit eyes.

Figure 48:

This figure documents the consensual effect of (-) timolol maleate on the untreated eyes. As indicated mean intra-ocular pressure dropped 20% from 24.5 to 19.5 mm Hg in this group of eyes over the first hour. Specific contrast between the treated eyes and the untreated eyes was not statistically significant ($p < 0.30$) confirming systemic absorption with a consensual effect on the untreated second eyes. Over the 2 - 12 hour period of the study the drug treated I.O.P.'s were significantly higher than the I.O.P.'s in the untreated eyes ($p < 0.0001$). This was due to much higher initial mean pressures in the drug treated group of eyes (See discussion).

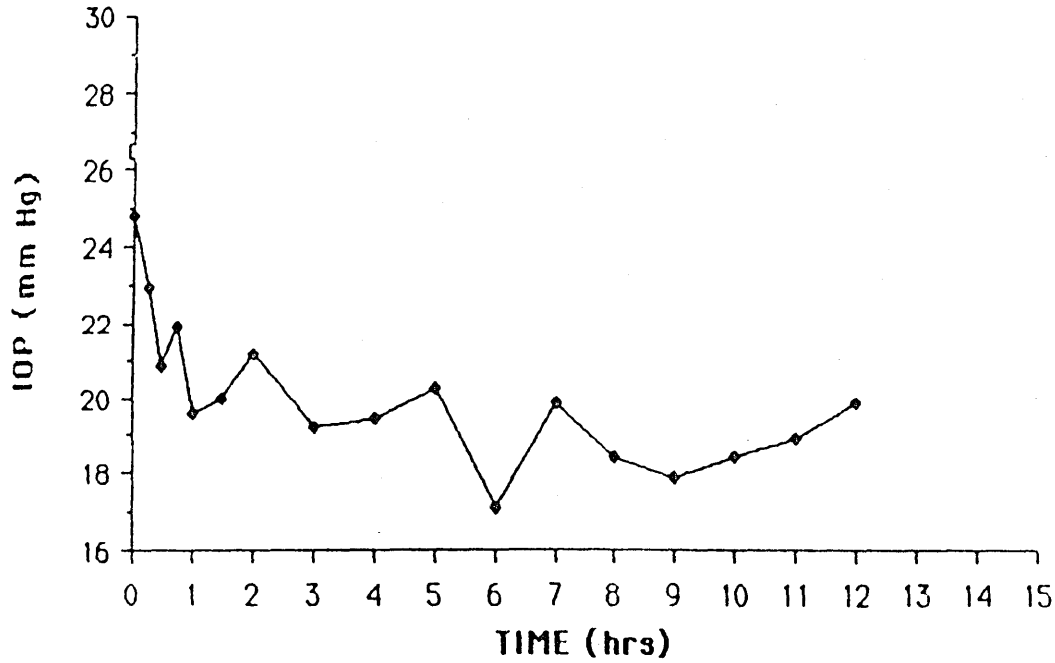


Figure 48: Consensual effect of (-) timolol maleate.

Figure 49:

This figure documents the effect of one 50 ul drop of 0.5% topical betaxolol hydrochloride (Betoptic) on bupthalmic rabbit intra-ocular pressure. As indicated mean intra-ocular pressure dropped 23.3% from 31.7 mm Hg to 24.3 mm Hg after 1 hour (-7.4 mm Hg). The initial change in intra-ocular pressure over the first 2 hours of the study was statistically significant when compared to the control group of eyes ($P < 0.01$).

The drop in intra-ocular pressure was sustained over the 2 - 12 hour period of the study with the final mean intra-ocular pressure reading 21.6 mm Hg. The contrast between the treatment and control group of eyes was statistically significant over this period of time ($P < 0.0001$).

Table 22: This table documents the mean ($\pm$ S.D.) intra-ocular pressure's in the betaxolol hydrochloride treated and control group of eyes over the 12 hour period of the study.

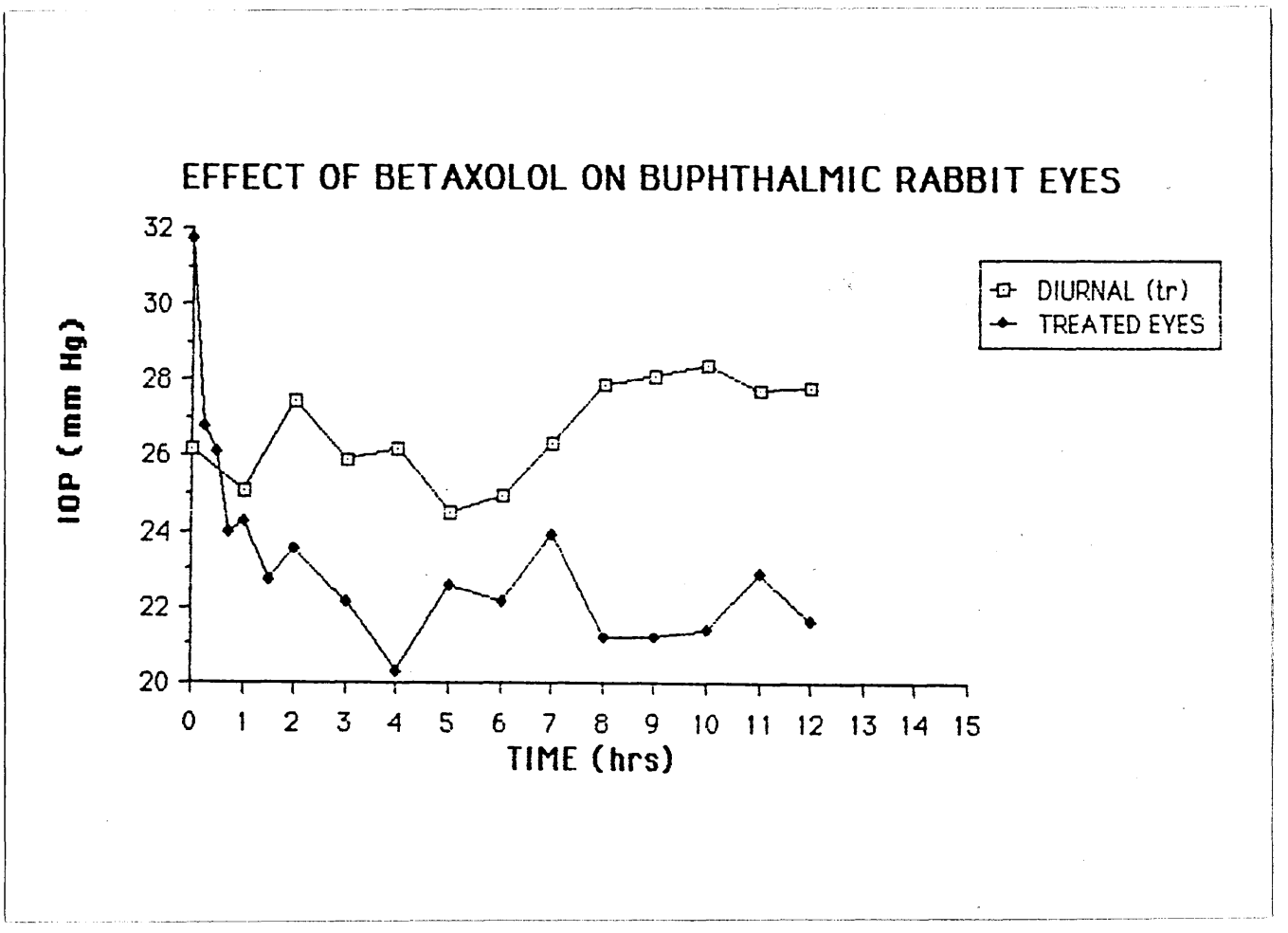


Figure 49: Effect of 1 drop of 0.5% betaxolol hydrochloride on Buphthalmic rabbit intra-ocular pressure.

Time	LI 32-468			
	Treated eyes (mm Hg)	S.D. (mm Hg)	Control eyes (mm Hg)	S.D. (mm Hg)
0 min	31.7	6.2	26.2	5.6
15 min	26.8	4.5	N/T	
30 min	26.1	7.4	N/T	
45 min	24.6	6.9	N/T	
1 hr	24.3	4.4	N/T	
1 1/2 hrs	22.7	5.4	25.1	6.2
2 hrs	23.5	5.6	27.4	7.1
3 hrs	22.1	6.6	25.9	6.5
4 hrs	20.3	5.1	26.2	6.2
5 hrs	22.6	4.8	24.5	9.2
6 hrs	22.1	4.5	24.9	7.0
7 hrs	23.9	6.9	26.3	7.2
8 hrs	21.2	2.5	27.9	7.2
9 hrs	21.2	2.2	28.1	4.7
10 hrs	21.4	3.5	28.4	5.0
11 hrs	22.9	3.9	27.7	4.7
12 hrs	21.6	2.4	27.8	7.2

Table 22: Mean intra-ocular pressure's (± S.D.) in the Betaxolol HCl and control treated group of eyes.

N/T = Not tested.

Figure 50:

This figure indicates that 0.5% betaxolol hydrochloride was systemically absorbed and had a consensual effect on the untreated group of eyes. This drug dropped mean intra-ocular pressure in the untreated eyes from 30 mm Hg to 22 mm Hg within 1 hour of treatment. There was no statistical difference between the treated and untreated eyes over the 0-2 hr period of the study ($P < 0.06$). Over the 2 - 12 hour period there was no statistical difference between the intra-ocular pressure in the drug treated versus the untreated group of opposite eyes ($P < 0.30$) indicating a long term consensual effect (See discussion).

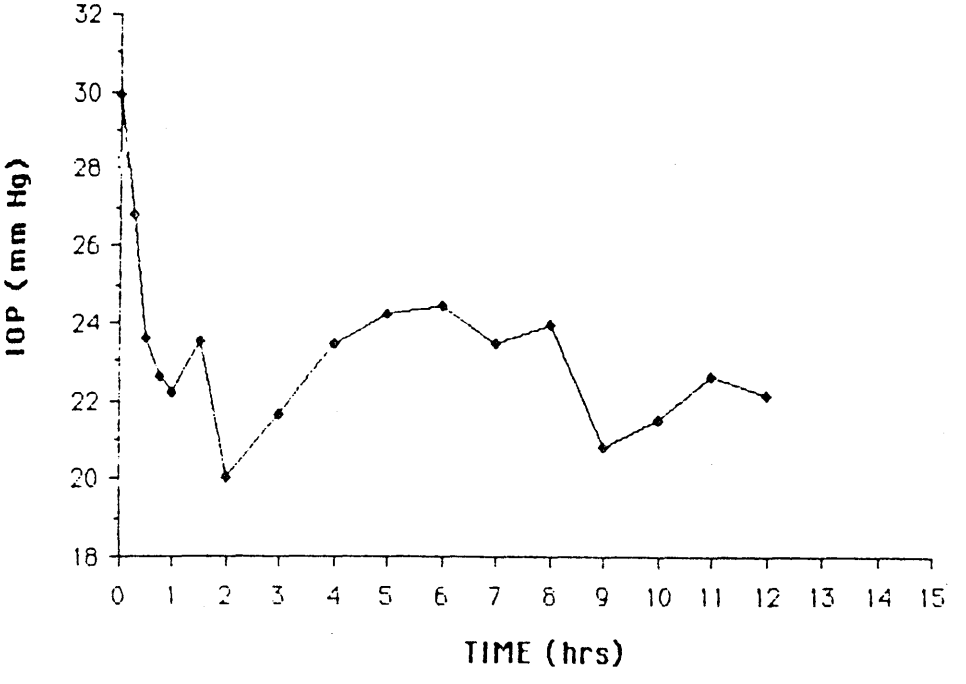


Figure 50: Consensual effect of 1 drop of 0.5% betaxolol hydrochloride.

DISCUSSION

I. Discussion of Intra-ocular Pressure Recovery Rate (Aqueous Humour Index) Results

The radioligand experiments reported in the developments section of this study support the view that beta-2 adrenoceptors exist in ciliary processes. The next goal of this project was to determine whether Beta-2 blockers inhibit aqueous production. Using the I.O.P. recovery rate technique we report that beta-2 blockers do inhibit production of this fluid.

Even though the I.O.P. recovery rate technique is an indirect method of measuring aqueous production, it is generally accepted as a good model (Chiou, 1982; Chang et al., 1985). This test depends on the intra-vascular hyperosmotic effect of saline. This hypertonicity causes ocular dehydration with passage of water from the eye via the semi permeable membranes of the blood-aqueous barrier (Flood, 1986). Varielles and Lotti have indicated that rehydration of the eye is mainly a function of aqueous production which can be monitored by measuring the intra-ocular pressure recovery rate (Varielles and Lotti, 1981).

A major criticism that can be leveled at this particular test model is that it is an indirect measurement of aqueous production. Advantanges of this technique include:

1) It is a simple in vivo technique to preform.
2) It is an inexpensive method of measuring aqueous production compared with the other techniques such as fluorophotometry (Coakes 1978).

3) It is non-injurious to the test animal eye and has no permanent ill effect on the experimental animals. Some of the other techniques used to study aqueous humour production and flow commonly involve invasive proceedures and/or the use of radioactive tracers (Stamper et al., 1986; Jones and Maurice, 1978; Seidehamel and Duncan, 1974).

As indicated on table 17, two of the beta-2 blockers namely IPS 339 and LI 32-468 inhibited aqueous production by 45% and 42% respectively. Timoptic and ICI 118551 inhibited IOP recovery by 27% and 22% respectively. Betaxolol hydrochloride only slightly inhibited aqueous humour recovery. It is interesting to note however, that all the drugs had a consensual effect on the buffer treated second eyes, thereby partially invalidating the comparisons between the drug treated and buffer treated eyes. This consensual drug effect however was predicted before the study commenced as it commonly occurs in animals and the human situation (Neufeld, 1979; Shin, 1986). Why it should occur however is not clear; various theories exist. 1) it could be due to systemic absorption from the treated eye or gastrointestinal tract (via the nasolactrimal duct) with blood born delivery of drug to receptors in the second eye (Woodward et al, 1987). 2) It could be due to systemic absorption producing a systemic hypotensive effect which secondarily decreases I.O.P. (Novack, 1987). 3) It could be due to neural mechanisms, wherein a decrease in I.O.P. in one eye results in reflex lowering of I.O.P. in the contralateral eye (Novack, 1987). 4) Finally it could be due to central effects (Liu and Neufeld, 1985). To obviate against this consensual phenomenon normal IOP recovery rate data

was collected in the same group of rabbits (control group) before the drug experiments were started. When the results of the drug treated eyes are compared with this control group, the overall results remain the same that is; beta-2 blockers inhibit IOP recovery rate more potently than non-specific and beta-1 specific drugs. As indicated in Table 17 IPS-339 and LI 32-468 inhibited I.O.P. recovery by 66% and 61%. ICI 118551 inhibited recovery by 59%. (-) timolol maleate and betaxolol hydrochloride inhibited recovery by 50% and 42% respectively.

As indicated in the developments section betaxolol HCl bound more potently to ciliary process beta-adrenoceptors than Racemic timolol maleate. (-) timolol maleate however is used to treat glaucoma and was the form used in this section of the study. It is therefore not surprising that betaxolol hydrochloride had slightly less effect than (-) timolol maleate on IOP recovery rate (42% and 50% respectively).

It is difficult to compare the results documented in this section of the study with other published data. As previously indicated this study is the first to report the effect of beta-2 blockers on aqueous humour production Varielles and Lotti, however reported the effects of 1% (-) timolol maleate ointment on IOP recovery in normal rabbits. These authors noted that 1% (-) timolol maleate ointment reduced IOP recovery rate by 40% (Varielles and Lotti, 1981). The slope of the 1% (-) timolol maleate IOP recovery curve reported by these authors was 0.077 mm Hg/min^2. The slope of the 0.5% (-) timolol maleate IOP recovery rate line in our study was similar (0.070 mm Hg/min^2). We noted a 26.8% difference between the drug vs. buffer group while Vareilles et al reported a 40% difference between their two groups. The

reason Varielles and our study report different recovery rates is probably due to the different absorption properties and hence consensual effects between drops and ointment.

It is interesting to note that the control data in both studies was similar. Varielles and Lotti reported a 0.110 mm Hg/min^2 slope for the IOP recovery rate, while we noted a 0.14 mm Hg/min^2 IOP recovery rate in our experimental control group.

As indicated in the methods and materials section of this study, the IOP recovery rate experiments were started approximately 50 min. after topical application of drug. It is therefore very interesting to note the effect of the various drugs on <u>normal</u> rabbit IOP. As indicated in the appropriate graphs, topical IPS-339, LI 32-468, (-) timolol maleate, ICI 118551 and betaxolol HCl all produced a drop in IOP of less than 1 mm Hg, 50 minutes after installation of 100 ul of appropriate drug. This data strongly supports the view that normal rabbit eyes are a poor model for studying the effects of drugs on rabbit IOP (Liu et al., 1984, Varielles, 1977, Bonomi et al., 1979).

In conclusion the results of this section of the study confirm that topical beta blockers inhibit aqueous humour production. Furthermore, the results indicate for the first time that beta-2 blockers inhibit aqueous production more potently than beta-1 and non-specific beta blockers. These results support the view that beta-2 adrenoceptors control aqueous production. The results also suggest that beta-2 blocker may lower I.O.P. in glaucomatous eyes via inhibition of aqueous production (see next section).

II) Discussion of Results on buphthalmic Rabbits.

The purpose of this study was to determine whether beta-2 blockers lower I.O.P. in glaucomatous rabbit eyes. As buphthalmic rabbits respond to anti-glaucoma drugs in a similar manner to patients with primary open-angle glaucoma (Variellas 1985, Auriccho and Wistrand, 1959) we felt such a study may indicate the potential clinical usefullness of this class of drug.

The results of the study were encouraging. As indicated in the results section, the beta-2 blockers IPS 339, LI 32-468, and ICI 138551 all significantly lowered buphthalmic rabbit IOP within one hour of treatment with a 50 ul drop of the appropriate drug. The mean drop in intra-ocular pressure was 23%, 21.8% and 16.6% respectively. Furthermore, all three drugs lowered intra-ocular pressure for the duration of the study namely 12 hours. These results are particularly encouraging when considered in relation to present day medication for primary open-angle glaucoma. Topical (-) timolol maleate (Timoptol/Timoptic) and betaxolol HCL (Betoptic) are the major drugs used to treat primary open-angle glaucoma in both Europe and North America (Novack, 1987, Leopold and Duzman, 1986; McGlaughion and Chiou, 1985). The data derived from this section of the study indicates for the first time that beta-2 blockers lower buphthalmic rabbit I.O.P. in a similar fashion to drugs presently used to treat P.O.A.G. Beta-2 blockers may therefore lower intra-ocular pressure in humans with POAG with less side effects than non-specific and beta-1 blockers (See later).

The only drug that has previously been studied in the buphthalmic rabbit eye is (-) timolol maleate. Varielles et al., reported a 24.2% drop in mean buphthalmic rabbit IOP 1 hour after a 100 ul eye drop of 1% (-) timolol maleate (Varielles et al., 1980). These authors also reported an effect on I.O.P. 5 hours later. As indicated above our results are very similar (a 23.3% drop at 1 hour) even though we used a smaller drop size (50 ul) and lower dosage (0.5%).

It is difficult to compare drug potencies on buphthalmic rabbit IOP's. As indicated in the results section, even though the same rabbits were used to test each drug, mean starting IOP levels differed from day to day. For example, the mean starting IOP level was 34.3 mm/Hg at 7 a.m. on the morning when (-) timolol maleate was tested, while it was 24 mm/Hg at the same time of day when ICI 118551 was tested. It is well known that the higher the initial IOP, the greater the drop in IOP after treatment (Lotti et al., 1984). This could explain why ICI 118551 had less of a short-term effect on IOP than the other beta-2 drugs. Despite this our results support the view that all 3 beta-2 blockers should be considered for trial in patients with POAG if ocular toxicological studies prove negative.

As indicated above one problem with using buphthalmic rabbits is the fact that the IOP varies from day to day. This fact makes interpretation of the control and experimental data difficult. For example, the diurnal curves performed before each drug was tested revealed initial IOP readings ranging between 24-28 mm/Hg. However, initial IOP readings on the day of the tests ranged between 24-34 mm/Hg. Despite these differences statistical analysis was possible between the

drug and control eyes.

As indicated in the results section, all the topical drugs tested produced profound consensual pressure reducing effects. This was particularly evident in the (-) timolol maleate study. In this study final mean I.O.P.'s were lower in the untreated eyes than the treated eyes. Clearly this was due to a combination of factors nl systemic absorption of drug and lower initial I.O.P.'s in the untreated group of eyes. This consensual effect has previously been documented in both animals and humans with glaucoma (Variellas et al., 1977; Woodard et al., 1987; Zimmerman and Kaufman, 1977; Shin 1986). Why the consensual effect occurs is not clear (Lotti, 1984). The various theories have been discussed in section I of this discussion.

The consensual effect together with the assymetry of pressures in rabbits with buphthalmos makes statistical comparison between the two eyes impossible. As a result Variellas et al., in their drug studies on buphthalmic rabbits used initial IOP as the control level and IOP at various time intervals thereafter as the comparison levels (Variellas et al., 1980). Clearly, a problem exists with this method, that is it presupposes that change in I.O.P. is only due to drug effect. This method neglects diurnal changes in I.O.P. and is therefore potentially misleading. Even though diurnal curves varied in our study, we used diurnal curve controls rather than initial I.O.P.'s or second eyes for reasons described above.

Some of the problems we experienced using the buphthalmic rabbit model are noted below:

1) Buphthalmic rabbits are not readily available and have to be specially bred.

2) The gene for buphthalmos is semi lethal and therefore the rabbits do not survive well unless carefully looked after.

3) Examination of the fundi of these animals is not possible due to the cloudy cornea. Therefore it is not possible to conduct ophthalmoscopic studies to determine whether a drug under investigation prevents optic nerve damage.

4) The raised intra-ocular pressure varies widely between individual rabbits and from day-to-day in the same rabbit eye.

5) The glaucoma although usually bilateral is often assymetric.

Despite these problems, for reasons previously stated, we believe that the buphthalmic rabbit model remains a useful model for studying anti-glaucoma drugs.

In conclusion, this section of the study supports both the in vitro binding data and in vivo I.O.P. recovery rate experiments that beta-2 adrenoceptors control aqueous production and I.O.P. Furthermore, the data derived from this section of the study confirms that beta-2 blockers lower I.O.P. in buphthalmic rabbits which strongly suggests that this class of drug may lower I.O.P. in <u>patients</u> with P.O.A.G.

III. Review of Evidence for and against beta-adrenoceptor involvement in Aqueous Production

The data presented in this thesis supports the evidence that beta-2 adrenoceptors play an important role in aqueous production and intra-ocular pressure control.

1) Evidence to support beta adrenoceptor involvement in aqueous production and IOP control.

As indicated earlier in this thesis, the ciliary processes are well innervated with sympathetic fibres (Ehinger 1964; Ehinger 1966; Matsuka 1981). Furthermore, it is now known that aqueous humour contains all three catecholamines (Cooper and Constable, 1984; Trope and Rumley, 1985; Trope et al., 1987). The exact origin of human aqueous catecholamines however remains uncertain. These amines may be derived from two different sources; 1) They may be derived from overflow from sympathetic nervous stimulation to receptors in the iris ciliary body diaphragm or 2) they may arise from an overflow from plasma. There is clinical evidence implicating circulating epinephrine in the production of aqueous humour (Topper and Brubaker, 1985). No matter the exact source of origin of aqueous catecholamines it seems likely that adrenaline exerts its effects on the ciliary processes via beta-adrenoceptors.

Ample evidence now exists that ciliary epithelium responds to beta (in particular beta-2) stimulants by producing cyclic AMP (Neufeld et al., 1972; Sutherland and Robinson, 1966; Cepelik and Cernohorsky, 1981; Nathanson, 1980; Nathanson, 1981; Elena et al., 1984). Evidence also exists that cyclic AMP is not produced by the ciliary processes in the

presence of beta blockers (Neufeld, 1979). This data plus in vitro fluid flow studies on rabbit ciliary epithelium (Green and Mayberry, 1983) also supports the view that beta-2 adrenoceptors control aqueous production via the adenylate cyclase system.

In addition to the above evidence, there is also in vivo evidence to support the view that ciliary process beta stimulation increases aqueous humour production. In 1970, Bill showed that intracameral isoproterenol (beta stimulant) increased aqueous humour production by 30% in monkey eyes (it also increased outflow facility by 55%), and that the non-specific beta blocker, propranolol, blocked this stimulatory effect (Bill, 1970). Bill's work has recently been confirmed in humans. Fluorophotometric observations by Townsend and Brubaker, have shown that adrenaline increases aqueous production (Townsend and Brubaker, 1980; Brubaker, 1986). Recently, Lee et al, indicated that it is the beta effect of adrenaline that increases aqueous humour production. Lee et al reported a 19% increase in aqueous humour production after the topical administration of adrenaline. This increase in aqueous production was maximal when the alpha effect of adrenaline was blocked with an alpha adrenergic blocker (Lee et al., 1983). Higgins and Brubaker reported that (-) timolol maleate blocked the aqueous stimulating effect of adrenaline (Higgins and Brubaker, 1980). Higgins and others have interpreted this data to mean that ciliary process beta-adrenoceptors control aqueous production (Potter, 1981; Higgins and Brubaker, 1980). We believe our binding data and animal experiments support this view and extend this knowledge to involve beta-2 adrenoceptors as the major receptor subtype controlling aqueous

production and I.O.P.

2) Evidence against beta-adrenoceptor control of aqueous production and I.O.P.

Despite the above published evidence some workers continue to believe that beta-blockers and beta stimulants do not exert their aqueous humour influencing effects via beta-adrenoceptors (Schmitt, 1981; Chiou, 1985; Varielles et al., 1977 and Bonomi, 1979). These workers often base their theories on animal work much of which has been performed using unsatisfactory animal models such as the water or glucose loaded rabbit (Lotti et al., 1984). As previously discussed, such models do not allow for accurate interpretation regarding receptor mechanisms involved in aqueous production.

An aspect of this subject that requires clarification is the role of adenylate cyclase on aqueous production. As previously indicated beta adrenergic stimulants exert their effect by stimulating beta receptors which in turn stimulate cyclic AMP production. Cyclic AMP is not produced by the ciliary processes in the presence of beta blockers. These facts all strongly support the theory as previously indicated that beta adrenergic stimulants and endogenous catecholamines act by stimulating beta-adrenoceptors and cyclic AMP to increase aqueous humour production. To test this hypothesis, Sears reported the effects of cholera toxin on the ciliary epithelium (Sears, 1981). Cholera toxin stimulates adenylate cyclase activity in many tissues. Sears reported that cholera toxin stimulated cyclic AMP production in rabbit eyes, but this production was associated with a <u>decrease</u> in both intraocular pressure and aqueous humour production. This surprising finding appears

inexplicable until one carefully reviews the original article in which this report appeared (Sears, 1981). Sears clearly indicated that the ciliary epithelial cells were pathologically swollen on light microscopy as a result of the cholera toxin administration. It is therefore not at all surprising that aqueous production (secretion) decreased with use of this toxin. Clearly, cells were capable of producing cyclic AMP but were so damaged as to be unable to secrete aqueous humour into the lateral intracellular spaces. Sears continues to believe that cyclic AMP decreases aqueous humour production (Caprioli and Sears, 1983; Sears and Condo, 1986). Sears has also attributed the claimed IOP lowering effect of Forskolin, a potent adenylate cyclase activator, to a decrease in aqueous humour production (Caprioli and Sears, 1983). The effect of Forskolin on aqueous production and I.O.P. however remains controversial (Potter et al., 1985). Brubaker and others have been unable to confirm an effect of Forskolin on human aqueous production and I.O.P. (Brubaker, 1986). Despite the controversy regarding the role of Forskolin on aqueous production and intra-ocular pressure control, Sears has proposed a new theory regarding aqueous production. He believes that ciliary epithelium cell polarity is reversed due to the invagination that occurs during embyrological development of the eye. With activation of adenylate cyclase, cyclic AMP activates intracellular protein kinase to stimulate fluid movement from the base of the cells to the apical surfaces of the non-pigmented epithelium. Sears goes on to suggest that fluid then moves via 'intra-apical ciliary channels' with eventual transudation of fluid across the pigment epithelium <u>away</u> from the posterior chamber. This theory has no scientific basis at present and

neglects the fact that the junctions of the cells i.e., between the pigmented and non-pigmented cells, are closed by dense junctional complexes (Cole, 1977). Sears agrees that considerable work is necessary to test this hypothesis (Sears, 1986). The proposed theory that beta-adrenoceptor stimulation activates adenylate cyclase leading to increased aqueous production remains a much more plausable theory.

One aspect of aqueous humour production that still requires further el ucidation is the all important relationship between cyclic AMP and ciliary processes membrane bound NA: K ATPase. Sodium pottasium ATPase is still believed to be the major enzyme controlling aqueous secretion. An in vitro study to determine the effect of cyclic AMP on ciliary process sodium pot asium APTase would significantly improve our knowledge regarding the physiology and pharmacology of secretion of aqueous humour and I.O.P. control (Rushton, 1983).

Another aspect so far not discussed is the possible role of beta adrenergic agents on choroidal and ciliary processes vasculature. There are some workers who believe that beta blockers may affect aqueous humour production and I.O.P. via a vascular or filtration mechanism of action (Smith, 1987; Langham and Craigie, 1979; Potter, 1981). It is known that many blood vessels contain $beta_2$ vasodilatory receptors (Breckenridge, 1983). Malik has reported that isoprenaline increases uveal perfusion in pigs (Malik, 1976). This finding suggests that beta-adrenoceptors may exist in uveal vasculature. If so, beta blockers could theoretically block uveal beta-2 vasodilatory receptors producing vasoconstriction as occurs in the peripheral circulation (Breckenridge, 1983). Confirmatory Evidence to support this theory is however non-

existant (Lotti et al., 1984).

The fact that betaxolol hydrochloride lowers I.O.P. in human eyes has further stimulated speculation regarding controlling receptor mechanisms (Novack, 1987). In 1984 we suggested that if an adequate concentration of a relative beta-1 selective agent is achieved in the anterior chamber, relative beta-1 selective blockers will block ciliary process beta-2 receptors (Trope and Clark, 1984). As indicated in table 15 K_i for betaxolol hydrochloride is 6.39×10^{-6} M. Phillips and others have reported that within 1 hour after topical instillation in humans and animals the concentration of 1 drop of 0.5% (-) timolol maleate is 1.2×10^{-6} M (Phillips, 1985; Ross et al., 1979; Schmitt, 1980). If one assumes that betaxolol hydrochloride is lipid soluble enough to achieve aqueous humour concentrations similar to (-) timolol maleate, it seems reasonable to suggest that ciliary process beta-2 adrenergic blockade will occur with topical instillation of this beta-1 antagonist in human eyes (Novack, 1987).

Other less plausible theories regarding control of aqueous production have been suggested, but definite proof regarding these theories is lacking (Potter, 1984; Phillips, 1987; Liu and Cho, 1981; Gregory, 1981; Yorio, 1985).

In conclusion I believe that the work reported in this thesis supports the traditional theory of beta-adrenoceptor control of aqueous production. Further work however is required to determine:

1) Whether aqueous humour can flow from the posterior chamber to ciliary processes vasculature as proposed by Sears.

2) The role of Na:K ATPase in aqueous production and the relationship between Forskolin, cyclic AMP and Na:K ATPase.

3) Whether beta-2 receptors exist in uveal tissue.

Answers to the above 3 questions will provide valuable insight into the physiology of aqueous production and I.O.P. control.

IV. Systemic Side-Effects of Non - Specific and Beta-1 Blockers

In 1978 (-) timolol maleate was introduced to treat POAG. (-) timolol maleate effectively controls I.O.P. for several years (Steinart et al., 1981; Strempel, 1982; Neilson, 1982; Villies, 1983 and McLaughin and Chiou, 1985). There is a tendency for I.O.P. to drift upward from the original maximum hypotensive effect overtime but seldom enough to warrant discontinuing (-) timolol maleate treatment (Steinart et al., 1981). In up to 25% of cases of P.O.A.G. additional drugs are needed to maintain satisfactory I.O.P. control (Gillis et al., 1983).

Topical (-) timolol maleate can produce significant local and systemic side effects. In fact (-) timolol maleate is the drug most frequently reported to the national registry of drug induced ocular side-effects in the U.S.A. (Van Buskirk and Fraunfelder, 1984). By far the most important and serious side-effects are respiratory and cardiovascular events attributed to this drug. By November 1986, over 450 case reports of serious and even fatal respiratory and cardiovascular events had been reported to the Food and Drug Administration in the U.S.A. (Neilson et al., 1986). By 1986 product labelling in the U.S.A. contained a warning in bold face type that "The same adverse actions found with systemic administration of beta-adrenergic blocking agents may occur with Topical administration" (Neilson et al., 1986). Shell reported that approximately 80% of topically administered eye drop volume drains through the nasolacrimal duct and is systemically absorbed (Shell, 1982). Neilson has reported that beta-adrenergic antagonists administered in eye-drop form may not undergo first-pass metabolism and therefore may behave like an

intravenous drug dose resulting in serious respiratory and cardiovascular side effects (Neilson et al., 1986). Other severe adverse effects reported with topical (-) timolol maleate eyedrops include; psychotic reactions, impotence, loss of libido, depression, personality changes and transfer in breast milk with apnoea in babies (Van Buskirk, 1980 and Munroe, 1985).

The Pharmaceutical industry have responded to these reports by trying to develop topical beta-blockers with decreased systemic side-effects. In 1982, Berrospi and Leibowitz, reported that betaxolol HCl lowered I.O.P in patients with glaucoma (Berrospi and Leibowitz, 1982). This drug was introduced into the North American and U.K. markets for use in P.O.A.G. in 1986. Betaxolol HCl binds more potently than propranolol to beta-1 receptors in cardiac tissue and has been shown to be able to decrease cardiac output significantly on systemic administration (Giudicelli et al., 1980).Initial reports suggested that betaxolol HCl was virtually as efficatious as (-) timolol maleate in lowering I.O.P. and had less cardiovascular and respiratory side-effects than (-) timolol maleate (Berry, 1984; Schoene et al., 1984). Recent data however indicates that betaxolol HCl does not lower I.O.P. as effectively as (-) timolol maleate (Allen et al., 1986) and does indeed produce significant systemic side-effects such as bronchospasm, heart failure and depression (Ball, 1987; Harris et al., 1986 and Orlando, 1986). In fact, 56 cases of serious adverse events have recently been reported to the F.D.A. (Neilson and Kuritsky, 1987).

Even though, betaxolol HCl is essentially a beta-1 blocker, as indicated on Table 14 this drug binds potently to beta-2 adrenoceptors

in the ciliary processes (K_i = 6.39 x 10^{-6} M). In fact it binds more potently than (±) timolol maleate to these receptor sites (K_i = 1.40 x 10^{-5} M). As previously indicated it is therefore not surprising that this drug lowers intra-ocular pressure in humans with P.O.A.G. Furthermore, our data shows that betaxolol HCl significantly inhibits rabbit aqueous humour production and lowers intra-ocular pressure in buphthalmic rabbit eyes. However, this drug did not bind as potently as the beta-2 antagonists to ciliary process beta-receptors (Table 15) nor does it inhibit aqueous production as potently as the three beta-2 blockers tested (Table 17).

In conclusion topical betaxolol hydrochloride is a useful drug in older patients with P.O.A.G., but should be used with caution if these patients suffer from cardiovascular or respiratory disease. This drug has relatively few advantages over topical (-) timolol maleate.

V. The potential role for beta-2 blockers in P.O.A.G.

As indicated above present medical management of P.O.A.G. is far from perfect, as both non-specific and beta-1 specific blockers are associated with significant local and systemic side-effects.

There are as yet no published reports on the effects of topical Beta-2 blockers in patients with glaucoma. A trial of topical beta-2 blockers for use in P.O.A.G. is indicated for the following reasons;

1) beta-2 blockers bind potently in vitro to beta-2 receptors in ciliary processes (this thesis),

2) Topical beta-2 blockers significantly inhibit aqueous production in rabbit eyes (this thesis),

3) Topical beta-2 blockers lower intra-ocular pressure in bupthalmic rabbit eyes (this thesis),

4) Beta-2 blockers bind more potently and inhibit aqueous production more potently than non-specific and beta-1 specific blockers (this thesis). Beta-2 blockers may therefore be more efficacious than non-specific and beta-1 blockers in patients with P.O.A.G.

5) In view of the relative cardio-protective effects of beta-2 blockers, the development of such drugs could lower the incidence of cardiovascular side-effects in elderly patients with P.O.A.G. The relative cardio-protective effect of beta-2 antagonists may also have beneficial implications regarding optic nerve perfusion in patients with P.O.A.G.

Two of the beta-2 drugs reported in this thesis have undergone extensive animal studies to determine their safety. Both ICI-118551 and Sandoz LI 32-648 are considered safe by the drug companies for clinical

trials. In view of the binding potency of Sandoz LI 32-648 (K_i = 2.91 x 10^{-8} M) it would seem that this drug would be ideal for clinical trials in patients with P.O.A.G.

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