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THE BOVINE PERFUSED EYE AS A MODEL FOR
PHARMACOLOGICAL INVESTIGATIONS

A thesis submitted to the
University of Glasgow
in candidature for the degree of
Doctor of Philosophy
in the
Faculty of Science
by
Stuart Robertson, B.Sc. (Hons.) in Physiology.

Neuroscience and Biomedical Systems
Institute of Biomedical and Life Sciences
University of Glasgow
January 1999
This thesis is dedicated to the memory of my gran,
who died on the 7th June 1999.
Abstract

The isolated perfused eye of many species has become a frequently used model in the study of ocular pharmacology. Due to its availability, cheapness and comfortable size for experimental use the bovine eye provides an attractive model for conducting perfusion experiments. Although initially rejected by Kishida et al. (1985), the bovine perfused eye has been proved to be a valid model for studying aqueous humour dynamics and the pharmacology of various antiglaucoma drugs, including the β-adrenoceptor antagonist, timolol, and the carbonic anhydrase inhibitor, MK-927 (Wilson et al., 1993). A general aim of this study was to develop the in vitro bovine perfused eye, to show whether it is a useful model for experimental work in studying drug mechanisms in the eye, whether from a pharmacodynamic or pharmacokinetic point of view.

Pharmacodynamic Study

The procedure for dissection and setting up of the constant flow method for the bovine perfused eye was initially described by Wilson and co-workers (1993). Bovine eyes, obtained from the local abattoir, were cannulated via the long posterior ciliary artery and perfused with Krebs’ solution. In order to monitor drug effects on intraocular pressure the anterior chamber was cannulated and connected to a water manometer. Since some drugs affect vascular resistance, the arterial perfusion pressure was continuously measured. Drug solutions or vehicles were administered by one of three routes; (i) by addition of drug to the perfusate reservoir at an exact
Abstract

concentration, (ii) as a bolus dose injected intra-arterially or (iii) as a bolus dose injected intracameraly.

Constriction of the pupil, in response to pilocarpine ($10^{-4}$ M), shown by a significant decrease in pupil diameter, indicated that following intra-arterial administration, drugs have access to the anterior segment of the eye, including the iris sphincter and therefore very probably the ciliary muscle, since their arterial supply is common.

To investigate the bovine perfused eye as a model for studying intraocular pressure and aqueous humour dynamics, we studied four different drugs known to alter either aqueous humour formation or aqueous humour outflow. The drugs used were pilocarpine, a cholinergic agonist; apraclonidine, an $\alpha_2$-adrenergic agonist; and the prostaglandins, $\text{PGE}_2$ and $\text{PGE}_3$.

Pilocarpine ($3 \times 10^{-7} - 5 \times 10^{-6}$ M) was found to cause a significant increase in intraocular pressure, suggesting that the drug might be increasing intraocular pressure by disrupting the integrity of the blood-aqueous barrier. In order to test this bovine serum albumin (1% w/v) was included in the perfusate and aqueous humour sampled at the end of the experiment was assayed for protein content. However, after including bovine serum albumin in the perfusate intraocular pressure was found to be reduced significantly by three concentrations of pilocarpine, $10^{-5}$ M, $3 \times 10^{-5}$ M and $10^{-5}$ M. Pilocarpine did not significantly increase protein content in the aqueous humour, indicating that the observed increases in intraocular pressure are not due to a breakdown in the blood-aqueous barrier. No satisfactory explanation for this
anomaly could be found.

Intra-arterial injection of high doses of apraclonidine (100 and 300 nmol) produced a biphasic response characterised by a significant large increase in intraocular pressure during the initial 30 min period after drug injection followed by a smaller increase over the next 60 min. Intracameral injection of apraclonidine did not significantly increase intraocular pressure. As vascular perfusion pressure was significantly increased following intra-arterial, but not intracameral injection of the drug, this suggests that the increase in perfusion pressure, presumably due to α-adrenoceptor mediated vasoconstriction, contributes to the concurrent increase in intraocular pressure.

Intra-arterial injection of PGF$_{2\alpha}$ (20 - 2000 nmol) failed to decrease intraocular pressure in eyes perfused with Krebs' solution or Krebs' solution supplemented with bovine serum albumin. PGF$_{2\alpha}$ also did not affect aqueous humour protein concentration, indicating that the blood-aqueous barrier remains intact following drug administration.

PGE$_{1}$, administered as bolus doses injected by the intra-arterial or intracameral route, shows a trend towards an elevated protein concentration in the aqueous humour. However, due to large variation this only produced statistical significance following intra-arterial injection of 60 nmol or intracameral injection of 600 nmol. No direct correlation was found between aqueous protein concentration and intraocular pressure. Expression of the data as a scatter plot of all the individual experiments
Abstract

indicates that more than one population of data exists. The data show that as intraocular pressure values increase above 165 mm H₂O, the associated protein concentrations either remain normal or increase markedly to a level indicating breakdown of the blood-aqueous barrier.

Although previous investigations, in a variety of species, have shown reductions in intraocular pressure in response to administration of pilocarpine, apraclonidine or prostaglandins, there were no intraocular pressure -lowering effects observed in the bovine perfused eye. The lack of a correlation between the bovine perfused eye and other species in the intraocular pressure response to pilocarpine, PGF₂α and PGE₂ is not surprising due to anatomy of the bovine eye. The lack of functional activity in the ciliary muscle (Prince et al., 1960) coupled with the observed IOP responses suggests that this preparation is not suitable for investigating IOP-lowering drugs which act primarily on AH outflow. However, the model is useful for investigating IOP-lowering drugs that act primarily by altering AH formation (Wilson et al., 1993).

Pharmacokinetic Study

The effects on ocular drug absorption of different formulations of the anti-viral drug, aciclovir, were studied utilising single time point determinations. Variation in absorption caused by experimentally induced corneal damage was also assessed. Different formulations of aciclovir were tested in three different vehicles: (i) a standard ocular ointment, white soft paraffin; (ii) a novel vehicle, polyvinylalcohol...
(PVA) film; (iii) an experimental aqueous gel. As well as leaving the cornea
undamaged, with the barrier function of the epithelium intact, the cornea was
damaged both physically, by removing the epithelium, and chemically, by alkali
burning, in an attempt to mimic the damage to the cornea associated with herpes
simplex virus infection.

180 min after topical application of the drug formulations, aqueous humour and
corneal tissue were removed from the eye and added to acid extraction media for
HPLC analysis and calculation of the level of drug absorption.

Physically removing the layers of the epithelium or exposing the cornea to alkali
soaked discs of filter paper provides two relatively reproducible methods of inducing
experimental damage to the cornea of the bovine perfused eye.

The results show that experimentally damaging the cornea, effectively removing the
barrier properties of the corneal epithelium, increases the amount of aciclovir
absorption in the cornea and aqueous humour compared with the undamaged cornea.
The observed increases in absorption of aciclovir following experimental damage are
much greater when the drug formulations have hydrophilic properties, such as the
PVA film and aqueous gel. From the present work, the formulations can be ranked
in order of corneal and aqueous humour absorption of aciclovir:

Ointment < PVA Film < Aqueous Gel
In this model the level of drug penetration in the cornea and aqueous humour can be measured without the complications associated with \textit{in vivo} studies. Due to the lack of \textit{in vivo} factors such as blinking, drug dilution, drainage and conjunctival absorption the levels of absorption found in the present model are likely to be exaggerated. Nevertheless, the bovine perfused eye provides a useful model for pharmacokinetic studies, which is perhaps superior to the isolated corneal preparation normally used to assess corneal drug absorption since conditions are more physiological and no edge-damage has been inflicted on the cornea.
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Corneal drug penetration

Movement of drugs across cellular membranes

Hydration of the cornea

Permeability of the cornea

Herpes simplex virus and corneal damage

Alkali burns and corneal ulcers

Drug absorption in the bovine perfused eye

Aims of the Project

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The bovine perfused eye preparation

Measurements of intraocular pressure

Criteria for accepting/rejecting eyes

Drug administration

Measurements of pupil diameter

The effects of pilocarpine, apraclonidine and PGF2α on IOP

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INTRODUCTION
GENERAL INTRODUCTION

Gross anatomy of the bovine eye

Through the process of evolution, the bovine eye has developed into a fluid-filled sphere, whose relative rigidity provides the optical alignment of the cornea, lens and retina, which is necessary for vision (Figure 1).

The globe of the eye consists of three layers. The outermost, protective layer is made up of the opaque sclera and the transparent cornea, which are joined together at the corneoscleral limbus. The middle layer, the uvea, is mainly vascular, consisting of the choroid, ciliary body and iris. The innermost layer, the retina, contains the essential nervous elements necessary for vision, the pigment epithelium, rods, cones and bipolar cells.

Within the eye three chambers are enclosed: the vitreous cavity, the anterior chamber and the posterior chamber. The vitreous cavity is the largest and is located behind the lens and ciliary body and adjacent to the retina. This chamber is filled with a clear jelly, the vitreous humour. AH, a transparent colourless fluid, which supplies nutrients to the avascular structures of the eye, fills the anterior and posterior chambers. The anterior chamber contains most of the aqueous and is located between the iris and the posterior surface of the cornea. The posterior chamber is smaller and is located between the iris and the lens. The two chambers communicate through the pupil.
Figure 1. Vertical section of the bovine eye, showing the prominent gross structures.

The eye is not drawn to scale. Dimensions: 28 - 30 mm antero-posteriorly; 30 mm vertically. Adapted from ‘Atlas of Bovine Anatomy’ (Pasquini, 1982).
The transparent cornea of the bovine eye covers the anterior surface of the globe and represents the major refractive component of the eye. The cornea is roughly oval-shaped with horizontal and vertical measurements ranging from 27 – 34.5 mm and 20 – 30.5 mm respectively (Doughty et al., 1995; Prince et al., 1960). Studies on bovine corneas have produced a range of central corneal thickness values generally falling in the range of 725 – 1450 μm (Doughty, 1997; Doughty et al., 1995; Lee & Davison, 1984; Merindano et al., 1998; Prince et al., 1960). Regardless of thickness, bovine corneal dimensions evaluated within 3 hr post mortem have a uniform thickness, within ± 3%, from centre to edge (Doughty et al., 1995). A positive correlation has also been shown between the horizontal corneal diameter and central corneal thickness (Doughty et al., 1995).

The bovine cornea consists of four layers (anterior to posterior): the epithelium, stroma, Descemet's membrane and endothelium (Samuelson, 1991).

The outermost corneal layer, the epithelium, is approx. 14 – 18 cell layers thick (Diesem, 1975; Prince et al., 1960) and usually measures about 90 μm in thickness (Prince et al., 1960). In the mammalian eye, intercellular tight junctions (zonula occludens) surround the most superficial cells of the corneal epithelium (Grass & Robinson, 1988b). In contrast to primates and humans, Bowman's layer, which is located beneath the basement membrane, is not present in the bovine cornea (Table 1).
The corneal stroma is a highly hydrophilic tissue containing mostly water, which constitutes the bulk of the corneal tissue in mammalian species (Maurice & Riley, 1970; Maurice, 1984; Samuelson, 1991). The stroma has long been recognised for its capacity to absorb water and is composed of an extracellular matrix of proteoglycans and lamellar arranged collagen fibrils running parallel to the corneal surface (Maurice & Riley, 1970; Maurice, 1984; Pepose & Ubel, 1992). The regular arrangement of collagen fibrils is essential for normal visual acuity (Järvinen et al., 1995).

Descemet's membrane located between the stroma and endothelium, ranges from 10 - 25 μm in thickness (Prince et al., 1960). This layer, consisting of type IV collagen, laminin and fibronectin, is highly resistant to proteolytic enzymes.

A single layer of endothelial cells, which adhere to Descemet's membrane, covers the posterior surface of the cornea. This layer is approx. 6 μm thick in the bovine cornea (Diescom, 1975; Prince et al., 1960). In mammals the endothelium is believed to constitute <1 % of the total corneal tissue (Grass & Robinson, 1988b; Maurice & Riley, 1970). Histological examination has shown that the bovine corneal endothelium consists of a continuous mosaic of uniformly polygonal cells (Doughty et al., 1995).

The lens is a transparent crystalline structure, located immediately posterior to the iris and is supported by a series of fibres called the suspensory ligaments, which are attached to the ciliary body. Doughty et al. (1995) calculated the lens wet mass to be
approx. 2.5g in a population of 20 bovine eyes.

The iris, the most anterior portion of the uvea, located between the cornea and the crystalline lens, is darkly pigmented and appears black or brown in colour. In the bovine eye, the pupil is oval and horizontally orientated (Diescm, 1975; Prince et al., 1960). As in the horse, the pupil of the bovine eye is held relatively immobile by radially arranged muscle fibres at the 3 and 9 o'clock positions. When the pupil is fully dilated it becomes almost circular. The iris is covered by two layers of epithelia, which are continuous forward extensions of the pigmented and non-pigmented epithelial layers of the ciliary body.

The ciliary body extends from the termination point of the retina, the ora ciliaris retinae, to the iris root (Prince et al., 1960). There is no evidence of any great deal of functional activity in the bovine ciliary body and the ciliary muscle is relatively long with very few circular or radial muscle fibres (Prince et al., 1960). This suggests that like other ungulates the ability for accommodation in the bovine eye is limited (Diescm, 1975). This is probably due to a combination of the rudimentary circular and radial ciliary muscle fibres (Prince et al., 1960), great size of lens and relative weakness of the lens capsule (Walls, 1963).

In contrast to primates, there is no scleral spur found in the bovine eye (Table 1). Instead the iris is supported against the contractile forces of the ciliary and sphincter (pupillary constriction) muscles, by the pectinate ligament (Walls, 1963). This tissue is very well developed in ungulates and consists of heavy connective tissue fibres,
which run from the limbal region of the fibrous tunic to the root of the iris.

Finger-like ridges, the ciliary processes, project from the ciliary body and occasionally the posterior surface of the iris. The large processes are each 3–5 mm in length and number approx. 90–110 in bovine and 70 in humans (Prince et al., 1960). Ciliary epithelium, lining the ciliary processes, are the cells responsible for aqueous humour (AH) production.

**Ocular circulation**

The bovine eye has a vascularisation where the majority of the blood flow is supplied by the ciliary artery (Figure 2) (Prince et al., 1960). This is in contrast with primate and human eyes, where the principal supply is derived from the internal ophthalmic artery (Table 1).

The vascular supply to the orbit is mainly provided by the external ophthalmic artery, the largest branch of the internal maxillary artery, a continuation of the external carotid artery. Branches of the external ophthalmic artery include the external arterial rete and lacrimal artery.

The ciliary artery, which originates from the external rete, bifurcates into the larger medial and smaller lateral ciliary arteries, which travel parallel to each other and the optic nerve. Both arteries branch into several short posterior ciliary arteries at the globe, which penetrate the sclera near the optic nerve at the posterior pole of the
Figure 2. Schematic diagram of the vascular blood supply to the bovine eye. Adapted from Prince et al. (1960).
Two branches of the medial ciliary artery each form a long posterior ciliary artery, which pass superficially within the sclera across the medial and lateral sides of the globe, penetrating fully before the equator is reached. The small internal ophthalmic artery, which travels along the surface of the optic nerve, anastamoses with the medial long posterior ciliary artery a few millimetres short of the posterior pole of the globe.

The short posterior arteries form a capillary layer adjacent to the retina known as the choriocapillaris, which is responsible for the nutrition of the outermost layer of the retina. After entering the sclera beyond the equator, the long posterior ciliary arteries run anteriorly through the choroid to the ciliary body where they anastomose with the anterior ciliary arteries to form the well developed major arterial circle at the periphery of the iris (Diescsm, 1975). The major arterial circle supplies the ciliary body and iris, as well as providing a minor contribution to the choroidal circulation in some species.

Blood from the choroid, iris and ciliary processes drain into the episcleral veins, which run through the sclera, continuing into the four vortex veins, which emerge through the sclera at each quadrant of the equator. Four vortex veins, one in each quadrant of the eye, drain blood from the anterior uvea of the eye. After leaving the globe the vortex veins join to the supraorbital vein, the inferior orbital vein and the external ret, which after anastomotic communication, join the orbital vein (Prince et al., 1960).
Table 1. Anatomical differences between the human and bovine eye

<table>
<thead>
<tr>
<th>Feature</th>
<th>Human eye</th>
<th>Bovine eye</th>
</tr>
</thead>
<tbody>
<tr>
<td>Globe dimensions</td>
<td>21-26 mm (antero-posterior) (23-25) mm (diameter)</td>
<td>28-30 mm (antero-posterior) (30) mm (diameter)</td>
</tr>
<tr>
<td>Main arterial blood supply</td>
<td>Internal ophthalmic artery</td>
<td>Ciliary artery</td>
</tr>
<tr>
<td>Distance from iris root to ora serrata/ ora ciliaris retinae</td>
<td>6 mm</td>
<td>6.5 mm</td>
</tr>
<tr>
<td>Ciliary muscle</td>
<td>Well developed</td>
<td>Poorly developed</td>
</tr>
<tr>
<td>Lens dimensions</td>
<td>(~4) mm (antero-posterior) (~10) mm (diameter)</td>
<td>(~13.3) mm (antero-posterior) (~19.5) mm (diameter)</td>
</tr>
<tr>
<td>Lens Volume ((\text{cm}^3))</td>
<td>0.2</td>
<td>2.2</td>
</tr>
<tr>
<td>Accommodation</td>
<td>Extensive</td>
<td>Limited</td>
</tr>
<tr>
<td>Termination point of retina/ start of ciliary body</td>
<td>Ora serrata</td>
<td>Ora ciliaris retinae</td>
</tr>
<tr>
<td>Scleral Spur</td>
<td>Present</td>
<td>Absent (well developed pectinate ligament)</td>
</tr>
<tr>
<td>Anterior Chamber Volume ((\text{cm}^3))</td>
<td>0.35</td>
<td>1.7</td>
</tr>
<tr>
<td>Ciliary Processes</td>
<td>(~70)</td>
<td>90–110</td>
</tr>
<tr>
<td>Pupil</td>
<td>Circular</td>
<td>Oval (transverse)</td>
</tr>
<tr>
<td>Corneal dimensions</td>
<td>(~11.7) mm (horizontal) (~10.6) mm (vertical)</td>
<td>(~27)–(~34.5) mm (horizontal) (~20)–(~30.5) mm (vertical)</td>
</tr>
<tr>
<td>Corneal thickness (total)</td>
<td>(~500) (\mu)m (central) (~1000) (\mu)m (peripheral)</td>
<td>(~725)–(~1450) (\mu)m (thickness relatively uniform)</td>
</tr>
<tr>
<td>Corneal epithelium</td>
<td>5–6 cell layers (50)–(60) (\mu)m thick</td>
<td>14–18 cell layers (90) (\mu)m thick</td>
</tr>
<tr>
<td>Bowman’s layer</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Descemet’s membrane</td>
<td>8–12 (\mu)m thick</td>
<td>10–25 (\mu)m thick</td>
</tr>
<tr>
<td>Corneal endothelium</td>
<td>1 cell layer \ Polygonal mosaic (5–6) (\mu)m thick</td>
<td>1 cell layer \ Polygonal mosaic (6) (\mu)m thick</td>
</tr>
</tbody>
</table>

Ciliary epithelium

The ciliary processes project inwards from ciliary body into the posterior chamber. Structurally, each process consists of a mass of capillaries surrounded by loose connective tissue (stroma), covered by an epithelial layer.

In the mammalian eye the ciliary epithelium, which separates the stroma of the ciliary processes from the AH and vitreous humour, consists of a bilayer of cells. The inner layer, in direct contact with the AH and vitreous humour, is non-pigmented epithelium (NPE). The outer layer is heavily pigmented (PE), and lies between the NPE and the ciliary stroma. In rabbits these epithelia show characteristics consistent with secretory epithelia, such as basal infoldings and interdigitations of the lateral surfaces of adjacent cells (Pappas & Smelser, 1958). These characteristics are found to be more developed in the NPE cells. Since the secreted AH must be derived from blood plasma and the extracellular fluid in the stroma of the ciliary processes, and secretion must occur across both epithelial layers, the relationship between the epithelial layers is very important. The PE and NPE cells lie apex-to-apex (Figure 3). Therefore, the basal surface of the PE cells faces the ciliary stroma, and the basal surface of the NPE cells faces the AH.

Several studies have demonstrated that the ciliary epithelium of the mammalian eye is densely populated by a variety of specialised intercellular junctions (Figure 3). In the rabbit and primate eye it has been shown that zonula occludenta, zonula adhaerens, gap junctions and desmosomes connect the NPE to one another, whereas
Figure 3. Schematic diagram of nonpigmented and pigmented epithelial cells, showing the apex-to-apex relationship. BI, basal infolding; BM, basement membrane; CC, ciliary channels; DES, desmosomes; FE, fenestrated capillary endothelial; GJ, gap junction; MEL, melanosome; MIT, microchondrion; RBC, red blood cell; RER, rough endoplasmic reticulum; TJ, tight junction. (Caprioli, 1992).
gap junctions, puncta adhaerentia and desmosomes connect PE cells to one another and to NPE cells (Raviola & Raviola, 1978). The ciliary epithelium is also the site of the attachment of suspensory ligaments. The physiological and anatomical functions necessary for maintaining the continuous flow of AH and the mechanical stability of the epithelium depend upon the specialised intercellular junctions.

The zonula occludenta, or tight junctions, found at the apex between NPE cells, represent the permeability barrier that prevents the diffusion of blood-borne macromolecules into the AH of rabbit and primate eyes (Raviola, 1974; Raviola, 1977; Smith & Rudt, 1973). Freeze-fracturing techniques show that zonula occludenta are only found connecting the lateral surfaces of the NPE cells. Although zonula occludenta between NPE cells are impermeable to macromolecules, it is believed they only restrict the paracellular movement of ions and small molecules without preventing it altogether (Raviola & Raviola, 1978).

Gap junctions are found at the interface between PE and NPE cells, interconnecting the lateral surfaces of the PE cells and, to a lesser extent, the lateral surfaces of the NPE cells (Lütjen-Drecoll, 1982). Gap junctions mediate the electrical and metabolic coupling between cells, therefore the extensive connections between cells in the ciliary epithelium suggests that epithelial cells function as a syncytium (Green et al., 1985; Gilula et al., 1972; Pappas et al., 1971; Wiederholt & Zadunaisky, 1986). It is therefore likely that gap junctions allow the passage of intracellular ions and small molecules between the epithelial layers (Gilula et al., 1972; Pappas et al., 1971; Raviola & Raviola, 1978).
Desmosomes are commonly found between the lateral surfaces of the NPE cells, and in small numbers between PE cells and the interface of the two epithelial layers. It is generally accepted that the function of desmosomes is intercellular cohesion (Raviola & Raviola, 1978). In the ciliary epithelium they probably provide the mechanical stability to withstand the forces exerted on the ciliary zonule by contraction and relaxation of the ciliary muscle during accommodation of the lens (Raviola, 1971).

**Blood-aqueous barrier**

The presence of a barrier, preventing the free movement of molecules from blood plasma into the AH is well known. This results in aqueous that is much different in composition than blood plasma or a simple ultrafiltrate of plasma.

Tight junctions between the apices of NPE cells of the ciliary epithelium and posterior surface of the iris represents the principal site of the blood-aqueous barrier (BAB). In the rabbit and primate eye the existence of this barrier has been observed experimentally by the impermeability of the NPE cell layer to the tracer molecule, horseradish peroxidase (Raviola, 1974; Raviola, 1977; Smith & Rudt, 1973).

The anterior surface of the iris is devoid of epithelia, permitting free exchange of macromolecules between AH circulating in the anterior chamber and the iris stroma. However, the blood vessels, in contrast with vessels in the ciliary processes, are lined
with an impermeable endothelial barrier (Raviola, 1977).

Under normal conditions the functional integrity of the blood-aqueous barrier excludes large molecules from the AH. However, the presence of a low level of protein in the AH of normal eyes (<1% of plasma protein concentration) demonstrates that the barrier function is not absolute (Novak & Leopold, 1988) or else that some proteins are secreted by the NPE. If IOP is experimentally depressed below venous pressure the direction of flow of AH out of the eye is reversed and large amounts of plasma proteins enter the anterior chamber from the canal of Schlemm which fills with venous blood (Raviola, 1974).

**Aqueous humour**

AH is a transparent colourless fluid formed from plasma by the epithelial cells of the ciliary processes. AH fulfils three main functions; the continuous formation and drainage of aqueous helps to: 1. maintain intraocular pressure; 2. meet the metabolic requirements of the avascular structures of the eye, the lens, cornea, drainage meshwork and anterior vitreous, by delivering oxygen and nutrients, and removing waste products; 3. maintain a transparent and colourless medium between the posterior cornea and the lens (Millar & Kaufman, 1995).
Aqueous humour formation

AH is formed continuously by the ciliary epithelium. The formation of AH in the mammalian eye is widely believed to occur by ultrafiltration of blood plasma into the stroma and then active transport of ions, followed by diffusion of water, across the PE and NPE into the posterior chamber (Bill, 1975; Brubaker, 1991).

It is now widely accepted that the active transport of solutes across the ciliary epithelium against a concentration gradient acts as the driving force behind AH formation, contrary to previous beliefs of a simple process of diffusion or ultrafiltration (Caprioli, 1992). In the mammalian eye the rate of AH formation is believed to depend on the rate of active transport by the ciliary epithelium (Cole, 1977).

Ultrafiltration favours the movement of water from the plasma into the ciliary stroma, but water cannot filter freely from the stroma into the posterior chamber (Bill, 1975). It was calculated that hydrostatic and oncotic pressures across the ciliary epithelium would favour reabsorption of AH into the ciliary processes if ultrafiltration was the driving force behind AH formation (Figure 4). Assuming an IOP of 15 mm Hg and a stromal oncotic pressure of 14 mm Hg due to protein leakage through the fenestrated capillaries, capillary hydrostatic pressure would have to exceed 29 mm Hg in order to drive an ultrafiltrate. Values for capillary hydrostatic pressure in the rabbit have been estimated between 27 - 28 mm Hg (Bill, 1975) and 25 - 33 mm Hg (Cole, 1977). It has been calculated that capillary
Figure 4. Schematic diagram of the hydrostatic forces involved in the production of AH in the mammalian eye (Caprioli, 1992).
hydrostatic pressure values exceeding 50 mm Hg would be required to drive ultrafiltration at a rate sufficient to produce normal AH flow (Green & Pedersen, 1972). Therefore the net pressures do not favour ultrafiltration as a driving force for AH formation.

Histochemical studies show a large population of active enzyme systems on the basolateral membranes of the rabbit NPE layer, including Na\(^+/K^+\)-ATPases, adenylate cyclase and carbonic anhydrase (Bhattachjee, 1971; Kaye & Pappas, 1965; Mishima et al., 1982; Shiose & Sears, 1966; Tsukahara & Maezawa, 1978). In the rabbit the NPE has also been shown to have a greater development of intracellular organelles and a higher metabolic rate than the PE (Cameron & Cole, 1963). Na\(^+/K^+\)-ATPase is a membrane-bound enzyme that catalyses the hydrolysis of ATP providing the energy to pump Na\(^+\) and K\(^+\). Cardiac glycosides, such as ouabain or digoxin, inhibit the action Na\(^+/K^+\)-ATPase and decrease the rate of aqueous formation by approx. 70% in a variety of species, including rabbits and primates (Becker, 1963; Bonting & Becker, 1964; Cole, 1977). Decreases in AH production due to inhibition of Na\(^+\)-transport by cardiac glycosides suggest that Na\(^+\) is the actively transported ion, with Cl\(^-\) or HCO\(_3^-\) following to maintain electroneutrality (Figure 5). Recent investigations into the role of ion channels in aqueous formation indicate that Na\(^+\)-K\(^+\)-Cl\(^-\) cotransporters are responsible for entry of ions into the PE from the ciliary stroma in the bovine (Edelman et al., 1994) and human eye (Jacob & Civan, 1996; Millar & Kaufman, 1995; Von Brauchitsch & Cook, 1993). After entry into the syncytium, the ions are free to diffuse into the NPE cells via gap junctions, where they are transported across the basolateral membrane (Edelman et
Figure 5. The transport and movement of elements responsible for the secretion of salts and water into the AH. Gap junctions (gj) connect pigmented (PE) and non-pigmented (NPE) ciliary epithelial cells, forming a functional syncytium (Jacob & Civan, 1996).
There is also evidence of a basolateral Na\(^{+}\)-K\(^{+}\)-Cl\(^{-}\) cotransporter located in human NPE cells which may play a role in AH formation (Crook et al., 1992; Von Brauchitsch et al., 1990). However, administration of bumetanide, an inhibitor of the Na\(^{+}\)-K\(^{+}\)-Cl\(^{-}\) cotransporter, did not affect AH formation, questioning its contribution to aqueous formation (Gabelt et al., 1997).

Measurements of electrical potential across the rabbit and bovine isolated ciliary epithelia indicate that the aqueous is positive with respect to the stroma (Cole, 1961a; 1961b; 1962). Ouabain has been shown to reduce the transepithelial potential virtually to zero (Cole, 1961a; Krupin et al., 1984; Sears et al., 1991). These studies confirm the hypothesis that active transport of Na\(^{+}\) is responsible for generating the transepithelial potential and is the primary driving force behind AH secretion.

Chloride secretion is now believed to play an important role in AH formation (Chen & Sears, 1997). Early experiments, in the rabbit eye, indicated that active transport of Cl\(^{-}\) may occur, although the magnitude of this transport was thought to be small compared with Na\(^{+}\) (Cole, 1969). Active Cl\(^{-}\) transport across the rabbit and cat isolated ciliary body preparation has been shown, indicating that the transport of Cl\(^{-}\) may be an important part of the secretory process (Holland & Gipson, 1970; Kishida et al., 1983). It has been suggested that Cl\(^{-}\) entry into the rabbit and bovine RE is due to Na\(^{+}\)-K\(^{+}\)-Cl\(^{-}\) cotransport and Cl\(^{-}\)/HCO\(_3\) exchange (Carré et al., 1992; Heilbig et al., 1988). The identification of Cl\(^{-}\) channels in the NPE and Cl\(^{-}\) currents across the NPE suggests that Cl\(^{-}\) channels may play a major role in the secretion of...
Cl\(^-\) into the rabbit, bovine and human aqueous (Coca-Prados et al., 1995; Chen & Sears, 1997; Edelman et al., 1995; Jacob & Civan, 1996). The secretion of K\(^+\) through selective channels is thought to be critical in providing the electrical potential to drive Cl\(^-\) secretion (Edelman et al., 1995; Jacob & Civan, 1996). Na\(^+\)-K\(^+\)-Cl\(^-\) cotransporters and Cl\(^-\)/HCO\(_3\) exchangers located in NPE cells are also believed to contribute to Cl\(^-\) secretion into the rabbit and human AH (Crook & Polansky, 1994; Matsui et al., 1996; Murakami et al., 1992).

Acetazolamide is a potent inhibitor of carbonic anhydrase (CA), which catalyses the reaction H\(_2\)O + CO\(_2\) → HCO\(_3\)\(^-\) + H\(^+\). Systemic treatment with acetazolamide and other specific CA inhibitors decrease IOP through a reduction in the rate of aqueous secretion by 40 - 60% (Becker, 1959; Becker & Constant, 1955; Garg & Oppelt, 1970; Maren, 1967). Evidence suggests that acetazolamide directly affects the transport mechanisms of the ciliary epithelium (Cole, 1977). Acetazolamide decreases the rate of Na\(^+\) and HCO\(_3\)\(^-\) transport into the posterior chamber by equimolar amounts in dogs and primates (Maren, 1976). Transepithelial electrical measurements in the isolated rabbit iris-ciliary body indicate that Na\(^+\)/K\(^+\)-ATPase and HCO\(_3\)\(^-\) are required for active ion transport (Krupin et al., 1984). These studies suggest a coupling in the movement of Na\(^+\) and HCO\(_3\)\(^-\) into the posterior chamber. However, the exact mechanism is not known. Several hypotheses have been postulated: (i) inhibition of CA causes a decrease in HCO\(_3\)\(^-\) available for movement into the intercellular channels between the NPE cells to maintain electroneutrality, (ii) a decrease in H\(^+\) availability may inhibit Na\(^+\)/K\(^+\)-ATPase, (iii) decreased availability of H\(^+\) produced by CA catalysed reaction decreases Na\(^+\)/H\(^+\) exchange,
reducing the availability of intracellular Na+ to be transported into the intercellular channel (Caprioli, 1992).

**Intraocular flow of aqueous humour**

After secretion into the posterior chamber, AH moves by bulk flow through the pupil aperture into the anterior chamber, flowing in a radially symmetrical fashion towards the periphery of the anterior chamber and trabecular meshwork (Davson, 1990).

AH drains continuously from the anterior chamber of the eye via two main outflow pathways: (a) the trabecular or conventional route and (b) the uveoscleral or unconventional route (Bill, 1975).

(a) **Trabecular outflow** is the main outflow pathway for almost all mammalian species. At the angle between the iris and the corneo-scleral junction, referred to as the anterior chamber angle, AH passes into the trabecular or, in the cow, the reticular meshwork (Figure 6). The reticular meshwork of the calf eye differs considerably from the laminated, trabecular meshwork of primates (Erickson-Lamy et al., 1988). The network consists of an extensive uveal meshwork with relatively large open spaces known as the spaces of Fontana (Johnson et al., 1990). After passing through the uveal meshwork aqueous then passes through the reticular meshwork. This area is equivalent to the human corneo-scleral trabecular meshwork, but lacks the organisation found in the human eye (Grierson et al., 1985). From the meshwork, aqueous passes into the aqueous collector vessels of the angular aqueous plexus.
which comprises a system anatomically analogous to Schlemm's canal in humans (Johnson et al., 1990; Samuelson, 1991). The angular aqueous plexus gathers aqueous which drains into the vortex veins, via the intrascleral and episcleral veins (Samuelson, 1991).

Figure 6. Schematic diagram of the calf outflow system (Johnson et al., 1990).

(b) Uveoscleral (unconventional) outflow. As there is no epithelial barrier between the anterior chamber and the ciliary muscle, AH may freely pass between the ciliary muscle bundles. Aqueous flows through the iris roots, across the anterior surface of the ciliary body and into the interstitial spaces between the muscle bundles of the ciliary muscle. These spaces in turn open into the suprachoroidal spaces from which fluid can pass via the venous circulation through the sclera and into the episcleral tissues (Bill, 1975).
Although there does not appear to be any specific information on uveoscleral drainage in the bovine eye, previous literature shows that the contribution of uveoscleral drainage to total AH outflow varies greatly among different species. In humans, the importance of uveoscleral flow has been investigated in older patients. Approx. 5 - 20% of the total drainage was due uveoscleral outflow, however this value may be larger for younger patients due to ciliary muscle degeneration with age (Shabo & Maxwell, 1973). Indirect calculations estimate that the uveoscleral outflow value may contribute as much as 35% of the total drainage in young adults (Nilsson, 1997). Uveoscleral flow is greatest amongst primates, contributing 30 - 50% of total drainage (Bill, 1971). Cats also have a well developed ciliary muscle and uveoscleral outflow is believed to make a small contribution to total outflow (Bill, 1966). Rabbits have very poorly developed ciliary muscles resulting in practically no drainage of AH via the uveoscleral pathway (Bill, 1975).

After removal of the meshwork tissue between the anterior chamber and the canal of Schlemm the resistance to outflow of the bovine eye is reduced by approx. 52% (Erickson-Lamy et al., 1988). This is similar to the 40 - 75% decrease in resistance seen in the enucleated monkey (Peterson et al., 1971; Peterson & Jocson, 1974) and human eyes (Ellingsen & Grant, 1971; Ellingsen & Grant, 1972; Erickson-Lamy et al., 1991; Grant, 1963; Rosenquist et al., 1985). This indicates that the reticular meshwork contributes the main resistance to outflow and drainage through the sclera only has a small contribution to resistance to outflow.

In the mammalian eye contraction of the ciliary muscle by parasympathomimetic
Introduction: General
drugs, such as pilocarpine, decreases the resistance to outflow. As the ciliary muscle contracts the muscle bundles become more tightly packed resulting in shortening and thickening of the muscle. This pulls on the scleral spur which physically alters the spaces in the trabecular meshwork, increasing the drainage through Schlemm’s canal (Leopold & Duzmann, 1986; Tamm et al., 1992). At the same time uveoscleral drainage is almost completely stopped as tighter packing of the muscle bundles compresses the interstitial spaces within the ciliary muscle. Substances which produce a relaxation of the ciliary muscle, such as atropine, have been shown to increase drainage through the uveoscleral route due to increased spaces between the muscle bundles. However, drainage through the trabecular meshwork and Schlemm’s canal is reduced because of tight packing of trabecular meshwork pores (Bill, 1971). Although cholinergic and adrenergic nerve endings have been identified in the trabecular meshwork their role in the regulation of AH outflow is not known (Nomura & Smelser, 1974). There is now evidence of a direct pharmacological effect on the endothelium of the trabecular meshwork. Bárány (1962) suggested that trabecular meshwork cells might be contractile after observing that pilocarpine injected into the anterior chamber directly affected outflow facility. Studies with isolated trabecular meshwork strips have demonstrated contractile responses to cholinergic agonists, adrenergic agonists and endothelin (Lepple-Wienhues et al., 1991a; 1991b; Wiederholt et al., 1996) and relaxant responses to prostaglandins and nitric oxide (Krauss et al., 1997; Wiederholt et al., 1994). It has been suggested that the contractile responses of the trabecular meshwork is due to smooth muscle-like spindle cells containing actin filaments (Coronado et al., 1991; Flügel et al., 1991). Recently several different receptor types including cholinergic, \(\alpha\)-adrenergic,
β-adrenergic, endothelin and prostaglandin F<sub>2α</sub> receptors have been identified in cultured human and bovine trabecular meshwork cells (Anthony et al., 1998; Lepple-Wienhues et al., 1994; Sharif, 1996; Stamer et al., 1996). Using the bovine anterior segment perfusion model, where the anterior uvea has been completely removed, contraction of the trabecular meshwork itself in response to cholinergic agonists, adrenergic agonists and endothelin results in a decrease in outflow facility (Wiederholt et al., 1995). However, these compounds also contract the ciliary muscle and increase outflow facility in the intact primate (Bill, 1969; Kaufman and Bárány, 1976; Kaufman, 1985) and human eye (Erickson-Lamy et al., 1991). Therefore it is believed that the influence on outflow facility of trabecular meshwork contraction is dominated by ciliary muscle tone (Wiederholt et al., 1995).
Composition of aqueous humour

A number of differences exist in AH as compared with plasma (Table 2).

Table 2. The concentration of electrolytes, low molecular weight solutes and protein in bovine and human AH, and human blood plasma.

<table>
<thead>
<tr>
<th>Components</th>
<th>Bovine AH</th>
<th>Human AH</th>
<th>Human Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Electrolytes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na⁺ (mM)</td>
<td>143.8 - 149.5</td>
<td>142</td>
<td>130 - 145</td>
</tr>
<tr>
<td>K⁺ (mM)</td>
<td>4.5 - 7.1</td>
<td>4</td>
<td>3.5 - 5.0</td>
</tr>
<tr>
<td>Cl⁻ (mM)</td>
<td>116.3 - 124</td>
<td>131 - 136</td>
<td>92 - 125</td>
</tr>
<tr>
<td>HCO₃⁻ (mM)</td>
<td>36</td>
<td>20</td>
<td>24 - 30</td>
</tr>
<tr>
<td><strong>Organic solutes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascorbate (mM)</td>
<td>2</td>
<td>1.0 - 1.1</td>
<td>0.04 - 0.06</td>
</tr>
<tr>
<td>Lactate (mM)</td>
<td>7.6 - 8.2</td>
<td>4.5</td>
<td>0.5 - 1.9</td>
</tr>
<tr>
<td>Glucose (mM)</td>
<td>2.17</td>
<td>2.8 - 3.9</td>
<td>5.6 - 6.4</td>
</tr>
<tr>
<td>Protein (mg/mL)</td>
<td>0.2 - 0.8</td>
<td>0.3 - 0.7</td>
<td>60 - 70</td>
</tr>
</tbody>
</table>


Under normal conditions the tight junctions of the blood-aqueous barrier limit the aqueous protein concentration to less than 1% of plasma protein concentration (Davson, 1990; Pavao et al., 1989). The plasma protein concentration is approx. 73
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- 106 mg.ml⁻¹ in bovine (Pavao et al., 1989) and approx. 60 - 70 mg.ml⁻¹ in humans, whereas in AH protein concentration is approx. 0.2 - 0.8 mg.ml⁻¹ in bovine (Doughty, 1995; Pavao et al., 1989) and approx. 0.3 - 0.7 mg.ml⁻¹ in humans (Novack & Leopold, 1988). Although the aqueous protein concentrations are low, separation and identification of AH by electrophoretic techniques have shown that all plasma proteins are present (Davson, 1990).

The concentration of electrolytes in AH, compared to human blood plasma are shown in table 2. Na⁺ is the major cation found in AH in both bovine and human eye, being very similar in concentration to human plasma. However, the concentration of major anions shown some species differences. In bovine AH, HCO₃⁻ concentrations are higher and Cl⁻ lower compared with human AH. A similar relationship exists between anion concentrations in the aqueous of rabbits and humans (Kinsey, 1967).

Exchange of substances between the aqueous and surrounding tissues, due to diffusion or secretion, occurs continuously. Therefore the normal composition of AH depends on diffusion from blood plasma, secretion across the ciliary epithelium plus the passive and active exchange of substances between the aqueous and tissues it bathes. The corneal endothelium acts as a fluid pump, constantly secreting fluid into the AH to keep the cornea in a clear, deturgesced state. However, the small volume of fluid secreted does not contribute significantly to the flow of AH (Fischbarg & Lim, 1974).
Changes in the composition of AH have been reported in diseased human eyes. For example, greater levels of heavy molecular weight proteins, found only in trace quantities in the normal eye, have been detected in AH of patients with both phagocytic glaucoma and primary open-angle glaucoma (Epstein et al., 1978; Kijlastra et al., 1989). It has been suggested that the increased levels of high molecular weight proteins in the AH obstructs aqueous outflow pathways resulting in increased IOP.

**Intraocular pressure**

Intraocular pressure (IOP) is necessary for the maintenance of shape, stability and alignment of internal structures. IOP in the *in vivo* calf eye has been measured at 16.5 ± 5.5 mm Hg (Booffel, 1964). This is similar to the value found in healthy human eyes, where the mean pressure normally maintained is approx. 15 mm Hg (Davson, 1990). In the human eye there are no changes in mean IOP values between the ages of 10 and 70, or between the sexes.

The three main factors concerned with the maintenance of IOP are the rate of formation of AH, the ease with which AH exits the anterior chamber through the trabecular meshwork into the canal of Schlemm, and the pressure in the episcleral veins into which the canal of Schlemm empties. This results in a dynamic equilibrium where small variations in either rate of formation or rate of outflow from the eye can result in large changes in IOP.
AH flows from the posterior chamber through the pupil into the anterior chamber, exiting the eye by the main outflow pathways by bulk flow and this is pressure dependent. This has been incorporated into the modified Goldman equation:

$$F = C \left( P_i - P_J \right) \text{ or } F = \Delta P \cdot C$$

This equation relates aqueous flow ($F$) to the facility of outflow ($C$) and the pressure difference across the meshwork ($\Delta P$). The pressure head responsible for driving AH outflow is the difference between IOP ($P_i$) and episcleral venous pressure ($P_J$). Episcleral venous pressure is the pressure in the blood vessels into which AH finally drains. Increases in episcleral venous pressure decrease the pressure head for aqueous outflow, resulting in engorgement of intraocular vascular beds and increased IOP (Hart, 1992).

**The glaucomas and ocular hypertension**

The glaucomas are a complicated group of disorders often associated, although not always, with abnormally high IOP resulting in damage to the optic nerve head and loss of visual field (Leopold & Duzman, 1986; Serle, 1994). After cataracts and macular degeneration, glaucoma is the third leading cause of blindness in the United States (Adkins & Balfour, 1998). Recent estimates of worldwide prevalence predict that 67 million people will suffer from glaucoma by the year 2000 (Flanagan, 1998). Clinically the damage to the optic nerve head and associated loss of visual field is manifested as cupping of the optic disk and optic atrophy. If left untreated glaucoma can lead to blindness (Adkins & Balfour, 1998).
The boundaries between glaucoma, low-tension glaucoma and ocular hypertension are indistinct. Glaucoma is often associated with an IOP greater than 21 mm Hg. Low tension glaucoma is the condition whereby the pathological changes associated with glaucoma, such as cupping of the optic disc and loss of visual field, are present, but within the boundaries of normal IOP. Ocular hypertension is characterised by IOP exceeding 21 mm Hg, but without the pathological changes associated with glaucoma. Patients suffering from ocular hypertension are more likely to develop glaucoma and are often treated to lower IOP (Sommer, 1989; Jay, 1992).

Predisposing risk factors for the development of glaucoma include family history of the disease, myopia, diabetes mellitus, long term topical or systemic corticosteroid use and ethnic group (Adkins & Balfour, 1998).

There are two basic types of glaucoma determined by the adequacy of drainage of AH through the main outflow pathway, the trabecular meshwork: open angle (OAG) where the AH has free access to the trabecular meshwork in the anterior chamber angle, and closed-angle (CAG) where the peripheral iris is in contact with the cornea or trabecular meshwork impairing outflow of AH.
Parasympathomimetic agents

Parasympathomimetic agents include both direct- and indirect-acting drugs. Direct-acting drugs act like pilocarpine at the muscarinic receptors; indirect-acting drugs inhibit cholinesterase, allowing acetylcholine to accumulate at the parasympathetic nerve endings (Hurvitz et al., 1991).

The muscarinic agonist, pilocarpine, is the most commonly used direct-acting cholinergic agent and has been established as a glaucoma treatment for more than 100 years (Zimmerman, 1981). The primary mechanism for pilocarpine-induced IOP reduction is by increasing facility of outflow of AH through the trabecular meshwork. Cholinergic agonists also produces miosis and accommodation of the lens (Abraham, 1985). In primates and humans, pilocarpine contracts the ciliary muscle and this pulls on the scleral spur resulting in mechanical deformation of the trabecular meshwork causing an increase in AH outflow facility (Bill, 1967; Bill, 1971). The effect of pilocarpine on outflow is lost following functional disconnection of the ciliary muscle from the scleral spur (Kaufman & Bárány, 1976). Although enhancing drainage by the conventional route and lowering IOP, pilocarpine has been shown to stop uveoscleral drainage almost completely (Bill & Wållinder, 1966; Bill, 1967). Pilocarpine contracts the ciliary muscle, which then appears much tighter than relaxed muscle, compressing the interstitial spaces between muscle bundles (Bárány & Rohen, 1965). The effects of muscarinic
agonists can be reversed by muscarinic antagonists such as atropine, which relaxes the ciliary muscle tending to increase uveoscleral flow (Bill, 1971). Clinically, when the anterior chamber angle is shallow pilocarpine can cause a paradoxical increase in IOP by decreasing uveoscleral outflow (Bleiman & Schwartz, 1979).

There is evidence that muscarinic agonists exert some direct influence on AH formation. Bill and Wallinder (1966) demonstrated a reduction in AH formation by intracameral infusion of pilocarpine in cynomolgus and vervet monkeys. Topical pilocarpine has also been shown to lower AH formation rates in the cynomolgus monkey, although no significant change in IOP was observed (Miichi & Nagataki, 1983). On the other hand, perfused pilocarpine had no effect on AH formation rates in the arterially perfused enucleated rabbit eye (Kodama et al., 1985). Pilocarpine has been shown by fluorophotometry to increase AH formation in the normal human eye (Nagataki & Brubaker, 1982).

\( \alpha_2 \)-adrenergic agonists

When applied topically the \( \alpha_2 \)-adrenergic agonist, clonidine, is effective in reducing IOP (Harrison & Kaufman, 1977; Krieglstein et al., 1978). However, as topically administered clonidine penetrates the blood-brain barrier, systemic hypotensive side effects are often associated with this drug (Harrison & Kaufman, 1977; Krieglstein et al., 1978). The development of apraclonidine and brimonidine, derivatives of clonidine with minimal penetration of the blood-brain barrier, has significantly reduced the side effects whilst retaining clonidine’s IOP lowering properties.
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(Topically applied apraclonidine or brimonidine has been shown to lower IOP in rabbits (Burke & Potter, 1986), cats (Burke & Potter, 1986), primates (Burke & Potter, 1986; Gabelt et al., 1994; Manlapaz et al., 1997; Serle et al., 1991) and humans (Gharagozloo et al., 1988; Lee et al., 1984; Schuman, 1996; Toris et al., 1995a; 1995b). In monkeys, brimonidine was more potent and effective in reducing IOP although the effects of apraclonidine were longer lasting (Gabelt et al., 1994). It has been suggested that local vasoconstriction, mediated via α2-adrenergic stimulation, leading to a decreased blood flow to the ciliary processes could be responsible for the decreased aqueous flow and fall in IOP (Abrams et al., 1987; Burke et al., 1986; Kriegstein et al., 1978). It is now generally accepted that α2-adrenergic agonists act to lower IOP by reducing AH formation (Gharagozloo et al., 1998; Mittag & Tormay, 1985; Toris et al., 1995). However α2-adrenergic agonists may reduce IOP by altering more than AH formation. It has been suggested that brimonidine and apraclonidine augment the local release of prostaglandins, which may increase uveoscleral outflow, resulting in a reduction in IOP (Crawford & Kaufman, 1987; Gabelt & Kaufman, 1989; Nilsson et al., 1989; Poyer et al., 1992). The α2-adrenergic agonists brimonidine and oxymetazoline, but not apraclonidine have been shown to increase total AH outflow via the uveoscleral pathway (Toris et al., 1995; Wang et al., 1993). Brimonidine has no effect on trabecular outflow facility (Toris et al., 1995a). Contradictory results have been reported on the effect of apraclonidine on trabecular outflow facility. One study shows apraclonidine does not change tonographic outflow facility (Robin & Pollack, 1988), whereas another shows that apraclonidine increases fluorometric...
outflow facility (Toris et al., 1995). It was suggested that the difference in outflow facility findings determined by tonography and fluorophotometry may be attributed to fundamental differences between the two techniques (Toris et al., 1995). Differences in the relative selectivity of \( \alpha_2 \)-adrenergic agonists for \( \alpha_2 \)-adrenoceptor subtypes (Bylund, 1988) may account for the differences observed in their mechanisms of action (Toris et al., 1995a; 1995b). It has also been demonstrated that apraclonidine, but not brimonidine lowers episcleral venous pressure by approx. 1 mm Hg possibly due to decreased blood flow to the anterior uvea (Hurvitiz et al., 1991; Toris et al., 1995a; 1995b), resulting in an equivalent fall in IOP.

Receptor binding studies indicate a predominance of \( \alpha_2 \)- and \( \beta \)-adrenergic receptors in the rabbit iris-ciliary body cell membrane (Mittag & Tormay, 1985) and human iris and ciliary epithelium (Matsuo & Cynader, 1992). In vitro, brimonidine has been shown to inhibit isoproterenol-stimulated cAMP accumulation in NPE cell culture. This effect was prevented by pre-treatment with pertussis toxin, suggesting the involvement of inhibitory G proteins (Ogidigen et al., 1993). Apraclonidine has also been shown to inhibit isoproterenol-stimulated cAMP accumulation in the isolated rabbit ciliary epithelial bilayer (Horio et al., 1992) and rabbit whole ciliary processes (Bausher et al., 1989). This evidence suggests that the \( \alpha_2 \)-adrenergic agonists may act by binding to \( \alpha_2 \)-receptors in the ciliary epithelium which are negatively coupled to the membrane-bound enzyme adenylyl cyclase, reducing the level of cAMP production, which may lead to a reduction in AH formation (Gabelt et al., 1994; Mittag & Tormay, 1985).
Prostaglandins

Early studies on the effects of prostaglandins (PG) in the rabbit eye showed that high doses of topical application of exogenous PG reproduced most of the characteristic signs associated with acute ocular inflammation, such as increased IOP and breakdown of the BAB (Beitch & Eakins, 1969; Camras et al., 1977; Eakins, 1977; Kass et al., 1972). However, the eyes of cats and monkeys were found to be much more resistant and similar breakdown of the BAB was not observed after repeated topical application of prostaglandins (Bito et al., 1983). Increases in AH protein concentration after intracameral or topical administered PGE$_1$ or PGE$_2$ provided evidence for an increased vascular or epithelial permeability (Beitch & Eakins, 1969; Kass et al., 1972).

At lower dosage, PG have also been shown to cause a prolonged reduction in IOP. Intracameral injection of PGE$_1$ or PGE$_2$ lowered IOP in rabbits after pre-treatment with polyphloretin phosphate, an anti-hyaluronidase which prevents increased capillary permeability in response to trauma (Starr, 1971). When applied topically as a single-dose, the IOP lowering effect of prostaglandin PGF$_{2\alpha}$ has been demonstrated on several species, including normotensive rabbits (Camras et al., 1977; Kulkarni et al., 1985; Lee et al., 1984), cats (Bito et al., 1983; Lee et al., 1984), monkeys (Camras and Bito, 1981; Crawford et al., 1987; Lee et al., 1984) and humans (Lee et al., 1988). Multiple-dose studies have demonstrated that topical application of PGF$_{2\alpha}$ in cats, monkeys and glaucomatous monkeys produced a maintained IOP reduction for up to several months, without any tolerance or
tachyphylaxis observed after early multiple-dose experiments (Bito et al., 1983; Lee et al., 1988). Multiple-doses of topically administered PFG$_{2a}$ also produced significant IOP reductions in normotensive and glaucomatous human subjects (Lee et al., 1988; Villumsen & Alm, 1987). Recently, newly developed PFG$_{2a}$ analogues, such as latanoprost, have been shown to decrease IOP with less side effects in experimental animals eyes as well as in the human eye (Toris et al., 1997). In fact latanoprost is the only PFG$_{2a}$ analogue which has already been approved for clinical use (Bito, 1997).

The mechanism behind the IOP-lowering effect of PG has remained obscure. Studies on AH dynamics have demonstrated that PG do not decrease AH formation (Gabelt & Kaufman, 1989; Havashi et al., 1987; Lee et al., 1984; Villumsen & Alm, 1989) and have minimal effects on conventional outflow facility (Crawford et al., 1987; Gabelt & Kaufman, 1989; Gabelt & Kaufman, 1990; Havashi et al., 1987; Kaufman, 1986; Villumsen & Alm, 1989; Villumsen et al., 1989), which are not great enough to account for the large drop in IOP. In some experiments PG have been shown to slightly increase AH formation (Crawford et al., 1987; Nilsson et al., 1989). It was suggested that other parameters of AH dynamics such as uveoscleral outflow may be involved (Kaufman, 1986). Measurements of the rate of movement of intracamerally-infused tracers through the uvea were used to demonstrate that PFG$_{2a}$ and its analogues increase uveoscleral outflow in monkeys (Gabelt and Kaufman, 1989; 1990; Nilsson et al., 1989). As uveoscleral outflow is highly dependent on the degree of ciliary muscle tone, pilocarpine-induced contraction of the ciliary muscle blocks uveoscleral outflow completely, while atropine increases it
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(Bill & Walinder, 1966; Bill, 1969). Crawford and Kaufman (1987) provided evidence for the enhancement of uveoscleral outflow by PGF$_{2\alpha}$ in cynomolgus monkeys by blocking the PG effect with pilocarpine. This antagonistic effect of pilocarpine was prevented by pre-treatment with atropine. In accordance with Bill and Walinder (1966) it was suggested that pilocarpine pre-treatment contracts the ciliary muscle, narrowing the intra-muscular spaces and closing off the uveoscleral drainage pathway.

In vitro studies on trabecular outflow have demonstrated a small direct relaxation effect of PG on pre-contracted isolated bovine trabecular meshwork strips, possibly mediated via EP receptor stimulation (Krauss et al., 1997; Toris et al., 1997). It has also been suggested that PGF$_{2\alpha}$ relaxes the ciliary muscle, widening the extracellular channels between the ciliary muscle fibres and subsequently increasing the outflow of aqueous. This idea originated after it was demonstrated that pilocarpine contracted the ciliary muscle and blocked the PG-induced increase in uveoscleral outflow and decrease in IOP (Crawford et al., 1987; Nilsson et al., 1989). Further evidence supporting this hypothesis includes reports that PGF$_{2\alpha}$ relaxes isolated ciliary muscle strips of cats (Chen & Woodward, 1992; Goh et al., 1995) and monkeys (Poyer et al., 1995) which have been precontracted with carbachol.

Morphological studies of monkey ciliary muscle after multiple doses of PGF$_{2\alpha}$ over several days show narrowing of the muscle bundles, with widening of the spaces and a reduction in collagen between the muscle bundles (Lüttjen-Drecoll & Tamm, 1988). PG have also been shown to induce substantial changes in the extracellular
matrix around cultured human smooth muscle cells (Lindsey et al., 1997a; Lindsey et al., 1997b). These structural alterations may help explain the observed increase in uveoscleral outflow after PG administration. However, it is unlikely that such anatomical changes could account for the increases in outflow seen rapidly, 2-4 hours after single-dose topical administration of PGF

It appears that at least two mechanisms are involved in the increased aqueous drainage through the ciliary muscle, which accounts for the decrease in IOP in response to PG. It is likely that the initial IOP decrease results from ciliary muscle relaxation and the sustained IOP reduction results from restructuring of the extracellular matrix (Toris et al., 1997).

Recently PG binding sites, of the EP and FP receptor type with a high affinity for PGE

Agents which have a higher affinity for the FP receptor have very little effect on the integrity of the barrier (Protzman & Woodward, 1990). The PGF

No changes in the level of aqueous flare were
observed after topical application of latanoprost in rabbits or humans (Widergard et al., 1998), indicating that the drug has no effect on permeability of the BAB in these species.

There is now general acceptance of Kaufman’s suggestion that the mechanism of the IOP-lowering effect of \( \text{PGF}_{2\alpha} \) is due largely to increase in uveoscleral outflow of AH, with aqueous outflow being partially redirected from the trabecular to the uveoscleral route. It has been postulated that the uveoscleral outflow route acts as a supplementary drainage alternative to prevent dangerous elevations in IOP due to possible obstruction of the trabecular meshwork (Hurvitz et al., 1991).
OCULAR DRUG ABSORPTION

Corneal permeation of topically applied drugs

The cornea is an optically transparent tissue that acts as the principal refractive element of the eye (Järvinen et al., 1995), in continuity with the sclera, constituting the anterior sixth of the surface of the globe (Pepose & Ubels, 1992). Anatomically the cornea consists of a sheet of connective tissue, the stroma, covered on either surface by a cellular layer (Maurice & Riley, 1970). A tear film covers the outer anterior cellular layer, the corneal epithelium. The supply of nutrients and removal of metabolites from the avascular cornea is provided by exchange: across the corneal limbus with blood capillaries in peripheral conjunctiva and sclera; across the endothelium with AII circulating in the anterior chamber; across the epithelium with the tear film (Maurice & Riley, 1970). The cornea also exchanges O₂ and CO₂ directly with the atmosphere (Maurice & Riley, 1970).

Influences on corneal drug penetration

After instillation of ophthalmic drug solutions the primary route into the eye for many drugs is transcorneal permeation (Friedrich et al., 1993). The quantity of drug that reaches the internal tissues of the eye is determined by two main factors: (i) pre-corneal drug loss such as drainage by the nasolacrimal system and absorption by the conjunctiva, much of which is then likely to be lost to the systemic circulation (Lee & Robinson, 1979), and (ii) the ability of the drug to penetrate the layers of the
Movement of drugs across cellular membranes

To traverse cellular barriers such as the cornea drugs have to cross lipid membranes. Drugs cross lipid membranes by two main mechanisms: passive diffusional transfer and carrier-mediated transfer.

The main factor which determines the rate of passive diffusional transfer across membranes is lipid solubility, with molecular weight being a less important factor. Non-polar substances which dissolve in lipids and pass through the cell membranes freely, are referred to as lipophilic. However, many drugs are weak acids or bases whose state of ionisation varies with pH according to the Henderson-Hasselbach equation. With weak acids or bases only the unionised species can diffuse freely across lipid membranes. The ionised species have a very low lipid solubility and are referred to as hydrophilic.

Hydration of the cornea

Generally the level of corneal hydration is expressed as the quantity of water associated with unit dry weight of the tissue (g H₂O.g⁻¹). Alternatively, the relative water content can be expressed as a percentage for corneal tissue (Doughty et al., 1996). Bovine corneal hydration has been calculated between 3.4 - 3.5 g H₂O.g⁻¹ dry weight (Davson, 1949: Duane, 1949) or 78 - 80% (Doughty et al., 1995; Doughty
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These values correlate well with most other mammalian species where water constitutes between 2.95 - 5.7 g H₂O.g⁻¹ dry weight (Maurice & Riley, 1970) or 75 - 80 % of the whole cornea (Maurice, 1983; Maurice & Riley, 1970). This value increases linearly with increasing corneal thickness. The corneal stroma swells readily with H₂O, which binds to proteoglycans in the extracellular matrix. No significant differences were found between the hydration of the intact cornea and epithelial-denuded stroma in monkeys (Ehlers, 1966) or rabbits (Otori, 1967). Oedema of the cornea leads to alteration of its structure and loss of transparency (Maurice & Riley, 1970). When moderate amounts of water are taken up or lost from the cornea, there is an alteration in the thickness (Hedbys & Mishima, 1966). Therefore measurement of corneal thickness can be a convenient and accurate way of assessing corneal hydration in the living eye (Lee & Davison, 1984; Mishima & Hedbys, 1967).

In the living mammalian eye normal thickness is controlled within narrow limits (Maurice & Riley, 1970). However, if the excised mammalian eye is stored at 4°C, then the cornea loses some of its transparency and increases in thickness due to absorption of water from the AH (Davson, 1955; Doughty, 1989; Doughty, 1997; Doughty et al., 1995; Harris & Nordquist, 1955). In the bovine eye this results in an increase from 3.4 g H₂O.g⁻¹ dry weight to 4.55 g H₂O.g⁻¹ dry weight (Davson, 1990), or an increase in central corneal thickness from 1007 ± 73 μm to 1218 ± 62 μm (Doughty, 1997). Transferring the mammalian eye to a moist chamber at 37°C can reverse this swelling (Davson, 1955; Harris & Nordquist, 1955). This "temperature reversal" effect still occurs after the epithelium is removed suggesting
the pump removing fluid from the corneal stroma is located in the endothelium (Harris & Nordquist, 1955). When the cornea is treated with ouabain, which specifically inhibits the transport of Na', it is found to swell, indicating that active transport of Na' and associated H₂O movement due to osmosis is involved in maintaining corneal dehydration (Trenberth & Mishima, 1968). The active transport of Na' is responsible for forming a small potential across the endothelium of approx. 0.76 mV, which is reduced to zero by ouabain, indicating the presence of Na'/K'-ATPase (Lim & Ussing, 1982). Na' transport is known to be accompanied by a net flux of HCO₃⁻ out of the stroma, into the AH (Fischberg & Lim, 1974; Hull et al., 1977). It has been suggested that active transport of HCO₃⁻ coupled to an energy source in a manner similar to Na'/K'-ATPase, may provide the osmotic gradient necessary for movement of water from the stroma (Riley, 1977).

**Permeability of the cornea**

The cornea is a unique tissue, which has an aqueous middle region, the hydrophilic stroma, between two hydrophobic or lipophilic layers, the epithelium and endothelium. This has great importance for drug penetration, restricting the corneal permeability of hydrophilic and macromolecular compounds (Morimoto et al., 1987). Usually the corneal epithelium provides the main barrier to drug absorption into the eye (Maurice & Mishima, 1984). Drugs penetrate across the epithelium via the transcellular or paracellular pathway. Generally lipophilic drugs penetrate through the transcellular route, whereas hydrophilic drugs penetrate through the paracellular route (Järvinen et al., 1995).
The permeability characteristics of the cornea are of great clinical interest as the cornea must be crossed in order that topically applied drugs reach their target tissue in adequate concentrations. Topically applied drugs face losses via four routes; drainage of AH back into the blood, uptake into the vascular system of the anterior uvea, flow of tears into the nasolacrimal duct and drug absorption into the conjunctiva. Corneal permeability experiments estimated that the endothelium was ~100 times more permeable to Na⁺ than the epithelium, indicating that the epithelium acts as a semi-permeable membrane, permeable to H₂O but not to solutes in tears (Maurice, 1951). Later it was calculated that the endothelium offers ~1700 times more resistance to diffusion than the same thickness of H₂O. Endothelial permeability studies using the isolated cornea with the epithelium removed suggest that in general permeability decreases as molecular weight increases (Kim et al., 1971; Mishima & Trenborth, 1968). In these experiments using the isolated corneal preparation, the permeability of Descemet's membrane and the corneal stroma was separately measured, indicating that they do not offer a significant barrier to diffusion. The relative resistance to diffusion of small electrolytes provided by the corneal epithelium, stroma and endothelium has been determined to be 2000:1:10 (Maurice, 1951; Mishima & Hedbys, 1967). The permeability of metabolically important substances such as glucose and amino acids is much greater than predicted from their size, due to carrier-mediated facilitated transport across the endothelial cell membrane (Davson, 1990).
It is generally accepted that the epithelium represents the major barrier to diffusion of compounds across the cornea into the anterior segment of the eye (Grass & Robinson, 1988b). The location of the barrier function is generally attributed to the outermost layer of the epithelium, which is characterized by tight junctions between adjacent epithelial cells (Tonjum, 1974). This barrier is often considered to vary in magnitude according to the solubility characteristics of the drug (Sieg & Robinson, 1976). In 1942, Swan and White found that substances which are soluble in lipid as well as in water penetrate the epithelium rapidly. A strong correlation has been reported between the oil-water partition coefficient of a compound and the ease with which it crosses the epithelium (Araie & Maurice, 1987). It is now widely accepted that lipid solubility, as reflected by the oil-water partition coefficient, is the major, but not the only factor influencing drug penetration into the cornea and AH (Jankowska et al., 1986; Schoenwald & Haung, 1983; Wang et al., 1991). This was highlighted experimentally, where despite an enormous difference in lipid solubility only a three-fold difference in absorption between methazolamide and benzolamide was found in the isolated intact cornea (Edelhauser & Maren, 1988). Other physicochemical drug properties including aqueous solubility, molecular size and shape, charge and degree of ionization are now thought to influence the route and rate of drug penetration (Grass & Robinson, 1988b; Haung et al., 1989; Maren & Jankowska, 1985; Rojanasakul et al., 1992; Sieg & Robinson, 1977).

Drugs which are soluble in water are generally unable to penetrate the epithelial barrier, therefore the rate-limiting barrier for ocular penetration of highly hydrophilic drug is the lipophilic corneal epithelium (Järvinen et al., 1995). However lipid-
soluble drugs which penetrate easily find difficulty in leaving; therefore some degree of solubility in both aqueous and lipid media is required for optimum transcorneal permeation (Swan & White, 1942; Kupferman et al., 1974).

Today, most ophthalmic drugs are available in a solution which is topically applied to the eye. Most drugs are ionizable and hence are present in a chemical equilibrium between the ionic (anion or cation) and non-ionic (free base) forms in aqueous solution (e.g., cation $\leftrightarrow$ free base $+ \text{H}^+$). This greatly complicates the mechanism of drug penetration through the cornea. Permeation of ionizable drugs (weak acids and weak bases) depends on the equilibrium between the ionized and unionized drug in the eyecrop and eventually the lacrimal fluid (Friedrich et al., 1993). The epithelium and endothelium would present the greatest barrier to corneal penetration if the drug was predominantly in the hydrophilic (ionized) form, whereas the stroma would theoretically present the greatest barrier to a drug in its lipophilic (unionized) form (Friedrich et al., 1993). As the epithelium and endothelium are lipid in nature the ionized form of the drug would not readily penetrate these barriers. However, the paracellular pathway does allow a small degree of penetration of ionic drugs through the epithelial and endothelial barriers (Klyce & Crosson, 1985). Unionized, lipophilic forms of a drug pass across the epithelium and endothelium by transcellular diffusion directly through the cell membranes. The rate of penetration via the transcellular route is much higher than via the paracellular route (Friedrich et al., 1993). However the ionised drugs exist at equilibrium with their unionized forms. Once an ionizable drug is applied to the tear film, the unionized and ionized form begin to penetrate across the epithelium. As the transcellular path is only
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available to the unionized form, the ionized form will accumulate at the interface between the epithelium and tear film. As the unionized and ionized forms are in equilibrium in the tear film, there is an imbalance at the interface. This imbalance pushes the equilibrium towards the unionized form. This equilibrium reaction is reversed as the drug reaches the hydrophilic stroma. This results in an enhanced overall permeability across the epithelium and endothelium to ionizable drugs, compared to the permeability predicted by lipid-solubility (Friedrich et al., 1993).

In the case of ionizable drugs, the charge on the molecule also affects their corneal penetration (Rojanasakul et al., 1992). At the physiological pH of 7.4 hydrophilic cationic charged compounds permeate the cornea more readily than anionic species (Rojanasakul et al., 1992). At acidic pH the cornea is selectively permeable to negatively charged molecules, however acidic formulations are not tolerated by the cornea and formulations for ophthalmic use seldom have a pH < 5.5 (Rojanasakul & Robinson, 1989).

Sieg and Robinson (1976) have investigated the mechanism of corneal penetration using the antiglaucoma drug, pilocarpine, which appears to possess optimum solubility characteristics being soluble in both polar (aqueous) and non-polar (lipid) solvents. The corneal epithelium is the rate limiting tissue and acts as both a barrier to drug penetration and as a reservoir of pilocarpine in the cornea (Sieg & Robinson, 1976). Removal of the epithelium prior to dosing produced a seven to eight fold increase in drug levels in the AH. The endothelium did not appear to offer any significant resistance to transcorneal permeation of pilocarpine. When the epithelium
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is removed there is also an absence of drug build up in the stroma and endothelium, eliminating the reservoir effect of the cornea. These results led the investigators to suggest that in the case of transcorneal permeation of pilocarpine the stroma, endothelium and AH almost act as a single phase.

Following topical application of drugs in vivo, there is rapid absorption across the cornea. Drugs with dissimilar physicochemical properties often show similar corneal absorption profiles. It has been shown that the drug contact time from 0 - 5 min postinstillation is critical for drug absorption into the cornea and AH (Sieg & Robinson, 1976), with drug concentrations in the AH peaking after 20 - 30 min (Makoid & Robinson, 1979; Sieg & Robinson, 1976). These early peak times are due to rapid elimination from the precorneal area (tear film), drug dilution in the tear film, drug loss via drainage into the nasolacrimal duct and absorption via the conjunctival sac into the systemic circulation, resulting in a rapid decrease in the concentration gradient between the precorneal area and the anterior corneal epithelial surface (Makoid & Robinson, 1979). Therefore the amount of drug absorbed is very small relative to the total amount instilled (Sieg & Robinson, 1976). Typically <5% of the applied dose reaches the eye following application of topical solutions (Järvinen et al., 1995).

It is very important to maximise the amount of drug absorption into the cornea and AH following application of a topical dose.
Ocular absorption of topically applied drugs may be improved by increasing their lipophilicity via prodrug derivatives (Lee & Li, 1989), ion-pair formation (Wilson et al., 1981) and optimized solution pH for the unionized form of the drug (Podder et al., 1992). Increasing the drug contact time in the precorneal area has also been shown to increase drug penetration in rabbits (Chrai & Robinson, 1974; Patton & Robinson, 1975) and humans (Saettone et al., 1982).

Soluble polymers, such as methylcellulose, hydroxypropyl methylcellulose and polyvinyl alcohol are commonly used in eyedrops to increase the viscosity of the solution, which decreases the dilution in the tear film and rapid initial drainage rate, resulting in an increased contact time in the precorneal area which sustains to some extent the initial tear concentration of the drug (Adler et al., 1971; Benedetto et al., 1975; Shell, 1985).

Ophthalmic ointments are a commonly used formulation for the delivery of drugs to the eye which also offer the advantage of prolonged medication from each instillation (Shell, 1985). Drug bioavailability has been found to peak later with ointment vehicles than with solutions resulting in a significantly greater bioavailability to the eye (Seig & Robinson, 1975). The superior bioavailability of drug from ointment formulations is believed to be due to several factors: (1) higher effective concentration of drug in the formulation (Seig & Robinson, 1975; Shell, 1985), (2) increased tissue contact time (Seig & Robinson, 1975; Shell, 1985), (3) inhibition of dilution by tears (Seig & Robinson, 1977) and (4) resistance to nasolacrimal drainage (Norn, 1964). The mechanical shearing action of blinking is also believed
to significantly enhance drug release from ointments by continually exposing new surface areas of the formulation to the precorneal area, continually changing the thickness of the diffusional layer and dispersing droplets of the drug in a water-in-oil emulsion (Seig & Robinson, 1979). On the other hand ophthalmic ointments present mixing problems between the ointment vehicle, which is lipophilic, and the tear film, which is hydrophilic (Sieg & Robinson, 1975).

Eyedrops, in the form of a topical emulsion or suspension have also been reported to prolong the therapeutic effect compared to a topical solution of the same drug (Mazor et al., 1979; Ticho et al., 1979). In an emulsion formulation, drug can be bound to a polymeric material, resulting in a suspension of drug-polymeric complex with a low viscosity which is readily delivered as an eyedrop (Shell, 1985). In vitro studies have indicated that the prolonged therapeutic effect is due to both an increased drug permeation and to a prolonged drug release from the vehicle (Mazor et al., 1979).

Increased vehicle contact time does not increase the epithelial concentration above its initial value, which is controlled by the concentration of the applied dose (Sieg & Robinson, 1976). Instead it maintains the epithelial concentration for an extended time, effectively increasing the time taken to reach peak drug concentration in the AH and the total amount of drug which penetrates into the AH. Increasing the contact time has been shown to moderately increase the absorption of pilocarpine by a factor of two to three (Chrai & Robinson, 1974; Patton & Robinson, 1975).
Several studies on ocular drug bioavailability indicate that drug solubility in the vehicle dictates the success of a drug formulation in increasing bioavailability and prolonging drug release (Sieg & Robinson, 1977). Drugs which are lipophilic and therefore soluble in lipophilic ointments, show greater absorption and prolonged release when applied as an ointment formulation. In contrast it was suggested that more appropriate vehicles for drugs which do not dissolve readily in lipophilic ointments would be aqueous or perhaps an oil-in-water emulsion (Sieg & Robinson, 1977).

Transcorneal permeability varies among different species. In vivo epithelial diffusion studies showed that chloramphenicol, fluorescein and gentamycin penetrate less readily in humans than in rabbits (Maurice & Mishima, 1984). In vitro studies also show a significant difference between the permeability characteristics of rabbit and human cornea (Edelhauser & Maren, 1988). When the epithelium was intact the permeability of hydrophilic compounds was much greater in human cornea. This difference between the species disappeared when the epithelium was removed, leading Edelhauser and Maren to suggest that the rabbit epithelium is more of a barrier to hydrophilic drugs, which probably pass through the cornea via aqueous channels. This agreed with previous reports and the general principle that the epithelium is a barrier to hydrophilic compounds (Maren et al., 1983). The permeability to lipophilic compounds was essentially the same in corneas from both species with intact or removed epithelium, indicating that the lipid-barrier component of the epithelium functions in the same way for both rabbit and human (Edelhauser
Compounds such as benzalkonium chloride and EDTA which are commonly added to topical drug solutions are known to disrupt the integrity of corneal epithelial barrier, which may affect ocular drug absorption (Podder et al., 1992). Total corneal débridement is reported to increase penetration of hydrophilic (water-soluble) drugs, but not lipophilic (lipid-soluble) drugs into the cornea (Chien et al., 1988; Hull et al., 1974; Lesar & Fiscella, 1985). The relationship between drug penetration and corneal epithelial integrity was investigated using the antifungal drug, saperconazole (Johnson et al., 1995). This drug has lipophilic properties and its penetration into the cornea is strongly influenced by the presence of an intact epithelium (O'Day et al., 1992). The study showed that débridement of the corneal epithelium increases penetration of the antifungal drug into the rabbit cornea and AH. The relationship between epithelial defect size and drug penetration was not linear. A plateau value between 25% and 50% of total epithelial débridement was found to exist, beyond which no greater drug penetration was seen. This work agrees with a previous report where a positive correlation was shown between epithelial defect size and penetration of fluorescein into the human cornea and anterior chamber (Berkowitz et al., 1981).

The poor solubility of saperconazole in aqueous solution was enhanced by the addition of a hydrophilic carrier molecule to the antifungal agent (Johnson et al., 1995). Therefore the saperconazole complex behaves as a hydrophilic substance. This helps to explain the increase in penetration into the cornea and aqueous of the lipophilic compound after removal of the epithelium, a layer which is permeable to lipophilic substances. Clinically, situations may arise where débridement of a
porportion of the corneal epithelium may be performed in an effort to increase penetration of drugs into the cornea and anterior chamber (Johnson et al., 1995).

**Herpes simplex virus and corneal damage**

Herpes simplex virus (HSV) is one of the most damaging infectious agents that can attack the eye. The virus is the most common infectious cause of corneal ulcers and corneal blindness in developed countries, and has reached epidemic proportions in the United States with nearly 300,000 new cases reported annually (Pavan-Langston & Boisjoly, 1985).

Clinical manifestations of primary HSV infection of the eye include tearing, conjunctivitis and epithelial keratitis. Primary herpetic keratitis is generally self limiting and usually heals within 4 weeks without scarring (Verdier & Kracher, 1984). However, the virus can remain latent with periodic recurrence producing clinical manifestations of secondary herpetic keratitis. In primary herpetic keratitis the damage is generally limited to the surface epithelium, whereas in secondary keratitis the damage often extends to the stroma and endothelium and may persist for months producing irreversible morphologic changes which may result in permanent blindness (Hughes et al., 1993a; 1993b).

Herpetic keratitis can be treated with topical antiviral compounds, such as aciclovir (Pavan-Langston & Boisjoly, 1985). Unlike other DNA-inhibiting antiviral agents, aciclovir acts preferentially on HSV-infected cells and is activated by the virus-coded
enzyme, thymidine kinase (Abel et al., 1975).

The major barriers to the treatment of herpetic keratitis are frequent administration of topical solutions and the failure of the active drug to achieve minimum inhibitory concentrations in ocular tissues (Hughes et al., 1993a; 1993b). Aciclovir has low ocular bioavailability due to its hydrophilic nature causing low epithelial permeability and rapid dilution and drainage from the precorneal area. Therefore it is important to maximise the amount of active drug which penetrates the epithelial barrier of the cornea.

**Alkali burns and corneal ulceration**

Alkali burns of the eye are among the most disastrous of ocular injuries, characterised by corneal opacification, vascularization, chronic irritation and conjunctival overgrowth. Burns commonly result in repeated ulceration or perforation of the cornea (Brown et al., 1969a). The viability and future integrity of the alkali-burned cornea was found to be linked to the extent of neovascularization (Brown et al., 1969a). When the corneas were completely vascularized, wounds healed as well as those of normal controls and perforations were rare (Brown et al., 1969a). Investigations indicated that all the cells of the cornea were destroyed immediately after exposure to alkali (Brown et al., 1972). Seven to eighteen days after exposure, regrowing epithelial cells and cellular elements that are repopulating the stroma produce collagenase which ultimately ulcerates the cornea (Brown et al., 1969a; 1969b; Mai Phan et al., 1991).
Alkali burn models have been extensively used to investigate the formation of corneal ulcers in rabbits (Berman et al., 1983; Levinson et al., 1976; Mai Phan et al., 1991; Pfister & Paterson, 1977). After deep anaesthesia was induced, alkali burns were made by either: placing disks of filter paper, saturated with 4N sodium hydroxide on the central corneal surface for 2 min (Berman et al., 1983; Mai Phan et al., 1991); or placing a circular Lucite well on the cornea and filling the well with 1N sodium hydroxide for 20 sec (Levinson et al., 1976; Pfister & Paterson, 1977; Pfister & Paterson, 1980). Alkali-burned corneas were then irrigated with 0.9% saline solution. In these models the corneal epithelial defect, induced by the alkali-burn, initially closes at a steady rate. This process usually ceases 3 - 5 days after the burn, with secondary epithelial breakdown eventually resulting in corneal ulceration. Thermal burns, formed by application of a thermokeratophore probe at a temperature of 130 °C to the cornea for 2 sec, have also been used to induce corneal ulceration (Phillips et al., 1983).

Treatment with a variety of agents including hypertonic ointments (Pfister, 1983), epidermal growth factor (Singh & Foster, 1987), collagenase inhibitors (Brown & Weller, 1970), ascorbic acid (Levinson et al., 1976; Pfister & Paterson, 1977; Pfister et al., 1978) and the corticosteroid, prednisolone (Phillips et al., 1983) helps to reduce the frequency of corneal ulcer formation in eyes with epithelial defects induced by alkali-burns. It has been reported that treatment of corneal ulcers with corticosteroids increases the activity of collagenase, which may be associated with a rapid perforation of the corneal epithelium (Brown, 1971). Topical fibronectin has
been reported to accelerate corneal epithelial wound healing in rabbits (Mai Phan et al., 1991; Nishida et al., 1984).

**Drug absorption in the bovine perfused eye**

Utilisation of the perfused bovine eye as an *in vitro* model for the study of drug absorption allows the study of drug penetration into the cornea and AH without the complications of blinking, drug dilution in the tear film, tear drainage, drug loss to the systemic circulation, or influences of the CNS and CVS associated with *in vivo* models (Figure 7a), while maintaining the eye under controlled physiological conditions (Zhu et al., 1996).

In the isolated bovine eye, drug molecules permeating the cornea are partitioned in the AH and then distributed into surrounding tissues such as the iris and lens. As the AH is the most accessible compartment for drug molecules diffusing across the cornea and only a small amount of drug molecules are distributed into the surrounding ocular tissues, it is assumed that the AH is the major compartment for elimination and distribution of drug (Zhu et al., 1996). Figure 7b describes the route of absorption following topical application of drug to the isolated perfused bovine eye. Under non-perfused conditions, corneal hydration of the freshly collected bovine eye remains satisfactory up to an hour after death (Zhu et al., 1996). Commencement of arterial perfusion with Krebs’ solution, within an hour after death, has been shown to maintain corneal hydration without significant alterations in corneal thickness for several hours (Zhu et al., 1996).
Introduction: Ocular Drug Absorption

**DRUG IN TEAR FLUID**

**OCULAR ABSORPTION**
- (<5% OF THE DOSE)
  - **CORNEAL ROUTE**
    - PRIMARY ROUTE
    - SMALL, LIPOPHILIC DRUGS
  - **CONJUNCTIVAL AND SCLERAL ROUTE**
    - LARGE HYDROPHILIC DRUGS
  - **AQUEOUS HUMOUR**

**SYSTEMIC ABSORPTION**
- (~50-100% OF THE DOSE)
  - ROUTES:
    - CONJUNCTIVA
    - NOSE
    - AQUEOUS HUMOUR

**OCULAR TISSUES**

**ELIMINATION**

**Figure 7a.** Flow diagram representing the theoretical route of drug absorption following topical application *in vivo.*
**Figure 7b.** Flow diagram representing the theoretical route of drug absorption following topical application *in vitro.*
AIMS OF THE PROJECT

A general aim of this project was to develop the in vitro bovine perfused eye, to show whether it is a useful model for experimental work in studying drug mechanisms in the eye, whether from a pharmacodynamic or pharmacokinetic point of view.

There are two sections to the current work and their ultimate aims are: A). the development of safer and more effective drugs for the treatment of glaucoma by improving our understanding of the mechanisms of existing drugs. The present work sets out to show whether this model will be useful in predicting antiglaucoma drug activity in man; and B). the development of a simple model allowing the study of corneal drug absorption under physiological and pathological conditions.

A. The effects of pilocarpine, apraclonidine and prostaglandins, drugs which are known to have IOP-lowering properties in other species, will be studied on IOP in the bovine perfused eye. Once the IOP-lowering properties of drugs have been confirmed in this model an attempt will be made to try to elucidate the mechanisms by which these drugs may alter IOP, by measuring facility of outflow, uveoscleral outflow or rate of formation of AH.

A further intention is to ascertain how various routes of drug administration might alter the effect of these drugs upon IOP. It is most likely that drugs which influence IOP via an alteration in aqueous formation in the ciliary body will be most effective.
by the intra-arterial route, whereas drugs influencing IOP by altering outflow are more likely to do so following administration via the intracameral route.

Where there is evidence that a given drug may affect the integrity of the BAB, the perfused bovine eye model will be used as a means of testing the effect of various drugs on BAB permeability by the inclusion of the high molecular weight substance, bovine serum albumin (1% w/v) in the arterial perfusate and measuring its leakage into the secreted AH.

B. Studies of ocular drug absorption will utilise the bovine perfused eye as an *in vitro* model to investigate the absorption of different formulations of the anti-viral drug, aciclovir. The other major experimental variable will be chemical and physical damage to the cornea attempting to mimic the corneal damage associated with FSV. Clinically, damage to the corneal epithelium associated with HSV, results in perforations in the epithelial layer which if severe enough may lead to the formation of corneal ulcers. As it is not feasible to keep the perfused eye model viable for the duration of time required for corneal ulceration to develop, then the development of a rapid, reproducible method of damaging the cornea may be helpful in studying absorption of aciclovir formulations.

High performance liquid chromatography (HPLC) will be used to analyze and quantify the amount of aciclovir which is absorbed in acid extracts of the cornea and AH.
MATERIALS AND METHODS
MATERIALS AND METHODS

The bovine perfused eye preparation

The procedure for dissection and setting up of the constant flow method for the bovine perfused eye was initially described by Wilson and co-workers (1993). Bovine eyes were obtained from the abattoir, and transported to the laboratory. Temperatures during transportation depended on seasonal variation, ranging from 2 - 20°C. The eyes were not transported on ice since the resulting solidity of the orbital fatty tissue seriously hampered dissection and cannulation (Wilson et al., 1993). It was also felt that the return of the eyes’ core temperature to 37°C on commencing perfusion would be delayed considerably by cooling the whole eye on ice (Wilson et al., 1993). Immediately upon return to the laboratory, excess adnexal tissue was trimmed from the eye, taking care not to damage blood vessels running over the posterior surface of the globe. The two posterior ciliary arteries were located, one of which was carefully cleared of fatty and connective tissue, and cannulated distal to the ophthalmic artery but proximal to the dark blue pigmentation which appears in the arterial wall prior to entering the sclera. This portion of the artery is relatively large and robust, allowing easy cannulation and manipulation.

After cannulation, the eye was placed in a warming jacket maintained at 37°C and insulated with a plastic cover (Figure 8). The cannulated long posterior ciliary artery and thus the blood vessels of the anterior uvea were then perfused with a Krebs’ solution at 37°C, containing (mM): NaCl, 118; KCl, 4.7; MgSO₄, 1.2; CaCl₂,
Figure 8. The bovine isolated eye, showing perfusion of the uveal vasculature through one long posterior ciliary artery under condition of constant flow rate. Perfusion pressure and IOP were both monitored as shown (Wilson et al., 1993).
2.5; NaHCO₃, 25; KH₂PO₄, 1.2; glucose, 11.5; ascorbate, 0.05. The solution was bubbled with 95% O₂ - 5% CO₂ to adjust the pH to 7.4. Flow of Krebs' through the vasculature was induced via a Watson-Marlow peristaltic pump. Arterial perfusion pressure was recorded via a Grass FT03C transducer, bridge box amplifier and Linseis (LS 52-2) pen recorder. Arterial flow of Krebs' was commenced at 0.2 ml.min⁻¹ and increased in approx. 10 increments to 2.25 - 2.5 ml.min⁻¹, over a 40 min period. In some eyes that showed a low initial perfusion pressure, optimum flow rate was reached much earlier than this, sometimes within 20 min of the start of perfusion. During this period arterial perfusion pressure often fluctuated, usually due to poor alignment of the cannula with the artery, the twisting of the artery to produce partial blockage of the lumen or excessively tight ligatures around the cannula. After 40 min, when AH secretion had restarted and the anterior chamber was firm, it was cannulated with a 23G needle connected via silicon rubber tubing (internal diameter 0.8 mm) to a water manometer for measurement of IOP.

**Measurement of intraocular pressure**

IOP, expressed as mm H₂O, was measured manually from the water manometer at 5 or 10 min intervals depending on the duration of the experiment. When recording IOP for 90 min, measurements were taken at 5 min intervals; when recording IOP for 180 min, measurements were taken at 10 min intervals. In order to minimise the effect of capillary surface tension on IOP, fine steel wire was used to disturb the H₂O meniscus approx. 30 sec prior to taking measurements. IOP can be easily converted
Materials and Methods

from mm H₂O to mm Hg by dividing the value obtained from the water manometer by 13.546, the specific gravity of mercury (specific gravity H₂O = 1).

Criteria for accepting/rejecting eyes

The criteria for proceeding further with each experiment, following an equilibration period of a further 30 - 60 min, were as follows (Wilson et al., 1993):

- maintenance of a stable IOP, with fluctuations of less than ±2 mm H₂O over a 15 min period, within the range 95 - 165 mm H₂O (7 - 12 mm Hg).
- maintenance of a stable arterial perfusion pressure within the range of 20 - 60 mm Hg.
- free flow of perfusate from at least 2 vortex veins.

Eyes were also rejected for further study if any of the following were observed:

- air bubbles moved from the heating coils into the vasculature resulting in a sharp increase in IOP, >20 mm Hg, which failed to return to its previous value within 5 min.
- perfusion pressure increased above 100 mm Hg at any point of the experiment.
- damage to the globe resulting in leakage of perfusate from the long posterior ciliary artery.

Drug administration

Once IOP had stabilised, drug solutions or vehicles were administered by one of three routes: (i) by addition of drug to the perfusate reservoir at an exact concentration, (ii)
as a bolus dose injected intra-arterially or, (iii) as a bolus dose injected intracameraly. Bolus doses of drug solution were injected in volumes of 3 - 10 µl using a microsyringe. Intra-arterial injections were administered immediately proximal to the arterial cannula through re-sealable rubber tubing. Intracameral injections were administered by cannulating the anterior chamber with a second 23G needle connected via silicon rubber tubing to a microsyringe. This secondary cannulation of the anterior chamber was carried out approx. 5 min after insertion of the manometer needle in order to minimise the disruption of IOP associated with mechanically inserting needles through the cornea. Drug solutions were injected once IOP had stabilised after the disturbance of piercing the cornea. The time of administration of the drug was designated as zero time.
Measurements of pupil diameter

The effects of a miotic agent on pupil diameter was tested in order to confirm that the isolated bovine perfused eye delivers drug, administered arterially, to the anterior uvea. Pilocarpine causes miosis by directly acting on receptors of the pupillary muscle sphincter (Zimmerman, 1981). The diameter of the pupil was measured externally before, and 90 min after, drug administration, using a Vernier Caliper.

Effects of pilocarpine, apraclonidine and PGF\textsubscript{2a} on IOP

In order to develop further the isolated bovine arterially perfused eye as an \textit{in vitro} model for the study of the mechanisms by which drugs lower IOP, several drugs known for their IOP lowering properties in other species were tested for their effects on IOP. Drug effects on IOP are expressed as changes, compared to control eyes, in the mean slope of the regression line drawn on IOP v time. Microsoft Excel calculates the statistics for a line by using the "least squares" method to calculate a straight line that best fits the data, and returns a slope value (mm H\textsubscript{2}O.min\textsuperscript{-1}) that describes the line.

Pilocarpine

The cholinergic agonist, pilocarpine was administered to the eye in the perfusate at concentrations of $10^{-7}$ M, $10^{-6}$ M, $3 \times 10^{-6}$ M and $5 \times 10^{-6}$ M. The vehicle for pilocarpine was distilled water. The effect of pilocarpine on IOP was also tested in eyes perfused with a solution of Krebs' containing bovine serum albumin (BSA) (1%
Materials and Methods

w/v). The drug was administered in the perfusate at concentrations of $10^{-7}$ M, $10^{-6}$ M, $3 \times 10^{-6}$ M and $10^{-5}$ M. At the optimum flow rate of 2.25 ml min$^{-1}$ approx. 10 min was allowed for the drug to reach the eye due to the flow of the perfusate from the reservoir through a heating coil via connecting rubber tubing. IOP was monitored for 90 min after the approximate time when the drug reached the eye with readings taken every 5 min.

**Apraclonidine**

The $\alpha_2$-adrenergic agonist, apraclonidine was tested for its effects on IOP. The doses used for apraclonidine were 10 nmol, 30 nmol, 100 nmol and 300 nmol. The vehicle for apraclonidine was distilled water. Drug solution or vehicle in volumes of 3 or 10 $\mu$l was injected as bolus doses intra-arterially into the perfusate as described previously, or intracamerally directly into the anterior chamber. IOP was monitored for 90 min after injection with readings taken at 5 min intervals.

**PGF$_{10}$**

The prostaglandin, PGF$_{2\alpha}$ was tested for its effects on IOP when the eye was perfused with Krebs' solution or Krebs' solution containing BSA (1% w/v). The doses used for PGF$_{2\alpha}$ was 20 nmol, 60 nmol, 200 nmol, 600 and 2000 nmol. The vehicle for PGF$_{2\alpha}$ was distilled water. Drug solution or vehicle (in these cases distilled H$_2$O) in volumes of 2 or 6 $\mu$l was injected as bolus doses intra-arterially into the perfusate as described previously. IOP was monitored for 180 min after injection with readings taken at 10 min intervals.
**Measurement of vascular effects**

After drug administration, the magnitude of vascular responses, indicated by changes in perfusion pressure, was measured after exclusion of injection artefacts.

**The effects of selective α-adrenergic antagonists on the vascular response to agonists**

The effects of selective α₁- and α₂-adrenergic antagonists were investigated on (i) the short term (< 10 min) vascular response observed after intra-arterial injection of a bolus dose of agonist and (ii) the sustained vascular response observed after continual intra-arterial perfusion of agonist.

Once a flow rate of 2.25 ml.min⁻¹ had been established and perfusion pressure was constant for a period of 20 min, control dose-response curves to the agonists, apraclonidine (10⁻⁸ - 10⁻⁶ mol) or noradrenaline (10⁻⁹ - 10⁻⁶ mol), were constructed by intra-arterial injection of bolus doses. The effect of the agonists on peak short term vascular perfusion pressure responses was expressed as the percentage increase in basal tone.

(i) Dose-response curves for the agonists were constructed in the presence and absence in the perfusate of the selective α₁-adrenergic antagonist, prazosin (3 x 10⁻⁶ M), or the selective α₂-adrenergic antagonist, yohimbine (3 x 10⁻⁶ M) (30 min pre-incubation). The effect of the agonists on peak short-term vascular perfusion pressure
responses, in the presence or absence of the antagonists was expressed as the percentage increase in basal tone.

(ii). The effect of the selective α₁-adrenergic antagonist, delequamine (gift from Prof. J.C. McGrath), on the sustained vascular response was investigated by perfusing the vascular beds of the isolated bovine eye with Krebs' solution containing apraclonidine at concentrations of $10^{-8}$ M, $3 \times 10^{-8}$ M or $10^{-7}$ M. After adding apraclonidine to the reservoir, 10 min was allowed for the drug to reach the eye. The vascular response to apraclonidine was measured after a further 30 min perfusion (total of 40 min after drug administration). Delequamine was then added to the perfusate at a concentration of $10^{-8}$ M and the effect on vascular response was measured after 40 min. This process was repeated for concentrations of $10^{-7}$ and $10^{-6}$ M delequamine.

The effect of the α₁-adrenergic antagonist, prazosin on the sustained vascular response was also investigated by perfusing the eye with Krebs' solution containing apraclonidine at concentrations of $3 \times 10^{-8}$ M or $10^{-7}$ M. The vascular response was measured after 40 min. Prazosin was added to the perfusate at a concentration of $10^{-8}$ M and the effect on the vascular response was measured after 40 min. This process was repeated for concentrations of $10^{-7}$ and $10^{-6}$ M prazosin.
Sheep eyes

Due to difficulties in the supply of bovine eyes during part of the period of this project, eyes from sheep (ovine) were used as alternatives to bovine. The dissection and cannulation was as described previously except that perfusion flow rates were restricted to a maximum of 1.5 ml.min\(^{-1}\) in order to consistently maintain vascular perfusion pressure between 20 to 60 mm Hg. Pilocarpine, the only drug tested on the ovine eye, was administered, at a concentration of \(10^{-4}\) M, by the addition of the drug to the perfusate reservoir. IOP was monitored for 90 min after the approximate time when the drug reached the eye with readings taken every 5 min.
Testing the integrity of the blood-aqueous barrier of the isolated eye after drug administration

In order to test the integrity of the blood-aqueous barrier after drug administration the perfusate was supplemented with 1% BSA to mimic the osmotic pressure of plasma. At the end of each experiment, samples of AH up to a volume of 1.5 ml were withdrawn from the anterior and posterior chambers of the eye. In order to remove AH the existing cannulation needle for measuring IOP was used, being inserted first behind, then in front of the iris. AH samples from each eye were transferred to Eppendorf tubes for storage and assay. The amount of protein contained in the AH was measured by assay. IOP was measured as described earlier throughout these experiments.

Protein assay

The amount of protein in the AH was determined by the Lowry (1951) method. The protein assay was done using a Dynatech Plate Reader, employing a 96-well plate. The product of this assay, a complex between copper, phenol and protein has a distinct blue colour and absorbs strongly at a wavelength of 750 nm. The intensity of this colour, determined spectrophotometrically, is proportional to the concentration of protein present in the well at low concentrations. At high protein concentrations standard curves determined by the Lowry method cease to remain linear, resulting in a plateau. In order to accurately calculate the concentration of protein present in AH, samples may have to be diluted to fit onto the linear portion of the standard curve.
Standard curve preparation

A blank and 5 protein standards were prepared in Eppendorf tubes using BSA (200 μg.ml⁻¹) and distilled H₂O as follows (Table 3):

Table 3. Preparation of protein standards

<table>
<thead>
<tr>
<th>Tubes</th>
<th>BSA (μl)</th>
<th>Distilled H₂O (μl)</th>
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<tbody>
<tr>
<td>Blank</td>
<td>0</td>
<td>1000</td>
</tr>
<tr>
<td>Standard 1</td>
<td>200</td>
<td>800</td>
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<tr>
<td>Standard 2</td>
<td>400</td>
<td>600</td>
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<td>Standard 3</td>
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<tr>
<td>Standard 5</td>
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</table>

A linear standard curve of absorbance values versus concentration of BSA was calculated to determine protein concentrations in the AH. An example of a linear standard curve is shown in figure 9.
Figure 9. Standard curve for protein concentration. The graph was linear with a correlation coefficient, $r = 0.995$ in the concentration range 0 - 200 $\mu$g.ml$^{-1}$. 
**Plate set-up**

Each plate consists of 96 wells, with each well containing a maximum volume of 300 µl. Each sample was assayed in duplicate. A blank (distilled water) and 5 protein standards were included in the plate design (Figure 10).

**B** - blank

**S** - protein standard (1 - 5)

**T** - AII samples

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<td>T21</td>
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<td>T29</td>
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<td>T37</td>
</tr>
<tr>
<td>D</td>
<td>S3</td>
<td>S3</td>
<td>T6</td>
<td>T6</td>
<td>T14</td>
<td>T14</td>
<td>T22</td>
<td>T22</td>
<td>T30</td>
<td>T30</td>
<td>T38</td>
<td>T38</td>
</tr>
<tr>
<td>E</td>
<td>S4</td>
<td>S4</td>
<td>T7</td>
<td>T7</td>
<td>T15</td>
<td>T15</td>
<td>T23</td>
<td>T23</td>
<td>T31</td>
<td>T31</td>
<td>T39</td>
<td>T39</td>
</tr>
<tr>
<td>F</td>
<td>S5</td>
<td>S5</td>
<td>T8</td>
<td>T8</td>
<td>T16</td>
<td>T16</td>
<td>T24</td>
<td>T24</td>
<td>T32</td>
<td>T32</td>
<td>T40</td>
<td>T40</td>
</tr>
<tr>
<td>G</td>
<td>T1</td>
<td>T1</td>
<td>T9</td>
<td>T9</td>
<td>T17</td>
<td>T17</td>
<td>T25</td>
<td>T25</td>
<td>T33</td>
<td>T33</td>
<td>T41</td>
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<tr>
<td>H</td>
<td>T2</td>
<td>T2</td>
<td>T10</td>
<td>T10</td>
<td>T18</td>
<td>T18</td>
<td>T26</td>
<td>T26</td>
<td>T34</td>
<td>T34</td>
<td>T42</td>
<td>T42</td>
</tr>
</tbody>
</table>

**Figure 10.** The layout of a 96 well plate for spectrophotometric analysis of protein content.
Assay procedure

The following reagent (sol$^A$ A) was prepared:

50 parts 4% (w/v) sodium carbonate dissolved in 0.1N NaOH.
1 part 1% (w/v) potassium tartate dissolved in distilled H$_2$O.
1 part 0.5% (w/v) copper sulphate dissolved in H$_2$O.

55 µl of blank, protein standard or diluted AH sample was added in duplicate to each well in accordance with the plate design.

109 µl of sol$^A$ A was added to each well containing blank, protein standard or AH sample and left for 10 min.

Following this 16 µl of 1N Folin-Ciocalteau reagent was added, mixed thoroughly and allowed to stand for 30 min.

The plate was read at a wavelength of 750 nm using a plate reader.

Calculation of results

The plate reader calculated the average absorbance of the duplicate samples, then subtracted the absorbance readings of the blank wells from the absorbance readings from the standards and samples from rest of the plate. The concentration of protein in each sample was calculated by identifying the absorbance reading on the Y-axis of the standard curve and reading the corresponding value on the X-axis. Any sample dilution factors were taken into account to find the concentration of protein in the AH.
Absorption of aciclovir in the cornea and aqueous humour

The absorption of the antiviral drug, aciclovir was tested across the damaged cornea in the isolated bovine arterially perfused eye. Absorption was tested using three different formulations of aciclovir: (i) a standard ocular ointment, white soft paraffin; (ii) a novel vehicle, polyvinylalcohol (PVA) film; (iii) an experimental aqueous gel. The cornea was damaged both physically, by removing the epithelium, and chemically, by alkali burning, in an attempt to mimic the damage to the cornea associated with herpes simplex virus.

Bovine eyes were dissected, cannulated and perfused as described previously. In contrast to the previous experiments, the anterior chamber was not cannulated as the recording of IOP was not necessary.

Absorption of aciclovir was tested on the undamaged (control), physically damaged and chemically damaged bovine cornea.

Physical damage

Physical damage to the cornea was induced by complete removal of the epithelium exposing the surface of the underlying stroma. A 14 mm diameter circle was marked on the epithelium with a steel trephine 14 mm in diameter. Epithelium within the circle was removed with a corneal scalpel.
Materials and Methods

Chemical damage

Chemical damage to the cornea was induced by burning the cornea with alkali. 14 mm discs of #1 filter paper soaked in 4N NaOH were placed on the cornea for 3 min, after which the filter paper was removed and the cornea was irrigated with 10 ml of 0.9% NaCl solution to wash away any residual NaOH from the cornea. The damaged epithelium, within the 14 mm diameter, was then removed with a corneal scalpel to expose the damaged corneal stroma.

Formulation and application of aciclovir

Three formulations of the anti-viral drug, aciclovir were used to assess drug delivery to the eye across the cornea. All drug formulations were applied carefully to the surface of the cornea within the area of damaged tissue or within a 14 mm diameter area of undamaged tissue for control purposes.

PVA film

PVA is an H2O soluble polymer in which drug can be dissolved and drawn into strips forming a film. This was applied to the cornea as discs 11 mm in diameter. As the amount of drug per cm² of film was known and easily manipulated, the amount of drug applied per disc of film could be easily controlled.
e.g. amount of drug/ cm² of film = 4 mg.

\[
\text{amount of drug/11 mm diameter disc} = \pi r^2 \times 4 \text{ mg} \\
= 0.95 \times 4 \\
= 3.8 \text{ mg}
\]

An example of preparation of PVA film containing aciclovir (20% w/w):

- PVA solution (% PVA w.r.t. H₂O, % aciclovir w.r.t. PVA) containing 85% H₂O, 12% PVA and 3% aciclovir.
- Weigh out the required amount of PVA and H₂O.
- Dissolve the PVA in the H₂O by heating to <95°C, whilst continually mixing and checking the temperature with a thermometer.
- Leave the dissolved solution overnight, allowing air bubbles to escape.
- Weigh out the amount of aciclovir required and add to the PVA solution, mixing carefully and slowly.
- Leave the mixture for 2 - 3 hr, allowing the release of any air trapped during mixing.
- Clean TLC glass plate with alcohol, allow to dry and apply a thin layer of repelcote, leaving to dry for 3 - 4 min.
- Draw mixture into strips along glass plate, allowing to dry overnight.
**White soft paraffin**

Aciclovir ointment (3% w/w) or ‘Zovirax’ is currently used clinically to treat herpes simplex virus in the eye. The vehicle for drug delivery, white soft paraffin has a melting point of 40°C and a density of 0.9 with respect to water (*Merck, 1989*). In order to apply consistent volumes and therefore consistent amounts of drug to the surface of the cornea, the formulation was melted in a water bath at a temperature of approx. 55°C. Exact volumes of the formulation were rapidly withdrawn and applied to the corneal surface using a 50 μl microsyringe before the ointment cooled and resolidified. The amount of drug contained in a known volume of melted white soft paraffin could be easily calculated by converting volume to weight (weight = volume x density). In order to apply a smaller concentration of aciclovir (1% w/w) to the cornea the 3% aciclovir formulation was diluted three fold with additional white soft paraffin by triturations on a slab.

**Aqueous gel**

This proprietary formulation utilises an aqueous gel vehicle, based on carbomer and hydroxyethyl cellulose, containing aciclovir at a concentration of 1% w/w. This formulation of aciclovir was received in unit dose dispensers designed to deliver 30 μl drops containing 300 μg of aciclovir directly onto the surface of the cornea.

**Tissue sampling and extraction of aciclovir from cornea and aqueous humour**

180 min after topical application of the drug to the perfused eye, two separate tissue sampling procedures were conducted.
Firstly, approx. 1.5 ml of AH is removed from the anterior and posterior chambers of the eye using a 23G needle and 2.5 ml syringe. 0.5 ml of the AH was added to 1.5 ml of extraction medium in 3ml vials.

Extraction medium for AH (total volume = 2000 µl):

- 500 µl AH
- 500 µl 0.8 M Perchloric Acid
- 80 µl 1 M Potassium Hydroxide
- 720 µl distilled H₂O
- 200 µl internal standard (ganciclovir, 100 µg.ml⁻¹)

Secondly, the full thickness corneal button 14 mm in diameter was removed using a steel trephine. Cornea was weighed, finely chopped up to increase surface area, then added to 2 ml of extraction medium in 3 ml vials.

Extraction medium for cornea (total vol. = 2000 µl + H₂O content of cornea):

- Finely chopped cornea (approx. 0.2 g)
- 900 µl 0.1 M Hydrochloric Acid
- 900 µl distilled H₂O
- 200 µl internal standard (ganciclovir, 100 µg.ml⁻¹)

Aqueous humour and cornea samples were vortex mixed and left to incubate for 24 hr at room temperature (18 - 24°C). After incubation all samples were stored at -20°C until HPLC analysis.
HPLC analysis

Prior to analysis all frozen samples were defrosted thoroughly, then centrifuged at 10000G for 5 min. The resulting supernatant was sampled for HPLC analysis.

Samples were analysed using a Gilson 712 automated liquid chromatograph comprising a 506 B system interface module, a 412 autosampler, two 306 pumps, a 805 manometric module, 811 C dynamic mixer and 118 UV-vis detector linked to a Viglen 486 PC utilising Gilson 712 software. Reversed phase separations were carried out at room temperature using a 150 x 3.20 mm i.d., 5 µm ODS-hypersil C₁₈ column fitted with a 30 x 3.20 mm, 5 µm ODS-hypersil C₁₈ guard column (Phenomenex, Cheshire, England) and an isocratic mobile phase comprising 20 mM ammonium acetate containing 1.2% acetic acid adjusted to pH 3.6, eluted at a flow rate of 0.5 ml/min. Column eluent was monitored at 254 nm. At this wavelength the limit of detection was 10 ng and a 10 - 200 ng linear calibration curve was obtained for the ganciclovir standard. With corneal and AH samples the homogeneity of the aciclovir and ganciclovir peaks was confirmed by co-chromatography. Absorption peaks and retention times were recorded on the PC and expressed as area under the peaks. The amount of aciclovir detected in each 20 µl sample was calculated by comparison of area under the peak corresponding to aciclovir with area under the peak corresponding to internal standard, ganciclovir. Dilution factors were taken into account in order to calculate the total amount and concentration of drug found in corneal and AH samples.
Materials and Methods

Calculation of concentrations and amounts of aciclovir

The following equations were used to calculate the concentration and amounts of aciclovir found in the cornea and AH after HPLC analysis:

Calculations for concentration of aciclovir per gram of cornea:

\[
[\text{aciclovir}] \, (\mu g \cdot g^{-1}) = \left[ \text{i.s.} \right] \times \frac{\text{area aci.}}{\text{area i.s.}} \times \frac{\text{total vol.}}{\text{tissue weight}},
\]

n.b. total vol. (ml) = vol. of extraction medium + H\textsubscript{2}O content of corneal sample

= 0.2 + (0.78 \times \text{tissue weight}).

Calculations for the total amount of aciclovir per 14 mm corneal button:

\[
\text{amnt. of aci. (ug)} = \left[ \text{i.s.} \right] \times \frac{\text{area aci.}}{\text{area i.s.}} \times \text{total vol.}
\]

Calculations for concentration of aciclovir per ml of AH:

\[
[\text{aciclovir}] \, (\mu g \cdot ml^{-1}) = \left[ \text{i.s.} \right] \times \frac{\text{area aci.}}{\text{area i.s.}} \times \text{dilution factor.}
\]

Dilution factor equals 8 for 0.5 ml AH samples diluted to a total vol. of 2 ml in aqueous extraction medium.

Calculations for total amnt. of aciclovir in the AH (total vol. in eye ~2 ml):

\[
\text{amnt. of aci. (ug)} = \left[ \text{i.s.} \right] \times \frac{\text{area aci.}}{\text{area i.s.}} \times \text{dilution factor} \times 2
\]
The concentration of internal standard present in cornea and AH extraction samples is dependent on the total vol. of the extraction media + sample. AH samples have a total volume of 2 ml, containing 0.2 ml of i.s. (100 μg.ml⁻¹), resulting in a ten fold dilution of i.s., and a final conc. in AH extraction medium of 10 μg.ml⁻¹. Corneal samples have a greater total volume due to H₂O content of the cornea, therefore the final concentration of i.s. is slightly less than 10 μg.ml⁻¹ (see equation below).

conc. of i.s. in corneal samples = vol. of i.s./total vol. x conc. of i.s.

= 0.2 / (2 + (0.78 x tissue weight)) x 100.

**Statistical analysis of data**

Results are expressed as the mean ± SEM of n experiments (n = number of individual eyes tested). Statistical comparisons were generally made by Student’s unpaired t-tests for IOP, protein concentration and corneal absorption data. Statistical comparisons for dose-response and concentration-response curves were made by one-way analysis of variance followed by the Bonferroni post-test. A value of p<0.05 was considered significant. Graphical representations and statistical analysis were performed using a computer-based programme (Graph Pad, Prism).
**Procurement and disposal of bovine and ovine eyes**

Bovine and ovine eyes obtained from a local abattoir were transported to the laboratory in a labelled container in accordance with the UK 'specified bovine material order 1997' and 'the heads of sheep and goats order 1996' (see Appendix IV). Material that was no longer required was stained with patent blue V colouring agent (0.5% w/v) and incinerated. A record of the weight of the specified material, place of origin, date of consignment, method and place of disposal shall be kept for 2 years.
RESULTS
THE EFFECTS OF DRUGS ON IOP AND BLOOD-AQUEOUS BARRIER INTEGRITY OF THE BOVINE EYE

Steady-state IOP

After cannulation of the anterior chamber, the IOP usually stabilised at a value between 95 and 160 mm H₂O. The steady-state or mean starting IOP was recorded at t = 0, prior to drug or vehicle administration, for eyes perfused with Krebs' solution in the presence or absence of 1% BSA (Table 4). Drugs were administered to the eye by intra-arterial or intracameral injection. Intracameral injection of drugs directly into the anterior chamber involves introducing a second needle through the cornea. The effect on steady-state IOP of piercing the cornea with that second needle is also shown in table 4, using data from all eyes tested (i.e., those destined to be injected with either a drug or vehicle only). The data in table 4 also indicates that inclusion of BSA in the Krebs' solution had no significant effect on the starting IOP value at t = 0 when the cornea was pierced with one needle only. Insertion of a second corneal needle caused a significantly higher starting IOP when perfusion was with Krebs' solution alone, whereas this manoeuvre appeared to have the opposite effect when BSA was included in the perfusate.
Table 4. Steady-state IOP values for eyes perfused with Krebs' solution or Krebs' solution plus BSA (1% w/v).

<table>
<thead>
<tr>
<th>Perfusate</th>
<th>Route for drug administration</th>
<th>n</th>
<th>IOP (mm H$_2$O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Krebs'</td>
<td>Intra-arterial</td>
<td>155</td>
<td>131.60 ± 1.19</td>
</tr>
<tr>
<td>Krebs'</td>
<td>Intracameral</td>
<td>49</td>
<td>136.43 ± 2.05*</td>
</tr>
<tr>
<td>Krebs' + BSA</td>
<td>Intra-arterial</td>
<td>138</td>
<td>126.71 ± 1.34</td>
</tr>
<tr>
<td>Krebs' - BSA</td>
<td>Intracameral</td>
<td>40</td>
<td>118.10 ± 2.13***</td>
</tr>
</tbody>
</table>

Steady-state values for IOP, at t = 0, from eyes perfused with Krebs' solution or Krebs' + BSA (1% w/v). All pressures (mm H$_2$O) are means ± SEM of the number of eyes shown (n). To convert IOP from mm H$_2$O to mm Hg, divide the value obtained from the water manometer by 13.546, the specific gravity of mercury (specific gravity H$_2$O = 1). Significance of differences (Student's unpaired t-test) among eyes prepared for intra-arterial vs intracameral drug administration: * 0.01 < p < 0.05, *** p < 0.001.
A histogram showing the range of steady-state or mean starting IOP values (t = 0) for eyes perfused with Krebs' solution or Krebs' solution + BSA (1% w/v) is shown in figure 11. The average starting IOP (mean ± S.D.) value for 204 eyes perfused with Krebs' solution alone was 132.76 ± 14.77 mm H₂O. When BSA was included in the perfusate the average starting IOP value for 178 eyes was 124.78 ± 15.60 mm H₂O. Conversion of IOP from mm H₂O to mm Hg, divide the value obtained from the water manometer by 13.546, the specific gravity of mercury (specific gravity H₂O = 1).
The effect of different vehicles and routes of administration on the IOP of eyes arterially perfused for control purposes

Variation in the IOP of the isolated perfused eye was tested using two alternative vehicles for drug administration, distilled H₂O or sodium bicarbonate solution (10 mg.ml⁻¹), injected via the intra-arterial or intracamereral route. Eyes were perfused with Krebs’ solution or Krebs’ solution plus BSA (1% w/v). Change in the vehicle or route of administration was not found to significantly alter IOP. Table 5 shows the results in terms of mean slopes for all control groups.

The effect on IOP of including BSA (1% w/v) in the perfusate was shown by comparing the mean slopes for all control eyes perfused with Krebs’ solution and all control eyes perfused with Krebs’ solution plus BSA. It was found that inclusion of BSA in the perfusate did not significantly affect IOP (Table 6).

Figure 12 shows the representative graphs of the mean IOP recordings at each time point in control eyes perfused with Krebs’ solution or Krebs’ solution plus BSA. Mean IOP values from -15 to 0 min show the steady-state pressure with fluctuations less than ±2 mm H₂O over this period. From 0 to 90 min changes in IOP occur in a linear manner allowing calculation of the slope value to be made.
Table 5. The effects of the vehicle and route of vehicle administration on control IOP responses.

<table>
<thead>
<tr>
<th>Perfusate</th>
<th>Vehicle</th>
<th>Volume (µl)</th>
<th>Route</th>
<th>n</th>
<th>Slope (mm H₂O/min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Krebs' nil i.a.</td>
<td>nil</td>
<td>24</td>
<td></td>
<td></td>
<td>-0.05 ± 0.02</td>
</tr>
<tr>
<td>Krebs' H₂O 6 i.a.</td>
<td>15</td>
<td>-0.02 ± 0.03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Krebs' H₂O 10 i.a.</td>
<td>20</td>
<td>-0.03 ± 0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Krebs' H₂O 10 i.e.</td>
<td>14</td>
<td>-0.04 ± 0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Krebs' + BSA nil i.a.</td>
<td>26</td>
<td>-0.02 ± 0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Krebs' + BSA H₂O 6 i.a.</td>
<td>8</td>
<td>-0.05 ± 0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Krebs' + BSA NaHCO₃ soln 6 i.a.</td>
<td>6</td>
<td>-0.01 ± 0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Krebs' + BSA NaHCO₃ soln 6 i.e.</td>
<td>8</td>
<td>-0.06 ± 0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The effects of the vehicle and route of vehicle administration on control IOP responses in the bovine isolated eye perfused with Krebs' solution or Krebs' solution plus BSA (1% w/v) shown as the mean slope (mm H₂O.min⁻¹) of the regression line drawn on IOP vs time (90 min). Each value is a mean ± SEM of the number of experiments shown (n). Significance of difference (Student's unpaired t-test) from control eyes perfused with Krebs' solution or Krebs' solution plus BSA: * not significant.
Table 6. The effect on control IOP responses of including BSA (1% w/v) in the perfusate.

<table>
<thead>
<tr>
<th>Perfusate</th>
<th>n</th>
<th>Slope (mm H₂O.min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Krebs'</td>
<td>73</td>
<td>-0.04 ± 0.01</td>
</tr>
<tr>
<td>Krebs' + BSA (1% w/v)</td>
<td>48</td>
<td>-0.03 ± 0.01</td>
</tr>
</tbody>
</table>

The effects of perfusion with Krebs' solution or Krebs' solution plus BSA (1% w/v) on IOP in control eyes, shown as the mean slope (mm H₂O.min⁻¹) of the regression line drawn on IOP vs time (90 min). Each value is a mean ± SEM of the number of experiments shown (n). Significance of difference (Student's unpaired t-test) from control eyes perfused with Krebs' solution or Krebs' solution plus BSA: "ns" not significant.
Figure 12. The changes in control IOP responses of eyes perfused with a) Krebs' solution or b) Krebs' solution plus BSA (1% w/v). The data is expressed as the mean ± SEM at each time point for >20 eyes.
Results: The Effects of Drugs on IOP...

a)  

IOP (mm H₂O)

175 - 150 - 125 - 100 - 75 - 50 - 25 - 0

Time (min)

-10 0 10 20 30 40 50 60 70 80 90

b)  

IOP (mm H₂O)

175 - 150 - 125 - 100 - 75 - 50 - 25 - 0

Time (min)

-10 0 10 20 30 40 50 60 70 80 90
Effect of pilocarpine on pupil size

Drug delivery to the anterior uvea of the isolated bovine eye was tested by measurement of the pupil diameter before and after 90 min of intra-arterial perfusion of pilocarpine (10^6 M). Pilocarpine was found to significantly reduce the diameter of the pupil. The results are shown in table 7.

**Table 7. The effects of pilocarpine (10^6 M) on pupil diameter.**

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Pupil size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before pilocarpine</td>
<td>5</td>
<td>16.5 ± 0.6</td>
</tr>
<tr>
<td>After pilocarpine</td>
<td>5</td>
<td>13.1 ± 0.2**</td>
</tr>
</tbody>
</table>

Each eye was perfused with Krebs’ solution at a flow rate of 2.25 ml.min⁻¹ for 20 min before the diameter (mm) of the pupil was measured using a Vernier calliper. Each eye was then perfused for 90 min with Krebs’ solution containing pilocarpine (10^6 M) before the pupil diameter was measured again. Each value is a mean ± SEM of the number of experiments shown (n). Significance of difference (Student’s unpaired t-test): ** 0.001 < p < 0.01.
The effect of pilocarpine on IOP

The effect of pilocarpine on IOP in the bovine isolated eye perfused with Krebs' solution was studied over a period of 90 min. Table 8a shows the results in terms of mean slopes for treated and control groups.

Unexpectedly pilocarpine caused a significant increase in IOP at all concentrations tested (3 x 10^-1 - 5 x 10^-8 M). These results suggested that pilocarpine might be increasing IOP by disrupting the integrity of the BAB. In order to test this the effects of pilocarpine were studied on eyes which were perfused with Krebs' plus BSA (1% w/v). IOP was recorded for 90 min as before and AH was sampled at the end of the experiment to determine whether protein had leaked from the vascular compartment.

After including BSA (1% w/v) in the perfusate IOP was found to be reduced significantly by three concentrations of pilocarpine, 10^-5 M, 3 x 10^-5 M and 10^-4 M. At 3 x 10^-7 M there was no significant change in IOP. The results are shown in table 8b.
Table 8. The effect of pilocarpine on IOP.

**a) eyes perfused with Krebs' solution**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Conc. (M)</th>
<th>n</th>
<th>Slope (mm H₂O.min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>24</td>
<td>-0.06 ± 0.02</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>3 x 10⁻⁷</td>
<td>6</td>
<td>0.16 ± 0.03***</td>
</tr>
<tr>
<td></td>
<td>10⁻⁶</td>
<td>12</td>
<td>0.01 ± 0.04**</td>
</tr>
<tr>
<td></td>
<td>3 x 10⁻⁶</td>
<td>6</td>
<td>0.35 ± 0.05***</td>
</tr>
<tr>
<td></td>
<td>5 x 10⁻⁵</td>
<td>6</td>
<td>0.23 ± 0.08***</td>
</tr>
</tbody>
</table>

**b) eyes perfused with Krebs' solution containing BSA (1% w/v)**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Conc. (M)</th>
<th>n</th>
<th>Slope (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>26</td>
<td>-0.02 ± 0.01</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>3 x 10⁻⁷</td>
<td>10</td>
<td>-0.03 ± 0.04**</td>
</tr>
<tr>
<td></td>
<td>10⁻⁶</td>
<td>8</td>
<td>-0.16 ± 0.02***</td>
</tr>
<tr>
<td></td>
<td>3 x 10⁻⁶</td>
<td>16</td>
<td>-0.10 ± 0.02**</td>
</tr>
<tr>
<td></td>
<td>10⁻⁵</td>
<td>12</td>
<td>-0.08 ± 0.02*</td>
</tr>
</tbody>
</table>

The effects of pilocarpine on IOP in the bovine isolated eye perfused with a) Krebs' solution and b) Krebs' solution plus BSA (1% w/v), shown as the mean slope (mm H₂O.min⁻¹) of the regression line drawn on IOP vs time from 20 - 90 min after drug administration. Each value is a mean ± SEM of the number of experiments shown (n). Significance of differences (Student's unpaired t-test) from control: "n" not significant; * 0.01 < p < 0.05; ** 0.001 < p < 0.01; *** p < 0.001.
The effect of pilocarpine on protein concentration in the aqueous humour

The effects of pilocarpine on the concentration of protein in the AH are shown in table 9. At $3 \times 10^{-7}$ M, $10^{-6}$ M and $3 \times 10^{-6}$ M pilocarpine there was no significant change in protein concentration. However at $10^{-5}$ M there was a significant reduction in protein concentration.

Table 9. Concentration of protein in the AH after perfusion with pilocarpine.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Conc. (M)</th>
<th>n</th>
<th>[protein] (µg.ml$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>19</td>
<td>$814.47 \pm 97.41$</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>$3 \times 10^{-7}$</td>
<td>10</td>
<td>$914.70 \pm 64.23^{**}$</td>
</tr>
<tr>
<td></td>
<td>$10^{-6}$</td>
<td>8</td>
<td>$581.63 \pm 30.72^{**}$</td>
</tr>
<tr>
<td></td>
<td>$3 \times 10^{-6}$</td>
<td>10</td>
<td>$628.00 \pm 78.15^{**}$</td>
</tr>
<tr>
<td></td>
<td>$10^{-5}$</td>
<td>9</td>
<td>$410.00 \pm 51.95^{*}$</td>
</tr>
</tbody>
</table>

Effects of pilocarpine on protein concentration in the AH, sampled 90 min after commencing drug perfusion, shown as µg of protein per ml of AH. Each value is mean ± SEM of the number (n) of experiments shown. Significance of difference (Students unpaired t-test) from control: ** not significant, * 0.01 < p < 0.05.
The effect of pilocarpine on IOP in the ovine eye

Pilocarpine ($10^{-5}$ M) had no effect on IOP in the ovine isolated eye perfused with Krebs' solution (Table 10).

Table 10. The effect of pilocarpine on IOP in the ovine eye.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Conc. (M)</th>
<th>n</th>
<th>Slope (mm H$_2$O.min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>6</td>
<td>$0.03 \pm 0.02$</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>$10^{-5}$ M</td>
<td>10</td>
<td>$-0.01 \pm 0.02$ ***</td>
</tr>
</tbody>
</table>

The effects of pilocarpine on IOP in the ovine isolated eye perfused with Krebs' solution, shown as the mean slope (mm H$_2$O.min$^{-1}$) of the regression line drawn on IOP vs time from 20 - 90 min after drug administration. Each value is a mean ± SEM of the number of experiments shown (n). Significance of differences (Student's unpaired t-test) from control: *** not significant.
The effect on IOP of apraclonidine injected intra-arterially.

The effects on IOP of four intra-arterial bolus doses, 10, 30, 100 or 300 nmol of apraclonidine, were studied for a period of 90 min. As the change in IOP in response to apraclonidine injection was non-linear over the 90 min period, regression lines were calculated over periods of linearity (Figure 13). This resulted in two slope values corresponding to the initial changes in IOP from 0 - 30 min after injection (Table 11a) and the delayed changes from 30 - 90 min after injection (Table 11b). 10 and 30 nmol doses of apraclonidine do not show a significant initial or delayed effect on IOP, whereas 100 and 300 nmol doses significantly increase the initial and delayed IOP response.
Figure 13. The change in IOP in response to a) intra-arterial injection and b) intracameral injection of 100 nmol bolus doses of apraclonidine. The data is expressed as the mean IOP at each time point for 8 eyes.
Results: The Effects of Drugs on IOP...

a) 175-
150
125
100
0
10 20 30 40 50 60 70 80 90
Time (min)

100 nmol apra (i.a.)

b) 175-
150
125
100
0
10 20 30 40 50 60 70 80 90
Time (min)

100 nmol apra (i.o.)
Table 11. The effect on IOP of apraclonidine injected intra-arterially.

a) Slope calculated from 0 - 30 min after drug injection

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (nmol)</th>
<th>n</th>
<th>Slope (mm H_2O.min^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>20</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>Apraclonidine</td>
<td>10</td>
<td>8</td>
<td>0.08 ± 0.03*</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>9</td>
<td>0.03 ± 0.05*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>8</td>
<td>0.26 ± 0.12*</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>8</td>
<td>0.26 ± 0.06***</td>
</tr>
</tbody>
</table>

b) Slope calculated from 30 - 90 min after drug injection

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (nmol)</th>
<th>n</th>
<th>Slope (mm H_2O.min^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>20</td>
<td>-0.04 ± 0.01</td>
</tr>
<tr>
<td>Apraclonidine</td>
<td>10</td>
<td>8</td>
<td>-0.00 ± 0.01*</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>9</td>
<td>-0.07 ± 0.03*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>8</td>
<td>0.03 ± 0.03**</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>8</td>
<td>0.19 ± 0.06***</td>
</tr>
</tbody>
</table>

The effects of bolus doses of apraclonidine injected intra-arterially on IOP in the bovine isolated perfused eye, shown as the mean slope (mm H_2O.min^-1) of the regression line drawn on IOP vs time; a). 0 - 30 min, b). 30 - 90 min. Each value is a mean ± SEM of the number of experiments shown (n). Significance of difference (Student’s unpaired t-test) from control (intra-arterial injections of 10 μl of H_2O): * not significant; * 0.01 < p < 0.05; ** 0.001 < p < 0.01; *** p < 0.001.
The effect of apraclonidine on protein concentration in the aqueous humour

Intra-arterial injection of a single bolus dose of apraclonidine (300 nmol) had no significant effect on the protein concentration of the AH. Aqueous samples taken 90 min after the injection of 300 nmol apraclonidine in 10 eyes contained protein at a concentration of $910.62 \pm 168.23 \, \mu g.mL^{-1}$ compared to a protein concentration of $814.47 \pm 97.41 \, \mu g.mL^{-1}$ for 19 control eyes. Statistical analysis was performed using a Student’s t-test for unpaired data.
The effect on IOP of apraclonidine injected intracamerally

The effects on IOP of four intracameral bolus doses, 10, 30, 100 or 300 nmol of apraclonidine, were studied for a period of 90 min. Changes in IOP over the 90 min period indicate linearity up to 30 min after drug injection and from 30 - 90 min after drug injection (Figure 13). A small but significant reduction in IOP was observed for the first 30 min after injection of the 30 nmol dose (Table 12a). No significant change in IOP was observed over this period for 10, 100 or 300 nmol doses. IOP was shown to significantly increase during the period 30 - 90 min after injection of 100 or 300 nmol doses (Table 12b). No significant change in IOP was shown for 10 or 30 nmol doses.
Table 12. The effect on IOP of apraclonidine injected intracamerally.

a) Slopes calculated from 0 - 30 min after drug injection

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (nmol)</th>
<th>n</th>
<th>Slope (mm H₂O.min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>14</td>
<td>0.05 ± 0.03</td>
</tr>
<tr>
<td>Apraclonidine</td>
<td>10</td>
<td>8</td>
<td>0.04 ± 0.02*</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>8</td>
<td>-0.06 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>8</td>
<td>0.04 ± 0.04*</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>6</td>
<td>0.08 ± 0.04*</td>
</tr>
</tbody>
</table>

b) Slopes calculated from 30 - 90 min after drug injection

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (nmol)</th>
<th>N</th>
<th>Slope (mm H₂O.min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>14</td>
<td>-0.08 ± 0.02</td>
</tr>
<tr>
<td>Apraclonidine</td>
<td>10</td>
<td>8</td>
<td>0.01 ± 0.02*</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>8</td>
<td>-0.13 ± 0.03*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>8</td>
<td>0.02 ± 0.03*</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>6</td>
<td>0.08 ± 0.05*</td>
</tr>
</tbody>
</table>

The effects on IOP of bolus doses of apraclonidine injected intracamerally in the bovine isolated perfused eye, shown as the mean slope (mm H₂O.min⁻¹) of the regression line drawn on IOP vs time; a) 0 - 30 min, b) 30 - 90 min. Each value is a mean ± SEM of the number of experiments shown (n). Significance of differences (Student’s unpaired t-test) from control (intracameral injections of 10 µl of H₂O): * not significant; * 0.01 < p < 0.05.
The effect of apraclonidine on vascular perfusion pressure

Intra-arterial injection of bolus doses of apraclonidine resulted in a dose-dependent increase in vascular perfusion pressure, characterised by an initial rapid increase in pressure, reaching a peak within 5 min post-injection. After the peak response the pressure decreased to stabilise at a level above the pre-injection pressure. Figure 14 shows examples of the vascular response to intra-arterial injection of apraclonidine. After excluding the injection artefact, characterised by a slight increase in perfusion pressure (< 5 mm Hg) followed by an immediate return to the basal level, intra-arterial injection of the drug vehicle, <10 μl distilled H₂O, did not alter vascular perfusion pressure.

Figure 14. Examples of changes in vascular perfusion pressure after intra-arterial injection of a) 30 nmol and b) 100 nmol of apraclonidine.
The effect of $\alpha$-adrenergic antagonists on the short term vascular response to apraclonidine and noradrenaline

Intra-arterial injection of bolus doses of the control agonists, apraclonidine (10 nmol - 1 μmol) and noradrenaline (10 nmol - 1 μmol) increased vascular perfusion pressure in a dose-dependent manner (Figure 15).

In the repeated experiments the presence of the $\alpha_2$-adrenergic antagonist, yohimbine ($3 \times 10^{-6}$ M) significantly decreased the vascular responses to apraclonidine (Figure 16a), but failed to affect the responses to noradrenaline (Figure 16b).

In the repeated experiments the presence of the $\alpha_1$-adrenergic antagonist, prazosin ($3 \times 10^{-6}$ M) significantly decreased the vascular responses to apraclonidine (Figure 17a) and higher doses of noradrenaline (Figure 17b).

It was noted that there were slight variations in the control responses to the agonists, apraclonidine and noradrenaline (Figure 15, 16 & 17), due to different populations of eyes.
Figure 15. Log dose-response curve following intra-arterial injection of bolus doses of the control agonists a) apraclonidine and b) noradrenaline. Each data point represents the mean ± SEM of >20 eyes.
Results: The Effects of Drugs on IOP...

(a) Effect of apraclonidine on IOP.

(b) Effect of noradrenaline on IOP.

% increase in basal tone

Log dose of agonist (mol)
Figure 16. Agonist dose-response curve in the presence and absence of yohimbine (3 x 10^-6 M) following intra-arterial injection of bolus doses of the agonists a) apraclonidine and b) noradrenaline. Each data point represents the mean ± SEM of > 10 eyes.

*** p < 0.001; ** 0.001 < p < 0.01: significance of difference between control and yohimbine pre-treated eyes (ANOVA, followed by Bonferroni analysis).
Results: The Effects of Drugs on IOP...

a) Graph showing the percentage increase in basal tone for apraclonidine and apraclonidine + yohimbine (3x10^-6 M).

b) Graph showing the percentage increase in basal tone for noradrenaline and noradrenaline + yohimbine (3x10^-6 M).
Figure 17. Agonist dose-response curve in the presence and absence of prazosin (3 x 10^{-6} M) following intra-arterial injection of bolus doses of the agonists a) apraclonidine and b) noradrenaline. Each data point represents the mean ± SEM of > 9 eyes.

*** p< 0.001; ** 0.001 < p < 0.01; * 0.01 < p < 0.05: significance of difference between control and prazosin pre-treated eyes (ANOVA, followed by Bonferroni analysis).
Results: The Effects of Drugs on IOP....

a) 

![Graph showing the effects of apraclonidine and apra. + prazosin (3x10^-6M) on the log dose of agonist (mol) vs. % increase in basal tone.]

- **apraclonidine**
- **apra. + prazosin (3x10^-6M)**

b) 

![Graph showing the effects of noradrenaline and NA + prazosin (3x10^-9M) on the log dose of agonist (mol) vs. % increase in basal tone.]

- **noradrenaline**
- **NA + prazosin (3x10^-9M)**
The effect of α-adrenergic antagonists on the sustained vascular response to apraclonidine

Perfusion of apraclonidine (10⁻⁴ - 10⁻³ M) produced a sustained increase in vascular perfusion pressure, represented by a "bell-shaped" curve (Figure 18).

The selective α₂-adrenergic antagonist, delequamine (10⁻⁶ and 10⁻⁵ M) did not affect the sustained vascular response to apraclonidine. Although delequamine (10⁻⁶ M) decreased the sustained vascular response to apraclonidine, this effect was not statistically significant.

Prazosin (10⁻⁵ - 10⁻⁶ M) had no effect on the sustained vascular responses to 3 x 10⁻⁴ or 10⁻³ M apraclonidine.
Figure 18. Log concentration-response curves showing the effect of delequamine (10^6 - 10^8 M) on contraction to apraclonidine (10^8 - 10^9 M) in the vascular beds of the bovine perfused eye. Each data point represents the mean ± SEM of > 8 eyes.
Figure 18. Log concentration-response curves showing the effect of delequamine ($10^{-8} - 10^{-6}$ M) on contraction to apraclonidine ($10^{-8} - 10^{-7}$ M) in the vascular beds of the bovine perfused eye. Each data point represents the mean ± SEM of > 8 eyes.
The effect of PGF$_{2\alpha}$ on IOP

The effects of five intra-arterially administered bolus doses, 20, 60, 200, 600 or 2000 nmol of PGF$_{2\alpha}$, on IOP in eyes perfused with Krebs' solution, were studied for a period of 180 min. All five doses failed to significantly influence IOP compared to control groups (Table 13a).

After the inclusion of BSA (1% w/v) in the perfusate, intra-arterial injection of 20, 200, 600 or 2000 nmol doses of PGF$_{2\alpha}$ did not significantly change IOP (Table 13b).
Table 13. Effect of PGF<sub>2α</sub> on the IOP

### a) eyes perfused with Krebs' solution

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (nmol)</th>
<th>N</th>
<th>Slope (mm H&lt;sub&gt;2&lt;/sub&gt;O.min&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>15</td>
<td>-0.02 ± 0.03</td>
</tr>
<tr>
<td>PGF&lt;sub&gt;2α&lt;/sub&gt;</td>
<td>20</td>
<td>7</td>
<td>-0.01 ± 0.03&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>8</td>
<td>0.05 ± 0.02&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>6</td>
<td>-0.01 ± 0.02&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>7</td>
<td>-0.07 ± 0.02&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>7</td>
<td>-0.03 ± 0.02&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

### b) eyes perfused with Krebs' solution containing BSA (1% w/v)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (nmol)</th>
<th>n</th>
<th>Slope (mm H&lt;sub&gt;2&lt;/sub&gt;O.min&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>8</td>
<td>-0.05 ± 0.01</td>
</tr>
<tr>
<td>PGF&lt;sub&gt;2α&lt;/sub&gt;</td>
<td>20</td>
<td>8</td>
<td>-0.12 ± 0.03&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>6</td>
<td>-0.03 ± 0.02&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>6</td>
<td>-0.08 ± 0.04&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>6</td>
<td>-0.07 ± 0.03&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The effects of bolus doses of PGF<sub>2α</sub> injected intra-arterially, on IOP in the bovine isolated eye perfused with a) Krebs' solution, b) Krebs' solution plus BSA (1% w/v), shown as the mean slope (mm H<sub>2</sub>O.min<sup>-1</sup>) of the regression line drawn of IOP vs time (180 min). Each value is a mean ± SEM of the number of experiments shown (n). Significance of differences (Student's unpaired t-test): * not significant.
**The effect of prostaglandins on the integrity of the blood-aqueous barrier**

The effect of intra-arterial (Table 14a.) and intracameral (Table 14b.) injection of 600 nmol of PGF$_2$α on the integrity of the BAB (expressed as changes in AH protein concentration) is shown overleaf. No significant change was observed in the protein concentration of the AH for PGF$_2$α treated eyes compared to control eyes.

The effect of intra-arterial (Table 14a.) or intracameral (Table 14b.) injection of PGE$_2$ on the integrity of the BAB was tested. Intra-arterial injection of 60 nmol PGE$_2$ significantly increased the concentration of protein detected in the AH. Paradoxically, the small increases in protein concentration detected after intra-arterial injections of 200 or 600 nmol PGE$_2$ were not statistically significant. Intracameral injection of 600 nmol PGE$_2$ significantly increased AH protein concentration. Smaller increases in AH protein concentration found following intracameral injection of 60 or 200 nmol PGE$_2$ were not statistically significant.
Table 14. The effect of prostaglandins on protein concentration in the aqueous humour.

a) intra-arterial drug administration

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (nmol)</th>
<th>n</th>
<th>[protein] (µg.ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>6</td>
<td>690 ± 63.4</td>
</tr>
<tr>
<td>PGF₃₀</td>
<td>600</td>
<td>6</td>
<td>769 ± 102.1</td>
</tr>
<tr>
<td>PGE₂</td>
<td>60</td>
<td>6</td>
<td>2490 ± 716.5</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>8</td>
<td>1140 ± 415.1</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>9</td>
<td>1556 ± 555.6</td>
</tr>
</tbody>
</table>

b) intracameral drug administration

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (nmol)</th>
<th>n</th>
<th>[protein] (µg.ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>5</td>
<td>652 ± 50.8</td>
</tr>
<tr>
<td>PGF₃₀</td>
<td>600</td>
<td>6</td>
<td>698 ± 87.4</td>
</tr>
<tr>
<td>PGE₂</td>
<td>60</td>
<td>5</td>
<td>1234 ± 574.8</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>13</td>
<td>835 ± 413.2</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>10</td>
<td>3490 ± 156.8</td>
</tr>
</tbody>
</table>

Effects of prostaglandins injected a) intra-arterially and b) intracameraly on protein concentration, shown as µg of protein per ml of AH in eyes perfused with Krebs' solution plus BSA (1% w/v). AH was sampled 180 min after commencing drug perfusion. Each value is mean ± SEM of the number (n) of experiments shown. Significance of difference (Students unpaired t-test) from control; * not significant; * 0.01 < p < 0.05; ** p < 0.001.
The effect of PGE\textsubscript{2} on IOP

The effect of PGE\textsubscript{2} on IOP cannot be expressed as the mean slopes for all eyes tested as these increases in IOP usually occurred in a non-linear manner and often continued above 300 mm H\textsubscript{2}O (the maximum value which could be registered on the water manometer). When these data for IOP are expressed as a function of the concentration of protein detected in the AH (Figure 19), there was no correlation between these parameters.

Inspection of the raw data indicates that while a substantial number of eyes suffer an apparent breakdown of the BAB, many others do not show a rapid IOP increase. In an effort to analyse the data for the latter population it was felt necessary to adopt an arbitrary criterion: those eyes which showed a rise in IOP > 0.5 mm H\textsubscript{2}O min\textsuperscript{-1} at 90 min after drug administration were rejected. For the initial 90 min period after drug administration, 1 eye was rejected following intra-arterial injection (Table 15a.) and 8 eyes were rejected following intracameral injection (Table 15b.). Calculation of the mean slope for the remaining eyes from 10 - 90 min following injection of the drug showed that PGE\textsubscript{2} has no significant effect on IOP (Table 15). Over the subsequent 90 min further eyes showed this rapid IOP response (Table 15).
Figure 19. The effect of a) intra-arterial and b) intracameral injections of PGE<sub>2</sub> on the protein concentration of the AH. AH samples were removed from the eye 180 min after drug injection. Each point represents an individual experiment. The x-axis represents the IOP expressed as mm H<sub>2</sub>O. Values exceeding 300 mm H<sub>2</sub>O are shown as 300 mm H<sub>2</sub>O. The y-axis represents the concentration of protein detected in samples of AH expressed as μg per ml of AH.
Results: The Effects of Drugs on IOP...

a.

![Graph a](image_a)

b.

![Graph b](image_b)
Table 15. The effect of PGE₂ on IOP, plus the number of eyes showing signs of breakdown in the BAB.

a) intra-arterial drug administration

<table>
<thead>
<tr>
<th>Dose (nmol)</th>
<th>Total No. of Eyes</th>
<th>Slope (10 - 90 min) (mm H₂O.min⁻¹)</th>
<th>No. of eyes showing an IOP response suggesting breakdown of the BAB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0 - 90 min</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>-0.01 ± 0.01 (6)</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>6</td>
<td>0.00 ± 0.05 (5)</td>
<td>1</td>
</tr>
<tr>
<td>200</td>
<td>8</td>
<td>-0.09 ± 0.03 (8)</td>
<td>0</td>
</tr>
<tr>
<td>600</td>
<td>9</td>
<td>-0.02 ± 0.04 (9)</td>
<td>0</td>
</tr>
</tbody>
</table>

b) intracameral drug administration

<table>
<thead>
<tr>
<th>Dose (nmol)</th>
<th>Total No. of Eyes</th>
<th>Slope (10 - 90 min) (mm H₂O.min⁻¹)</th>
<th>No. of eyes showing an IOP response suggesting breakdown of the BAB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0 - 90 min</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>-0.07 ± 0.02 (8)</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>5</td>
<td>-0.04 ± 0.03 (4)</td>
<td>1</td>
</tr>
<tr>
<td>200</td>
<td>13</td>
<td>-0.05 ± 0.02 (12)</td>
<td>1</td>
</tr>
<tr>
<td>600</td>
<td>11</td>
<td>0.01 ± 0.02 (5)</td>
<td>6</td>
</tr>
</tbody>
</table>

The effects of bolus doses of PGE₂ administered by a) intra-arterial or b) intracameral injection, on IOP, shown as the mean slope (mm H₂O.min⁻¹) of the regression line drawn of IOP vs time (10 - 90 min), for eyes which meet the criterion explained in the text. Each value is a mean ± SEM of the number of experiments shown (n). Column 2 shows the total number of experiments conducted. Columns 4 and 5 show the number of eyes that indicate an apparent breakdown of the BAB at 90 and 180 min following drug administration respectively.
Results: Absorption of Aciclovir....

**Absorption of Aciclovir in the Bovine Perfused Eye**

**HPLC Analysis**

Under the chromatographic conditions described in the methods, the internal standard, ganciclovir, was identified with a retention time of approx. 8 min (Figure 20a). Aciclovir in the cornea and AH was identified with a retention time of approx. 10.8 min (Figure 20b and c). The calibration graph for ganciclovir, obtained by the peak-area method, was linear with a correlation coefficient, $r = 0.999$ in the range 10-200 ng (Figure 21). The limit of detection of aciclovir in the cornea and AH was calculated from the limit of detection of ganciclovir per 20 μl injection. The limit of detection of aciclovir in corneal buttons varied according to the pre-treatment of the cornea (Table 16). The limit of detection in the AH was 2 μg/ml.

**Table 16. Limit of detection of aciclovir in the cornea.**

<table>
<thead>
<tr>
<th>Corneal pre-treatment</th>
<th>Limit of detection of aciclovir (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undamaged</td>
<td>1.056</td>
</tr>
<tr>
<td>Physically damaged</td>
<td>1.053</td>
</tr>
<tr>
<td>Chemically damaged</td>
<td>1.083</td>
</tr>
</tbody>
</table>

The limit of detection for aciclovir in 14 mm diameter full-thickness corneal buttons excised from pre-treated corneas.
Results: Absorption of Aciclovir.

Figure 20. Sample chromatograms following isocratic reversed phase HPLC analysis of: a) the internal standard, ganciclovir; b) a corneal sample; and c) an AH sample. Samples of cornea and AH were taken 180 minutes after application of 3% film formulation to the physically damaged cornea. U = unidentified peaks characteristic of b) corneal samples and c) AH samples. G = ganciclovir. A = aciclovir.
Figure 21. The calibration curve of ganciclovir following injection of 10, 20, 100 and 200 ng doses onto the HPLC column.
Absorption of aciclovir into the cornea and aqueous humour

Following topical application of different drug formulations, the level of aciclovir absorbed into the cornea and AH was measured in eyes which had been chemically damaged (4N NaOH burn), physically damaged (epithelium removed) or were undamaged (epithelium intact). An overview of all the results of the concentrations of aciclovir detected in the cornea and AH, for each formulation and corneal pretreatment, is shown in figures 22a and b. This overview indicates several main trends:

- Chemical and physical damage to the cornea greatly increases the concentration of aciclovir absorbed into the cornea and the AH.
- Generally, the level of absorption is greater after removal of the epithelium compared with alkali burning of the cornea.
- When the epithelium remains undamaged there is no detectable level of aciclovir found in the AH except at the highest concentration of drug applied (20% film).
- The concentration of aciclovir detected in the cornea and the AH varies according to the formulation in which it is applied, even when the dose is constant.

More detailed comparisons and statistical analyses of these data follow in figures 23 to 29.
Figure 22. A general overview of the results, showing the concentration of aciclovir detected in a) the cornea and b) AH, after application of different doses of aciclovir contained in various formulations, following three different pre-treatments of the cornea. Each bar represents the mean of at least 6 experiments.
Results: Absorption of Aciclovir.

a) Absorption of Aciclovir concentration (μg/g of cornea) with different formulations and epithelial conditions.

b) Absorption of Aciclovir concentration (μg/ml of aqueous) with different formulations and epithelial conditions.
The effect of increasing the concentration of aciclovir in the film formulation

Increasing the concentration of aciclovir in the film formulation increases the concentration of aciclovir detected in the cornea (Figure 23a) and AH (Figure 23b).

After chemical or physical damage to the cornea, each increase in film concentration resulted in a significant increase in the concentration of aciclovir detected in the cornea. In eyes with undamaged corneas, the apparent increases in the concentration of aciclovir detected in the cornea following each increase in film concentration were found not to be statistically significant.

After chemically or physically damaging the cornea, there were statistically significant increases in the concentration of aciclovir detected in the AH between the 1% and 1.6% film, the 1.6% and 20% film and the 3% and 20% film. The difference in concentrations between the 1.6% and 3% film was found not to be significant following either pre-treatment. Only at the highest aciclovir concentration of 20% was there any detectable level of absorption into AH where the cornea was undamaged.
Figure 23. The differences in drug absorption in a) the cornea and b) AH among increasing concentrations of aciclovir in the film formulation. Each bar represents the mean ± SEM of at least 6 experiments. Aciclovir concentrations represented here as zero, in fact indicate levels of drug < the limit of detection in the cornea and AH (see table 15).

### p< 0.001; ### 0.001< p< 0.01; # 0.01< p< 0.05: significance of difference among concentrations of aciclovir detected in eyes which have been chemically damaged (pre-treated with 4N NaOH).

*** 0.001< p< 0.01; * 0.01< p< 0.05: significance of difference among concentrations of aciclovir detected in eyes which have been physically damaged (epithelium removed).

++++ p< 0.001: significance of difference among concentrations of aciclovir in eyes with undamaged cornea (epithelium intact).
Results: Absorption of Aciclovir.

a.

![Graph showing Aciclovir concentration (μg/g of cornea) vs Concentration of drug. The concentration levels are 1% film, 1.5% film, 3% film, and 20% film. The graph includes bars and error bars for each concentration level. The legend indicates 4N NaOH, epithelium removed, and epithelium intact.]

b.

![Graph showing Aciclovir concentration (μg/g of cornea) vs Concentration of drug. The concentration levels are the same as in (a). The graph includes bars and error bars for each concentration level. The legend is the same as in (a).]
The effect of different pre-treatments on the weight of corneal buttons

Prior to acid extraction, full-thickness corneal buttons were removed from the eye and weighed. After chemically damaging the cornea with 4N NaOH the excised corneal buttons were found to be significantly heavier than corneal buttons from the physically damaged and undamaged cornea (Table 17). Undamaged corneal buttons were also significantly heavier than corneal buttons from physically damaged corneas where the epithelium has been removed (Table 17).

Table 17. The effect of different pre-treatments on the corneal button weight

<table>
<thead>
<tr>
<th>Corneal pre-treatment</th>
<th>n</th>
<th>mean ± SEM (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undamaged (epithelium intact)</td>
<td>57</td>
<td>0.143 ± 0.002</td>
</tr>
<tr>
<td>Physically damaged (epithelium removed)</td>
<td>49</td>
<td>0.135 ± 0.002**</td>
</tr>
</tbody>
</table>
| Chemically damaged (4N NaOH burn)           | 58 | 0.212 ± 0.004***||*

The effect of physically or chemically damaging the cornea on the weight (g) of excised 14 mm diameter corneal buttons. Each value is the mean ± SEM of the number of experiments shown (n).

Significance of difference (Student’s unpaired t-test) from undamaged corneal buttons: ** 0.001 < p < 0.01; *** p < 0.001.

Significance of difference (Student’s unpaired t-test) from physically damaged corneal buttons: *** p < 0.001.
The effect of different pre-treatments on the concentration of aciclovir detected in the cornea and aqueous humour

The effect of different pre-treatments on the level of aciclovir absorption following application of the film, ointment or gel formulation is shown in figures 24 and 25.

Application of the 1% film resulted in a significantly greater concentration of aciclovir in the cornea when it was undamaged compared to chemically damaged (Figure 24a). Physically damaging the cornea also resulted in a significantly greater concentration of aciclovir compared to chemically damaged. No difference was detected between the physically damaged and undamaged cornea following application of 1% film. Following application of 1.6% film there were no significant differences in the concentration of aciclovir detected in the cornea among all the pre-treatments. Compared to the undamaged or chemically damaged cornea, physically damaging the cornea resulted in a significant increase in the concentration detected in the cornea following application of 3% film. The difference between undamaged and chemically damaged cornea was not statistically significant. At the highest film concentration (20%), chemical or physical damage to the cornea resulted in a significant increase in the concentration of aciclovir detected in the cornea. There were no significant differences in the concentrations detected in the AH between chemically and physically damaged cornea following application of 1%, 1.6% or 3% film (Figure 24b). Following application of 20% film, chemical damage resulted in a significantly greater concentration of aciclovir in the AH than did physical damage. Compared to the undamaged cornea, either chemically or physically damaging the
cornea significantly increased the concentration of aciclovir in the AH following application of 20% film. A similar conclusion may also be drawn from the data for the films with lower concentrations of aciclovir, since absorption into the AH was undetectable in eyes with undamaged corneas.

Compared to the undamaged or chemically damaged cornea, physical damage significantly increased the concentration of aciclovir detected in the cornea following application of 1% gel (Figure 25a). Differences in absorption were even more pronounced when comparing aciclovir concentrations found in the AH of damaged eyes with that in undamaged eyes, where the level of drug was below the limit of detection (see table 16). There were no significant differences between the concentrations of aciclovir detected following chemical or physical damage in the AH.

Paradoxically, the concentrations of aciclovir detected in the undamaged cornea compared to the physically damaged cornea was significantly greater following application of 1% ointment (Figure 25a). No significant difference was found between the absorption levels in the damaged corneas following application of 1% ointment.

No significant differences were found in the concentrations of aciclovir detected in the cornea or AH between chemically damaged, physically damaged or undamaged cornea following application of 3% ointment (Figure 25b).
Figure 24. The effect of chemical or physical damage on the concentration of aciclovir detected in a) the cornea and b) AH following application of the film formulations. Each bar represents the mean ± SEM of at least 6 experiments. The data is identical to that in fig. 27; only the comparisons differ.

*** p < 0.001; ** 0.001 < p < 0.01; * 0.01 < p < 0.05: significance of difference from chemically damaged eyes.

+++ p < 0.001; + 0.01 < p < 0.05: significance of difference from physically damaged eyes.
Results: Absorption of Aciclovir.

(a)

Concentration of drug

(b)

Concentration of drug
Figure 25. The effect of chemical or physical damage on the concentration of aciclovir detected in a) the cornea and b) the AH following application of the gel or ointment formulations. Each bar represents the mean ± SEM of at least 6 experiments.

* 0.001 < p < 0.01: significance of difference from chemically damaged eyes.

** 0.001 < p < 0.01; † 0.01 < p < 0.05: significance of difference from physically damaged eyes.
Results: Absorption of Aciclovir.

a) 300.

$\text{co}^200$

$\text{II}$

$\text{§ 8}$

$\text{o}$

100

$\text{II}$

3% gel

Formulation

$\text{1% oint.}$

$\text{3% oint.}$

$\text{4N NaOH}$

$\text{epithelium removed}$

$\text{epithelium intact}$

b) 75-

$\text{50}$

$\text{25-}$

1% gel

1% oint.

3% oint.

Formulation
Expression of the data as concentrations or amounts of aciclovir

The difference between expressing the data as a concentration or an amount of aciclovir is shown in figure 26a and b.

Following application of the 3% film, the concentration of aciclovir found in the physically damaged cornea was significantly greater than the concentration found in either the chemically damaged or undamaged cornea. There was no significant difference in the concentrations of aciclovir found between the chemically damaged or undamaged cornea.

When the same data were recalculated as the amount of drug per 14 mm corneal button, the significance of these differences changed noticeably. Following application of 3% film, the amount of aciclovir detected in corneal samples that have chemically or physically damaged cornea was significantly greater than the amount detected in the undamaged cornea. There was no difference in the amount of aciclovir detected in physically damaged compared to chemically damaged cornea.

No significant difference in the concentrations or amounts of aciclovir were detected among the chemically damaged, physically damaged or undamaged cornea following application of 3% ointment, although these trends were very similar to those seen for 3% film.
Figure 26. Absorption of aciclovir in the cornea following application of 3% ointment or 3% film formulation expressed as a) the concentration of aciclovir and b) the amount of aciclovir. Each bar represents the mean ± SEM of at least 6 experiments.

* 0.01 < p < 0.05: significance of difference from chemically damaged eyes.

+ 0.01 < p < 0.05: significance of difference from physically damaged eyes.
Results: Absorption of Aciclovir

a. Aciclovir concentration (μg/g of cornea)

Formulation

- 4N NaOH
- epithelium removed
- epithelium intact

b. Aciclovir amounts (μg)

Formulation

- 3% oint.
- 3% film
Comparison of different formulations delivering an equivalent dose of aciclovir

Comparisons between the concentration of aciclovir detected in the cornea and AH following application of the ointment (1% w/v), film (1.6% w/v) and gel (1% w/v) formulations containing the same dose (300 µg) of aciclovir are shown in figures 27a and b.

Application of the gel formulation resulted in significantly greater concentration in corneal samples compared with the ointment or film formulation, for damaged corneas. The film formulation also showed significantly greater corneal concentrations compared to the ointment formulation following damage to the cornea. When the cornea was undamaged, the level of absorption was only significantly different between the gel and film formulations.

There was no detectable level of absorption in AH samples following application of the ointment formulation to eyes in any of the treatment categories (Figure 27b). There was also no detectable level of absorption in aqueous samples following the application of the film or gel formulation to the undamaged bovine cornea. Following chemical or physical damage to the cornea, significantly greater concentrations were detected in aqueous samples for the 1% gel formulation compared to the 1.6% film formulation.
Figure 27. The concentration of aciclovir detected in a) the cornea and b) AH following application of the 1% gel, 1% ointment or 1.6% film formulation, each containing an equivalent dose of aciclovir (300 µg). Each bar represents the mean ± SEM of at least 6 experiments.

*** p < 0.001; ** 0.001 < p < 0.01; * 0.01 < p < 0.05: significance of difference from 1% gel.

*** p < 0.001; ++ 0.001 < p < 0.01; + 0.01 < p < 0.05: significance of difference from 1.6% film.
Results: Absorption of Aciclovir.

(a) Absorption of Aciclovir (μg/g of cornea) across different formulations:

- **Formulation**
  - 1% gel
  - 1% oint.
  - 1.6% Film

- **4N NaOH**
- **Epithelium removed**
- **Epithelium intact**

(b) Absorption of Aciclovir (μg/ml of aqueous) across different formulations:

- **Formulation**
  - 1% gel
  - 1% oint.
  - 1.6% film
Comparison between concentrations of aciclovir detected in the cornea and AH following application of the ointment (3% w/w) and film (3% w/v) formulations containing the same dose (570 µg) of aciclovir are shown in figures 28a and b.

No significant difference was found between the 3% ointment and 3% film formulation in the concentration of aciclovir in the cornea for chemically damaged, physically damaged or undamaged cornea. The concentration detected in the AH when the corneal epithelium had been physically or chemically damaged was significantly greater after application of the 3% film. There was no detectable level of aciclovir in aqueous samples following the application of the 3% ointment or 3% film to the undamaged bovine cornea.
Figure 28. The concentration of aciclovir detected in a) the cornea and b) AH following application of the 3% ointment or 3% film formulation, each containing an equivalent dose of aciclovir (570 µg). Each bar represents the mean ± SEM of at least 6 experiments.

*** p< 0.001; ** 0.001 < p< 0.01: significance of difference between 3% ointment and 3% film.
Results: Absorption of Aciclovir...

a. 

Aciclovir concentration (μg/ g of cornea)

- 4N NaOH
- epithelium removed
- epithelium intact

Formulation

3% oint. 3% film

b. 

Aciclovir concentration (μg/ml of aqueous)

3% oint. 3% film

Formulation

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**Comparison between the 1% gel formulation and the 3% ointment formulation**

The concentration of aciclovir detected after application of the 1% gel formulation (300 µg dose) was compared against the concentration detected with a 3% ointment formulation (570 µg dose). No significant difference was found between the concentration of aciclovir detected in the cornea in eyes with physically or chemically damaged corneal epithelium (Figure 29a). Application of the 1% gel compared to the 3% ointment resulted in a significantly greater concentration of aciclovir in the aqueous of eyes with physically or chemically damaged corneal epithelium (Figure 29b).
Figure 29. The concentration of aciclovir detected in a) the cornea and b) AH following application of the 1% gel (300 μg dose) or 3% ointment (570 μg dose) formulation. Each bar represents the mean ± SEM of at least 6 experiments.

** * p<0.001; ** 0.001< p< 0.01: significance of difference between 1% gel and 3% ointment.
Results: Absorption of Aciclovir...

**a.**

![Bar chart showing aciclovir concentration (μg/g of cornea) for different formulations: 3% oint., 1% gel, with 4N NaOH and epithelium intact/removed conditions.]

**b.**

![Bar chart showing aciclovir concentration (μg/ml of aqueous) for different formulations: 3% oint., 1% gel, with 4N NaOH and epithelium intact/removed conditions.]

Formulation

- **3% oint.**
- **1% gel**

4N NaOH
- epithelium removed
- epithelium intact

---

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Total drug recovery from the cornea and aqueous humour

The total amounts of drug recovered from the cornea and AH for all formulations, following each pre-treatment, are shown in table 18. The amounts of drug recovered are expressed as a percentage of the dose applied.
Table 18. Total drug recovery for each formulation.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>a) undamaged cornea</th>
<th>b) physically damaged cornea</th>
<th>c) chemically damaged cornea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>% Drug Recovery (Mean ± SEM)</td>
<td>n</td>
</tr>
<tr>
<td>1% Aqueous Gel</td>
<td>7</td>
<td>2.02 ± 0.22</td>
<td>7</td>
</tr>
<tr>
<td>1% Ointment</td>
<td>8</td>
<td>0.81 ± 0.12</td>
<td>5</td>
</tr>
<tr>
<td>3% Ointment</td>
<td>7</td>
<td>1.54 ± 0.13</td>
<td>5</td>
</tr>
<tr>
<td>1% Film</td>
<td>6</td>
<td>2.29 ± 0.33</td>
<td>6</td>
</tr>
<tr>
<td>1.6% Film</td>
<td>7</td>
<td>1.35 ± 0.17</td>
<td>7</td>
</tr>
<tr>
<td>3% Film</td>
<td>11</td>
<td>1.27 ± 0.09</td>
<td>8</td>
</tr>
<tr>
<td>20% Film</td>
<td>11</td>
<td>0.69 ± 0.43</td>
<td>11</td>
</tr>
</tbody>
</table>

The total amount of drug recovered from the cornea and AH following application of the formulations to eyes with a) undamaged, b) physically damaged, or c) chemically damaged cornea. Each value is expressed as a percentage of the applied dose and is the mean ± SEM of the number of experiments shown (n).
DISCUSSION
In Vitro methods for the study of drug mechanisms on IOP

The arterially perfused eye has become a frequently used model in the investigation of ocular function (Niemeyer, 1981). Early studies investigating the physiology and pharmacology of AH dynamics utilised isolated eyes from the cat (Macri & Cevario, 1978a; 1978b; Chiou et al., 1980a; Liu & Chiou, 1980; Macri et al., 1980; van Alphen & Macri, 1981), rabbit (Kodama et al., 1983; 1985; van Pinnxteren & van Alphen, 1985) or bovine (Kishida et al., 1985). The pharmacological effects on facility of outflow in the perfused anterior segment of bovine (Erickson-Lamy et al., 1988) and human (Erickson-Lamy et al., 1991; Erickson-Lamy & Nathanson, 1992) eyes have been measured.

Due to its availability, cheapness and comfortable size for experimental use the bovine eye provides an attractive model for conducting perfusion experiments. Although they recommended the bovine perfused eye for biochemical studies, Kishida et al. (1985) judged this preparation to be unsuitable for the study of AH dynamics. It was demonstrated by Wilson et al. (1993) that the delay in commencement of perfusion beyond 1 hr post-mortem considerably reduces the probability of achieving a stable IOP, which may explain the dissatisfaction Kishida found with the preparation. Further investigation into the bovine perfused eye, where perfusion commenced before 1 hour post-mortem, suggests that the preparation offers a useful method for studying the mechanisms of action of drugs on IOP and AH formation, in isolation from CNS and CVS influences (Wilson et al., 1993). Several categories of drugs have now been shown to decrease IOP in this preparation.
Discussion: The Effects of Drugs on IOP...

(Shahidullah et al., 1995). The bovine arterially perfused eye has been kept viable for almost 5 days, indicated by utilisation of oxygen and glucose, when perfused with oxygenated serum-free Minimal Essential Medium under organ-culture conditions (de Coo et al., 1993).

In accordance with the experimental procedure of the constant flow method of Wilson et al. (1993), in the present work bovine eyes were cannulated and perfused with Krebs’ solution or Krebs’ solution supplemented with BSA. After cannulation, some eyes were rejected due to failure to fulfil the following criteria: perfusion pressure did not exceed 100 mm Hg at any stage during perfusion; perfusate was flowing freely from at least two vortex veins; perfusion pressure did not display any major fluctuations, such as might be caused by an air bubble or blood clot blocking an artery; establishment of a stable IOP. Failure to achieve anyone of these criteria resulted in the rejection of a substantial number of eyes before drug or vehicle administration.

In the present study, the mean steady-state IOP in 155 eyes perfused with Krebs’ solution was found to be 131.60 ± 1.46 mm H₂O (see table 3). This was consistent with previously reported values of 127.43 ± 1.90 mm H₂O for 88 eyes (Shahidullah, 1994) and 137.97 ± 4.33 mm H₂O for a series of 25 eyes (Wilson et al., 1993) perfused with Krebs solution. Introduction of a second needle through the cornea, cannulating the anterior chamber for the purpose of intracameral drug injection, resulted in a slightly greater steady-state IOP of 136.43 ± 2.05 mm H₂O for 49 eyes perfused with Krebs’ solution. It is possible the physical trauma of cannulating the
anterior chamber with a second needle may be sufficient to cause minor damage to the blood-aqueous barrier resulting in a significant elevation in IOP. Perfusion of Krebs’ solution plus BSA (1% w/v) in 138 eyes resulted in a steady state IOP of 126.71 ± 1.34 mm H$_2$O. Cannulation of the anterior chamber with a second needle for 40 eyes perfused with Krebs’ solution plus BSA resulted in a steady-state IOP value of 118.10 ± 2.13 mm H$_2$O, a value significantly lower than the IOP value for eyes perfused with Krebs’ solution plus BSA which were cannulated with only one needle. Unexpectedly, the value for steady-state IOP does not increase when the anterior chamber is cannulated for a second time in eyes which are perfused with Krebs’ solution plus BSA.

Various experimental inconsistencies may be responsible for the variation in steady-state IOP values. Values were recorded at t = 0 immediately prior to injection of the drug or vehicle. This measurement was taken at variable times after commencement of the perfusion due to the variation in a) the time taken to increase perfusion flow rate to the optimum level of 2.25 ml.min$^{-1}$ and b) the time taken after cannulation for eyes to achieve a steady-state IOP. On-going changes in meat hygiene regulations in response to the bovine spongiform encephalopathy (BSE) crisis have also resulted in a fall in the average age of cattle that pass through the abattoir. However there is no reported correlation in the literature between increases in age and IOP (Davson, 1990). There is also no control over the breed of cattle from which bovine eyes are obtained. As many as 20 different breeds or cross-breeds of cattle pass through the abattoir on a regular basis (Sandyford Abattoir, personal communication), which may contribute to the experimental variation.
Discussion: The Effects of Drugs on IOP.

We have found that the IOP responses, expressed as the mean slopes for the duration of control experiments were not significantly different irrespective of which vehicle or route of administration was used. This compares favourably with control responses for the same preparation found in the literature, which show that injection of different vehicles (e.g. H₂O, saline or DMSO), or injection via different routes, does not alter the mean slope of the IOP response (Bellashher, 1998; Shahidullah, 1994). The inclusion of BSA in the perfusion fluid, which has the advantage of providing retrospective information relating to the permeability of the blood-aqueous barrier (Wilson et al., 1993), was also found not to alter IOP over the duration of the control experiments.

Pilocarpine

Cholinergic agonists such as pilocarpine are widely used in treating glaucoma, and have been shown to decrease IOP in a variety of species including rabbits (Jianming et al., 1998), dogs (Chion et al., 1980b; Gwin et al., 1977), primates (Bill, 1962; Bill, 1967; Bill & Wållander, 1966; Crawford & Kaufman, 1987; Kaufman and Bárány, 1976) and humans (Bill, 1971). Pilocarpine acts by binding to cholinergic receptors, producing a contraction of the ciliary muscle, which in turn pulls on the scleral spur, opening the trabecular meshwork (Bill & Wållander, 1966). This increases the facility of AH outflow via the trabecular meshwork, resulting in a decrease in IOP.
In the present work a decrease in IOP in response to intra-arterial administration of pilocarpine was not observed in the bovine isolated eye, at least when it was perfused with Krebs’ solution. Instead a significant increase in IOP was observed at all concentrations of drug administered, although there was little sign of dose-dependence.

Constriction of the pupil, in response to pilocarpine, indicated that the drug had access to the anterior segment of the eye, including the iris sphincter and therefore very probably the ciliary muscle, since their arterial supply is common. In the mammalian eye contraction of the ciliary muscle would be expected to result in a decrease in resistance to outflow. However the ciliary muscle in the bovine eye is poorly developed with very little functional activity (Prince et al., 1960). The limited ability of the ciliary muscle to accommodate the lens is a general physiological trend found in other herbivores including rabbits, sheep and horses (Bill, 1975; Diescm, 1975; Prince, 1964; Prince et al., 1960). It is therefore not surprising that pilocarpine does not decrease IOP in the bovine eye as the contractile response of the ciliary muscle might not sufficiently alter the structure of the trabecular meshwork to significantly increase the facility of AH outflow.

The predominant overall effect of pilocarpine is an increased IOP, suggesting that a direct contractile effect on the trabecular meshwork, similar to that shown in bovine isolated trabecular meshwork strips (Weiderholt et al., 1995; 1996), may be involved. Lack of dose-dependence may be partly due to variable absorption of drug
Discussion: The Effects of Drugs on IOP...

into the avascular meshwork tissue. Influences such as a decrease in uveoscleral outflow, an increased production of AH, or a breakdown of the BAB may also contribute to the IOP response.

Contraction of the ciliary muscle in primate and human eyes, in response to pilocarpine, has been shown to decrease AH outflow via the uveoscleral route, due to a reduction in the spaces between ciliary muscle bundles (Bill, 1971; Bill & Wällinder, 1966; Crawford and Kaufman, 1987; Nilsson et al., 1989). However, the contribution of uveoscleral outflow to the total outflow in the bovine eye is unknown. It is possible that the bovine eye will act in a similar manner to the rabbit eye, where a rudimentary ciliary muscle results in practically no aqueous drainage via the uveoscleral route (Bill, 1975). If this is true then contraction of the ciliary muscle is unlikely to affect IOP.

Previous evidence in the literature concerning the effect of pilocarpine on AH production is contradictory and subject to species variation. Pilocarpine has been shown to have no effect on AH formation in rabbits (Kodama et al., 1985), decrease AH formation in primates (Bill & Wällinder, 1966; Miichi & Nagataki, 1983; Wällinder & Bill, 1969) and increase AH formation in humans (Nagataki & Brubaker, 1982). In order to determine the effect in the bovine eye, future experiments would investigate the effect of pilocarpine on AH formation determined by the fluorescein dilution technique (Millar et al., 1997; Shahidullah et al., 1995; Wilson et al., 1993).
The most likely explanation is a breakdown of the BAB, normally indicated by a continually rising IOP. Pilocarpine induced increases in BAB permeability to high molecular weight molecules (i.e. plasma proteins) have been reported in dogs (Krohne, 1994; Krohne et al., 1998) and humans (Mori et al., 1992). This theory was tested by the addition of a high molecular weight substance, albumin, to the perfusate. This substance is unable to cross the BAB under normal physiological conditions and so would remain undetected in the AH at the end of the perfusion. However if the BAB was not intact, then leakage of high molecular weight substances into the posterior chamber would occur. A correlation between breakdown of the BAB, continually rising IOP and leakage of albumin into the chamber has previously been reported (Wilson et al., 1993). After sampling and protein analysis of AH at the end of 90 min perfusion with Krebs' containing BSA, there was no significant increase in protein concentration at any concentration of pilocarpine. In fact, the protein concentration was significantly reduced at the highest pilocarpine concentration ($10^{-4}$ M). Examination of the IOP responses, recorded for the duration of the experiment, shows a trend where pilocarpine, at all except the lowest concentration, significantly decreases the IOP. This suggests that the presence of BSA in the perfusate of this preparation may play a permissive role in the IOP response to intra-arterially perfused drugs. The results may even indicate that pilocarpine-induced damage to the BAB is prevented by the inclusion of BSA in the perfusate, despite the fact that the addition of the high molecular weight substances, dextran (3% w/v) and albumin (0.5% w/v), does not significantly
increase the likelihood of achieving a stable IOP under control conditions (Wilson et al., 1993).

**Apraclonidine**

Apraclonidine, a derivative of clonidine, is an $\alpha_2$-adrenergic agonist with some $\alpha_1$ activity (Toris et al., 1995b). Topical application of clonidine, apraclonidine or brimonidine has been shown to lower IOP in a variety of species, including rabbits (Burke & Potter, 1986), cats (Burke & Potter, 1986; Chiou, 1983), primates (Burke & Potter, 1986; Gabelt et al., 1994; Serle et al., 1991) and humans (Gharagozloo et al., 1988; Lee et al., 1984; Toris et al., 1995a; 1995b). These $\alpha_2$-adrenergic agonists are believed to lower IOP primarily by reducing AH formation, presumably by acting on the NPR of the ciliary body (Adkins & Balfour, 1998; Burke & Potter, 1986; Gharagozloo et al., 1988; Mittag and Torinay, 1985; Toris et al., 1995b).

In the bovine isolated perfused eye, intra-arterial injection of apraclonidine increased the IOP in a non-linear manner. Analysis of the raw IOP data, expressed as the mean value for each time point plotted against time (see figure 17), showed an initial substantial linear increase in IOP for the first 30 min after drug injection, followed by a decline to a smaller rate of increase over the next 60 min. This suggested that the slopes of IOP vs time for each drug response should be calculated over the periods of linearity from 0 - 30 min and 30 - 90 min.
Intra-arterial administration of high doses of apraclonidine produced a significant increase in IOP characterised by a large increase over the first 30 min followed by a smaller increase over the following 60 min. Immediately after intra-arterial injection of the drug, vasoconstriction, presumably mediated via $\alpha_1$- or $\alpha_2$-adrenoceptors, resulted in a transient increase in vascular perfusion pressure, which decreased slightly to a plateau. This was sustained for the remainder of the experiment.

The increase in IOP, observed after intra-arterial injection of high doses of apraclonidine, is similar to the short term rise reported by Murray & Leopold (1985) following topical application of various $\alpha_1$- and $\alpha_2$-adrenergic agonists in the rabbit, although such findings do not correspond to the majority of reports which describe a decrease in IOP after topical application in vivo to primate and human eyes. *In vivo* responses to apraclonidine are generally measured over a longer time scale, starting from at least 1 hr after drug administration, and hence may fail to observe possible early influences on IOP. It is generally believed that apraclonidine lowers IOP primarily by decreasing the rate of flow of AH, plus a combination of increasing the facility of outflow and decreasing episcleral venous pressure (Gharagozloo et al., 1988; Toris et al., 1995b).

In order to determine any effect of apraclonidine on the permeability of the BAB, the perfusate was supplemented with BSA and AH was sampled 90 min after drug injection. Intra-arterial injection of 300 nmol apraclonidine did not significantly change the protein concentration in the AH, indicating that apraclonidine does not
affect the permeability of the BAB in the bovine perfused eye. This agrees with a previous result showing that in humans topical application of apraclonidine has no effect on BAB permeability (Gharagozloo et al., 1988).

It is possible that the increase in perfusion pressure occurring after intra-arterial injection of apraclonidine contributes to the increase in IOP. In vivo studies show that the α₁-adrenergic agonists have both local and systemic cardiovascular side effects including a decrease in systolic blood pressure (Lee et al., 1984), a decreased episcleral venous pressure (Krieglstein et al., 1978; Toris et al., 1995b) and a dose-dependent decrease in systolic and diastolic ophthalmic arterial pressure in patients with open-angle glaucoma (Heilmann, 1973). These factors plus the suggestion that apraclonidine constricts blood vessels to the anterior uvea resulting in a decrease in ocular blood volume (Chako & Camras, 1994), indicate that the vascular effects caused by α₁-adrenergic agonists are more likely to contribute towards a decrease in IOP, not an increase. However, the occurrence of vasoconstriction in the vascular beds of the anterior uvea of the perfused eye would not result in a decrease in ocular ‘blood’ volume, since perfusion is carried out using a constant flow method. Instead, resistance to flow would result in the observed increase in vascular perfusion pressure which might contribute to a short term increase in IOP by causing the distension of blood vessels proximal to the constriction and hence an increase in intraocular volume. Constriction of vascular beds may also redirect the flow of Krebs’ solution, which could result in an altered rate of perfusion in particular areas of the eye, although the total flow rate through the whole eye must remain constant. Again this is unlikely to contribute to an increase in IOP as even substantial changes
in the perfused flow rate of the bovine eye do not significantly alter the IOP (Wilson et al., 1993).

Unlike intra-arterial injection, there were no significant increases in IOP over the 30 min following intracameral injection of the drug (see table 11). Instead, after injection of 30 nmol apraclonidine, IOP was observed to decrease significantly over this period. In contrast, from 30 - 90 min after intracameral injection of high doses of apraclonidine there was a significant increase in IOP.

Administration of apraclonidine by intracameral injection delivers the drug directly into the anterior chamber, and hence rapid access to the trabecular and uveoscleral outflow pathways. When administered intra-arterially, the drug passes through the vascular system of the anterior eye, supplying the ciliary processes. Access of the drug to the AH and outflow pathways is relatively slow with aqueous production at less than 13 μl/min (Shahidullah et al., 1995; Wilson et al., 1993).

Comparison between the changes in IOP for apraclonidine administered via different routes, shows that relative to their controls, intra-arterial injection of high doses produces a significant rise in IOP over the initial 30 min, whereas intracameral injection does not. This suggests that the early rise in IOP is due to vasoconstriction as vascular perfusion pressure also increases during this period. In the longer term (30 - 90 min post-injection), the drug has time to diffuse to most ocular tissues regardless of the route of administration, producing a similar moderate rise in IOP by either the intra-arterial or intracameral route.
In the perfused eye, the peak short term increase in vascular perfusion pressure associated with intra-arterial injection of apraclonidine was inhibited by inclusion in the perfusate of the selective α₁-adrenergic antagonist, prazosin or the selective α₂-adrenoceptor antagonist, yohimbine. This suggests that the increased vascular perfusion pressure in response to apraclonidine is mediated via action on both α₁- and α₂-adrenoceptors. In order to confirm the selectivity of prazosin and yohimbine on α₁- and α₂-adrenoceptors, respectively, in the bovine perfused eye, their effects on the α₁-adrenergic agonist, noradrenaline were investigated. The short term increase in vascular perfusion pressure associated with intra-arterial noradrenaline was inhibited by prazosin, but not by yohimbine. Therefore it appears that in the bovine perfused eye, yohimbine at a concentration of 3 x 10⁻⁶ M is relatively selective for α₂-adrenoceptors. This confirms that the short-term vascular response to apraclonidine is mediated through both α₁- and α₂-adrenoceptors.

This corresponds with previous work by Murray and Leopold (1985), who showed that topical application of the α₁-adrenergic agonist, methoxamine or the α₂-adrenergic agonists, oxymetazoline or clonidine caused early dose-related rises in IOP, with peak responses occurring between 15 - 30 min after drug application. These hypertensive IOP effects were inhibited by pre-treatment with prazosin or with yohimbine. Various studies have also noted an initial hypertensive effect following topical or systemic application of the adrenergic agonists, adrenaline and noradrenaline in rabbits (Langham & Krieglestein, 1976; Langham & Palewicz, 1977) and humans (Kupfer & Ross, 1971). This increase in IOP was attributed to a
decrease in outflow facility, due to constriction of aqueous and episcleral veins (Langham & Palewicz, 1977; Wilke, 1974). It was also shown that the hypertensive IOP response to α-adrenergic agonists could be blocked by the α-adrenergic antagonist, phenoxybenzamine but not by the β-blocker, propranolol, suggesting the involvement of an α-adrenergic mechanism (Langham & Krieglestein, 1976). In the bovine perfused eye, Wilson et al. (1993) assume that as the vortex veins have been cut then pressure in the episcleral veins will be virtually zero. However, substantial vasoconstriction, occurring in the aqueous and episcleral veins, could still have an effect on resistance to outflow and hence on IOP (see ‘modified Goldman equation’, pg. 36). Direct measurement of episcleral venous pressure in the bovine eye would be necessary to establish whether vasoconstriction in these vessels may be the cause of the initial rise in IOP due to apraclonidine.

The sustained increase in vascular perfusion pressure, associated with apraclonidine was investigated by perfusing the eye with Krebs’ solution containing apraclonidine, and attempting to reverse the elevated perfusion pressure with prazosin or the selective α1-adrenergic antagonist, delequamine. Delequamine was chosen as its’ selectivity for α1-adrenergic receptors is higher than that of yohimbine. At 10^{-6} M, delequamine appeared to reduce the elevated vascular perfusion pressure associated with apraclonidine, though this effect was variable and not statistically significant. Prazosin did not have any effect on the sustained vascular response to apraclonidine. These results suggest that α1-adrenoceptors are not associated with the sustained increase in vascular perfusion pressure, which occurs after perfusion of the bovine
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eye with apraclonidine. The apparent decrease in perfusion pressure in response to delequamine indicates that $\alpha_2$-adrenoceptors may well be involved.

While many $\alpha_1$-adrenoceptors are located at presynaptic sites, they have also been observed at post-junctional locations in the vasculature of many species, including rats (Dyke & Widdop, 1987), rabbits (McGrath & McKean, 1981), dogs (Matthews et al., 1984) and humans (Thom et al., 1985; Jie et al., 1984; 1987), and are believed to be involved in the vasoconstriction induced by $\alpha$-adrenergic agonists (Langer & Hicks, 1984). However, in most vascular beds, the $\alpha_1$-adrenoceptor is the predominant receptor mediating vasoconstriction (Langer & Hicks, 1984). It has been postulated that the vascular smooth muscle contractile response differs between these two $\alpha$-adrenoceptor subtypes (McGrath, 1982). The $\alpha_1$-adrenoceptor response is rapid in onset, short-lived, utilises internal $Ca^{2+}$ and responds to short-term stimuli such as bolus injection of catecholamines; the $\alpha_2$-adrenoceptor response is slower in onset, longer-lived, utilises external $Ca^{2+}$ and responds to more prolonged stimuli such as circulating catecholamines. In the present work, the vascular responses to intra-arterial injection of apraclonidine correlate with the hypothesis of McGrath (1982), where stimulation of $\alpha_1$- and $\alpha_2$-adrenoceptors mediates different contractile responses in vascular smooth muscle.

Post-junctional $\alpha_1$-adrenoceptors have also been identified in the ciliary body, choriocapillaris, retina and iris of the rabbit (Huang et al., 1995), porcine (Wikberg-Matsson et al., 1996), bovine (Bylund et al., 1997) and human eyes (Matsuo & Cynader, 1992). Since there can be no neural activity in the isolated eye, it is
unlikely that presynaptic receptors could influence either vascular tone or IOP, indicating a possible involvement of post-junctional \( \alpha_2 \)-adrenoceptors.

The relationship between the early rise in IOP associated with stimulation of \( \alpha \)-adrenoceptors, possibly located in the blood vessels of the anterior uvea, could be investigated by pre-treating the eye with selective \( \alpha_1 \)- or \( \alpha_2 \)-adrenoceptor antagonists such as prazosin, yohimbine or delequamine and monitoring the IOP response to intra-arterial injection of apraclonidine.

In order to understand other possible mechanisms underlying the IOP response to apraclonidine in the bovine perfused eye, further experiments determining its effect on AH formation and facility of outflow would be required. In the present work, we chose not to investigate these parameters as the effects, which did occur, were often small, generally short-lived and consisted of an increase in IOP rather than a decrease. Such an effect clearly indicates a major difference from that in man, and did not merit further investigation as the usefulness of the bovine perfused eye model could not be furthered.

**Prostaglandins**

Previous studies have shown that prostaglandins, in particular \( \text{PGF}_{2\alpha} \) and its analogues, including latanoprost, lower IOP in rabbits (Camras et al., 1977), cats (Bito et al., 1983a), primates (Camras and Bito, 1981; Crawford et al., 1987) and humans (Lee et al., 1988). It is generally accepted that treatment with \( \text{PGF}_{2\alpha} \) does
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not induce a fall in IOP by lowering AH production (Brubaker, 1989; Camras et al., 1987; Lee et al., 1984; Toris et al., 1997). Instead PGF$_{2\alpha}$ acts by increasing AH outflow, via the uveoscleral outflow route (Crawford and Kaufman, 1987; Gabelt and Kaufman, 1989). The mechanism for the IOP reduction was unclear until it was demonstrated that pilocarpine antagonises PGF$_{2\alpha}$-induced ocular hypotension in primates (Crawford and Kaufman, 1987; Nilsson et al., 1989). PGF$_{2\alpha}$ is reported to alter the extracellular material between the ciliary muscle bundles, providing an alternative pathway for drainage of AH from the anterior chamber (Lütjen-Drecoll & Tamm, 1988; Tamm et al., 1989). Recent investigations indicate that prostaglandins can induce substantial changes in the extracellular material around human ciliary smooth muscle cells in vitro, supporting the hypothesis that prostaglandin-induced alteration in extracellular material between ciliary muscle bundles plays a role in increasing uveoscleral outflow facility (Lindsey et al., 1997a; 1997b; Weinreb et al., 1997).

In the present study PGF$_{2\alpha}$ had no effect on IOP when compared to control groups in eyes perfused with Krebs' solution or Krebs' solution plus BSA. Inclusion of BSA in the perfusate had no significant effect on the IOP response following intra-arterial injection of PGF$_{2\alpha}$. Intra-arterial injection of PGF$_{2\alpha}$ had no effect on vascular perfusion pressure, perhaps due to a lack of vascular tone in the perfused eye preparation. Possible explanations for these observations are that the contribution to total outflow provided by the uveoscleral route in the bovine eye may be minimal, due to the poorly developed ciliary muscle (Prince et al., 1960). However, PGF$_{2\alpha}$ has been shown to effectively lower IOP in rabbits which also has a poorly
developed ciliary muscle, as well as cats and humans where uveoscleral outflow accounts for considerably less than 50% of total aqueous drainage (Crawford and Kaufman, 1987). During in vivo experiments the drug is generally administered topically over a successive period of 3 - 5 days before a reduction in IOP is observed (Crawford and Kaufman, 1987; Gabelt and Kaufman, 1989). This suggested that administering \( \text{PGF}_{2\alpha} \) intra-arterially to the bovine perfused eye over a period of three hours may not be an ideal model for testing this type of drug. Over a three hour period the time scale may be insufficient to allow the physical widening of and loss of extracellular material from the connective tissue-occupied spaces between the ciliary muscle bundles demonstrated by morphological studies (Lütjen-Drecoll & Tamm, 1988). On the other hand, the drug has been shown to produce a 60% reduction in IOP, 3 hours after administration as a single dose in cynomolgus monkeys (Nilsson et al., 1989). Nilsson suggested that increased uveoscleral outflow, contributing up to 80% of total outflow facility compared with 40 - 50% in control eyes, was responsible for the fall in IOP. Interestingly, a larger IOP-reduction was observed following 3 - 5 days of daily treatment rather than a single dose treatment (Camras et al., 1987; Crawford et al., 1987), suggesting that the long term effect of prostaglandins is mainly structural, while the short term effect is mainly due to relaxation of the ciliary muscle (Nilsson et al., 1989).

Evidence in the literature suggests that high doses of prostaglandins, particularly \( \text{PGE}_1 \) and \( \text{PGE}_2 \), elevate IOP and AH protein concentration, indicating an increased permeability of the BAB (Beitch & Eakins, 1969; Kass et al., 1972; Waitzman & King, 1966). The presence of a low level of protein (300 - 700 \( \mu \text{g.mL}^{-1} \)) in the AH of
mammalian eyes demonstrates that the barrier function of the BAB is not absolute (Novack & Leopold, 1988). Protein concentrations from control eyes, where AH is sampled at the end of 180 min of perfusion with Krebs' solution, did not differ significantly from freshly excised bovine eyes where AH was sampled upon reaching the lab. The level of protein found in the bovine eye corresponded with a previous report in the literature (Doughty, 1995).

In the bovine perfused eye, the lack of a significant elevation in AH protein concentration in response to intra-arterial or intracameral administration of 600 nmol PGF$_{2a}$ indicates no BAB-disrupting effects at this dose. Other investigators have also shown negative or minimal effects of PGF$_{2a}$ on disruption of the BAB in primates (Crawford et al., 1987; Gabelt & Kaufman, 1989).

Intra-arterial and intracameral injection of PGE$_2$ shows a trend towards increase in protein concentration at all doses tested, though this is only significant following intra-arterial injection of 60 nmol PGE$_2$ or intracameral injection of 600 nmol PGE$_2$. The wide range of concentrations detected following administration of each dose indicate that although PGE$_2$ appears to increase the permeability of the BAB the effects are sporadic and non-reproducible in the bovine isolated perfused eye. In rabbits, increases in IOP and aqueous protein levels following administration of prostaglandins are thought to be due to a highly developed BAB breakdown mechanism, characteristic of animals with virtually 360° fields of vision (Bito, 1997). Responses observed in cats and primates, which do not have such a wide visual field, occur to a lesser degree. It is believed that the large species differences
which exist in response to ocular irritation and trauma, such as administration of prostaglandins or paracentesis, may be a result of the evolution of different types of visual systems (Bito, 1997).

Previous investigations have indicated that a direct correlation exists between elevations in IOP and aqueous protein concentration in rabbit (Beitch & Eakins, 1969) and bovine eyes (Wilson et al., 1993). Expression of all the raw data in the form of protein concentration as a function of IOP immediately prior to AH sampling (see figure 23) did not show a clear correlation between increases in protein concentration and increases in IOP for intra-arterial or intracameral injection of PGE₂. IOP values which increased above 300 mm H₂O in response to PGE₂ exceeded the maximum value on the water manometer scale and were necessarily represented as 300 mm H₂O in the scatter plot (figure #). What is clearly demonstrated by the scatter plots is that more than one population exists within the data. Both plots show that eyes, with IOP values which fall within the presumed normal range of 95 - 165 mm H₂O (Wilson et al., 1993), are associated with a low concentration of protein. There are no eyes found within this IOP range which are associated with an increased concentration of protein which suggests breakdown of the BAB. As IOP values increase above 165 mm H₂O associated protein concentrations either remain normal or increase to a level indicating breakdown of the BAB.

The IOP response to PGE₂ administration in the bovine isolated perfused eye differs greatly from other species. *In vivo* responses to PGE₂ are characterised by an initial
increase in IOP to a peak within 30 min of drug administration followed by a slow fall in IOP back towards the initial value. In rabbits the initial increase in IOP is large and is associated with breakdown of the BAB and protein leakage into the anterior chamber (Camras et al., 1977). However, other species show a less marked initial increase in IOP with little or no effect on the BAB (Nilsson et al., 1989). It is now generally thought that the unique sensitivity of the BAB to PG in rabbit make this species a poor model for the study of BAB integrity and AH dynamics (Bito, 1984; Toris et al., 1997). The bovine isolated perfused eye does not show a reproducible initial increase in IOP followed by a decrease back to the initial value. In the majority of eyes there is no significant change in IOP over the 90 min following drug administration.

The lack of a reproducible or dose-dependent response to PGE2 could result from several factors: the variation in the responses to prostaglandins due to species differences could account for the sporadic effect of PGE2 on permeability of the BAB in the bovine isolated perfused eye; the delay of any breakdown of the BAB may be highly variable (if the onset of the sudden rise in IOP is any indication), allowing very different concentrations of protein to accumulate in the anterior chamber by the end of the experiment; the volume of AH removed from the anterior and posterior chamber of the eye is not uniform. In the normal eye, up to 2 ml of AH can be removed. However, if the IOP has exceeded 300 mm H2O for the majority of the experiment the volume removed can be as little as 0.2 ml, presumably due to gross oedema of the anterior uvea. The experiments conducted suggest that the bovine isolated perfused eye does not appear to be an effective preparation for reproducibly
demonstrating drug induced permeability changes in the BAB. Measurement of aqueous flare in the anterior chamber might provide a more effective method of determining permeability changes in the BAB in the preparation, avoiding the reproducibility problem associated with AH sampling. This method may provide information regarding the time course of the breakdown response to PGE$_2$. 
Conclusion (i)

Although previous investigations have demonstrated IOP-reductions in response to several classes of drugs, including the carbonic anhydrase inhibitor, MK-927, β-adrenoceptor antagonists, timolol, oxprenolol and betaxolol (Wilson et al., 1993), the β-adrenoceptor agonist, terbutaline (Shahidullah et al., 1995) and vasodilator agents, including atriopeptin (Shahidullah et al., 1995), it was shown that neither pilocarpine, apraclonidine, PGF₂α or PGE₂ acted to lower IOP in this preparation.

The failure of pilocarpine, PGF₂α or PGE₂ to produce an IOP-lowering response might well have been expected due to the rudimentary development and lack of functional activity in the bovine ciliary muscle. The lack of correlation between the bovine eye and other species for drugs which increase trabecular or uveoscleral outflow suggests that this preparation is not suitable for screening anti-glaucoma drugs which act primarily on AH outflow. On the other hand, where a drug does lower IOP, the bovine eye offers a simple cheap system with which drug mechanisms can be studied.
The isolated perfused bovine eye as a model for investigation of corneal drug absorption

In the present study on the bovine perfused eye, the effects on ocular drug absorption of different drug formulations and experimentally induced corneal damage have been studied utilising single time point determinations. However, no pharmacokinetic data on drug absorption has been evaluated. A kinetic profile with minimal variability can only be constructed by measuring the AH drug concentrations from a sufficient number of time intervals to adequately characterise the absorption, distribution and elimination process (Qiu et al., 1993; Solanki & Morton, 1988; Tang-Liu & Burke, 1988). In general serial measurements are carried out in vitro on isolated corneal preparations as AH samples from the anterior chamber cannot be taken continuously in vivo (Richman & Tang-Liu, 1990). However, it has been shown that the bovine perfused eye model allows repetitive sampling from the anterior chamber, provided that 50 µl sample volumes are immediately replaced with an equivalent volume of AH substitute (Wilson et al., 1993). This allows the kinetics of absorption to be followed with relative ease under controlled physiological conditions (Zhu et al., 1996). Under normal physiological conditions, drugs which are absorbed across the cornea into the anterior chamber will be concentrated adjacent to the posterior surface of the cornea as mixing in the anterior chamber occurs very slowly, being influenced by aqueous flow from the posterior chamber, outflow of aqueous via the trabecular and uveoscleral drainage pathways and convection currents due to temperature gradients. In order to obtain an accurate measurement of AH drug concentrations from 50 µl aqueous samples, mixing of the
anterior chamber contents has to be increased. In the present study this complication is avoided, at the expense of pharmacokinetic data, by single time point determinations where all the available AH from the anterior chamber is sampled at the end of the perfusion. A more important reason for single sampling was that HPLC would not have been able to detect aciclovir in many of the small samples taken at intervals. However, this method of analysis does appear to be adequate for the present purpose of comparing the effects of different formulations and pre-treatments on corneal absorption.

HPLC analysis

In the present study, HPLC provided efficient and clean separation of aciclovir and the internal standard, ganciclovir, from samples of cornea and AH. The ganciclovir and aciclovir peaks, eluted at 8 min and 10.8 min respectively were not subject to interference by any coeluting compounds, allowing the concentration or amount of aciclovir absorption to be calculated accurately.

The retention time identified for ganciclovir compares favourably with previously reported retention times under similar HPLC conditions (Arevalo et al., 1995; Kupperman et al., 1993).
The effect of increasing the concentration of aciclovir in the film formulation

As expected, increasing the concentration of aciclovir dissolved in the PVA film resulted in an increase in the concentration of aciclovir detected in the cornea and AH, by increasing the gradient between the medicament and the surface of the cornea, thus accelerating diffusion of drug into the eye.

When the corneal epithelium is undamaged, intercellular tight junctions located between the apices of adjacent superficial epithelial cells remain intact and form the major barrier to ocular drug absorption (Grass & Robinson, 1988b; Tonjum, 1974). The barrier property of the epithelium is highlighted following chemical or physical damage to the cornea. With each increase in aciclovir film concentration the concentration of aciclovir detected in the cornea increased significantly in a concentration-dependent fashion. This relationship was not so clear when the cornea was undamaged, probably due to the limitations in the sensitivity of the HPLC system. Increases in aciclovir film concentration generally produced significant increases in aciclovir absorption into the AH following damage to the cornea. However, when the cornea was undamaged no detectable level of absorption was found in the AH except at the highest concentration of 20% aciclovir.

The barrier property of the corneal epithelium is often considered to vary in effectiveness according to the solubility of the drug (Sieg & Robinson, 1976). The lipophilic epithelium is relatively impermeable to water-soluble drugs, whereas lipid-soluble drugs penetrate easily (Järvinen et al., 1995). A previous study,
investigating the physicochemical properties of aciclovir, has shown that the drug is relatively hydrophilic (octanol: water partition coefficient \( \approx 0.06 \pm 0.01 \)) and would thus be expected to have low epithelial permeability (Hughes et al., 1993). Due to the physicochemical properties of aciclovir and the 14 - 18 cell layer thickness of the bovine corneal epithelium (Diescem, 1975; Prince et al., 1960), it is not surprising that the drug is poorly absorbed into the cornea and AH following application of the film formulation to eyes with undamaged corneas.

Physical or chemical damage to the cornea removes the barrier function of the epithelium, resulting in direct contact between the medicament and the hydrophilic corneal stroma. Aciclovir in the hydrophilic film will readily move down its concentration gradient into the stroma. In order for the drug to enter the anterior chamber it must pass through Descemet's membrane and the lipophilic corneal endothelium. Although tight junctions are present between adjacent endothelial cells, they are not as tight as those found in the epithelium, resulting in a "leaky" barrier between the AH and corneal stroma (Grass & Robinson, 1988b). It has also been suggested that in the human eye, the endothelium does not offer any significant resistance to hydrophilic drugs such as pilocarpine (Sieg & Robinson, 1976). After removal of the epithelium there was an absence of drug accumulation in the corneal stroma and endothelium following topical application of pilocarpine. Sieg and Robinson suggested that in the case of transcorneal permeation of pilocarpine the stroma, endothelium and AH act as a single phase, allowing free movement of hydrophilic molecules. In the bovine eye, the 10 - 25 \( \mu m \) thickness of Descemet's membrane, is approx. double that found in man (Diescem, 1975; Prince et al., 1960).
and might cause a relative decrease in the ease of drug movement into the anterior chamber. However, it is reasonable to suggest that aciclovir, due to its hydrophilic nature, will behave in a similar manner to pilocarpine and move with relative ease from the stroma into the anterior chamber. This mechanism explains the detection of substantial amounts of aciclovir in the AH following physical or chemical damage to the cornea.

The effect of different pre-treatments on the concentration of aciclovir detected in the cornea and aqueous humour

After application of different formulations at various doses following different pre-treatments to the eye, comparison between the concentration of aciclovir detected in the cornea and AH highlighted several trends. Generally, much larger concentrations of drug were absorbed in the cornea and AH following damage to the eye. Application of 1% film and 1% ointment formulations provided exceptions to this trend, with greater concentrations of aciclovir being absorbed in the undamaged cornea. However, the drug concentrations detected in these two examples approaches the limit of detection for this system (see table 16) and consequently may not be an accurate measure of the level of aciclovir. No detectable absorption was found in AH samples following application of the ointment or gel formulation when the epithelium remained undamaged. These trends confirm that the corneal epithelium is a major barrier to drug absorption through the cornea. We have shown that removal of the corneal epithelial barrier, as a result of physical or chemical damage, generally results in an increase in the concentration of drug absorbed. This
is in good agreement with previous data in other species showing that removal of the epithelial barrier results in a significant increase in absorption of topically applied hydrophilic, but not lipophilic drugs (Edelhauser & Maren, 1988; Hull et al., 1974; Lesar & Fiscella, 1985; Maren et al., 1983; Sieg & Robinson, 1976).

Comparison between the drug concentration detected in the cornea following chemical or physical damage indicates a trend where there is an apparent increase in absorption in corneas which have been physically damaged. These apparent increases are statistically significant following application of the film formulations containing 1%, 3% or 20% aciclovir. There were no differences between the aqueous drug concentrations except after application of 20% film formulation where the concentration of aciclovir detected in the chemically damaged cornea was significantly greater. Although there is no conclusive reason explaining the differences in absorption between the two pre-treatments, it is important to realise the differences in the nature of the experimentally induced damage. When the cornea is physically damaged the epithelium is débrided exposing the underlying stroma. Corneal transparency and thickness do not appear to alter. However, when the cornea is chemically damaged with sodium hydroxide, the underlying stroma rapidly loses its transparency and swells, as indicated by a visible thickening and approx. 50% increase in the weight of excised corneal buttons (see table 17). This effect is presumably due to sodium hydroxide-induced damage to the endothelial cells, resulting in a loss of the endothelial regulatory function on corneal hydration (see ‘Hydration of the cornea’, page 41). These observations agree with previous reports where severe alkali burns of the rabbit eye resulted in corneal opacification and
increased stromal hydration (Mai Phan et al., 1991; Pfister et al., 1971). It is therefore reasonable to suggest that the nature and severity of chemically induced damage to the cornea may affect the level of drug absorption, resulting in the apparent differences between chemically and physically damaged corneas. The alkaline environment, induced by NaOH pre-treatment, may also influence vehicle and drug stability in the eye. However, very little evidence was found in the literature to suggest that alkaline hydrolysis of aciclovir occurs under such experimental circumstances.

Experimental damage to the cornea was performed in an attempt to mimic the corneal damage associated with HSV infections, which in humans is often characterised by epithelial keratitis which may lead to corneal ulceration (Grayson, 1983; Newell, 1996; Pavam-Langston & Biosjoly, 1985; Verdier & Krachmer, 1984). This results in damage to the cornea ranging from the appearance of multiple microscopic pits on the epithelium to digestion of areas of epithelial and stromal tissue by enzymes (Grayson, 1983; Newell, 1996; Pavam-Langston & Biosjoly, 1985; Verdier & Krachmer, 1984). It is likely that such damage to the cornea would greatly limit the barrier function of the epithelium, resulting in an increase in absorption of topically applied hydrophilic drugs. It is purely speculative to consider how well the two models of experimentally induced corneal damage mimic the damage associated with HSV infections. Detailed morphological examination of the various types of damage would be required before this could be assessed. However, the pathological manifestations of severe HSV infection, such as enzymatic digestion of corneal tissue, are linked with the later stages of alkali burns where collagenase
production in the epithelium and stroma results in corneal ulceration which if left untreated can lead to corneal 'melting' (Brown, 1971). The NaOH model used in the present work would doubtless show severe disruption especially of the epithelium and superficial stroma since NaOH dissolves both lipid and protein structures. On the other hand, exposure was brief and the experiment was too short for collagenase release to occur. In the present work, we have confirmed that chemically or physically damaging the cornea provides two relatively reproducible methods of inducing experimental damage to the cornea, resulting in the loss of the barrier function of the corneal epithelium, a situation which also appears to occur during HSV infections.

**Differences between the expression of the data as concentrations or amounts of aciclovir**

The data shown in figures 22 - 25 and 27 - 29 have been expressed as the concentration (µg.g⁻¹ of cornea or µg.ml⁻¹ of AH) of aciclovir detected in the cornea and AH. When the data is expressed as an amount (µg) then the interpretation of the levels of corneal absorption may alter. This is highlighted in the figures 26a and b. Concentration in the cornea is influenced by the weight of the corneal button from which the level of absorption is calculated. In the chemically damaged cornea, the tissue is swollen with water from the AH resulting in a dilution in the concentration of drug contained in each sample. In the case of the 3% film there is no difference in the amounts of aciclovir found between chemically and physically damaged cornea, whereas the difference in concentration between them is statistically significant. The
same applies in another way to the difference between amounts and concentrations following 3% film application in corneas which are chemically damaged or undamaged. The difference in the amounts of drug found between the two pre-treatments is statistically significant, whereas no difference is found between the concentrations. On the other hand, changing the expression of the data for the 3% ointment from concentrations to amounts did not change the relationship between pre-treatments.

It is clear that chemical damage to the cornea increases the weight of the sampled corneal buttons presumably due to an increased water content of the stroma. When the data for all pre-treatments is expressed as concentrations, the swelling of the cornea after chemical damage results in a decrease in concentration of drug relative to the other treatments. The difference between two treatments may thus appear to alter according to whether the data are expressed as concentrations or amounts of drug.

The effect of aciclovir formulations on corneal absorption

This study shows that permeation of aciclovir into the cornea and AH of the perfused bovine eye is greatly influenced by the formulation of the applied drug. Topical application of aqueous gel, PVA film and ointment formulations, each containing an equivalent 300 μg dose of aciclovir, resulted in significantly greater drug concentration in corneal and aqueous samples with the aqueous gel than with the other formulations (Figure 27a and b). When the cornea was experimentally
Absorption of Aciclovir in the eye

damaged the film formulation also showed significantly greater absorption in cornal samples compared to the ointment formulation. There was no detectable absorption in AH samples following application of the ointment formulation. This is not surprising as the very small concentrations of aciclovir which are detected in corneal samples were approaching the limit of detection. Differences in the levels of absorption among the formulations were calculated in eyes which had undergone identical pre-treatments.

The variations in the levels of absorption found among the formulations could be explained by the physicochemical properties of the drug and the vehicles. Aciclovir is hydrophilic in nature, with an octanol: water partition coefficient of $0.06 \pm 0.01$ (Hughes et al., 1993). Therefore the drug will dissolve most readily in an aqueous vehicle. Once dissolved in the vehicle the formulation can provide drug readily. If a drug is insoluble in the vehicle then only drug molecules in immediate contact with the tear film can dissolve and become available for absorption. It has been reported that drug solubility in the vehicle dictates the success of a drug formulation in increasing bioavailability and prolonging drug release (Sieg & Robinson, 1977). In the bovine perfused eye, prolonging the presence of the medicament is not an important consideration since in vivo factors, such as blinking, dilution in the tear film and drug loss via conjunctival absorption and nasolacrimal drainage, are not present. As each application of medicament contains an equivalent dose of aciclovir, initial concentration gradients between the formulation and the cornea will be similar. This leaves the ability of the formulation to release aciclovir as the most important factor.
The low level of absorption in the cornea and undetectable level in the AH found when using a lipophilic ointment vehicle containing 1% aciclovir agrees with previous reports where low levels of corneal absorption are found when formulations combine hydrophilic drugs with lipophilic vehicles (Sieg & Robinson, 1977).

The film formulation is a hydrophilic vehicle containing a high percentage of PVA, a soluble polymer which is commonly used in eyedrops to increase the viscosity of the solution, resulting in an increased contact time in the precorneal area. However, as mentioned above, increasing the contact time does not affect drug absorption in the bovine perfused eye. The increased corneal and AH absorption when compared with the 1% ointment formulation is likely to be due to the hydrophilic nature of the drug and PVA film formulation. When the levels of absorption from the 3% film and 3% ointment were compared, there was no significant difference between corneal samples following any of the pre-treatments. However, the concentration of drug in AH samples was significantly greater for chemically and physically damaged corneas. Although for the 3% film and 3% ointment there were no significant differences between the drug concentrations in corneal samples, the overall trend agrees with that observed for the 1% film and 1% ointment as the total amount of drug absorbed into the cornea and AH is greater for the 3% film (Table 18). When the total amount of drug recovered from the cornea and AH is calculated and expressed as a percentage of the applied dose (570 µg), the amount of drug detected is significantly greater following application of the 3% film to both the physically (p = 0.029*) and chemically (p < 0.001***) damaged cornea. It is also possible that
other factors may influence the level of absorption from the film formulation. Saettone et al. (1982) have shown that from a group of equiviscous polymeric vehicles, PVA significantly increased the transcorneal penetration of pilocarpine, indicating that non-viscous factors such as a direct effect on corneal permeability may be responsible for the increase in penetration.

The proprietary aqueous gel formulation was a new formulation developed to combine the adhesive properties of carbomer and hydroxyethyl cellulose polymers in a preservative-free gel which will be more acceptable and better-tolerated in the eye (Rao, personal communication). This formulation demonstrated the highest absorption in the cornea and AH. Since this is a new formulation, no reference in the literature was available for direct comparison with our results. The enhanced absorption compared with the ointment is likely due to the hydrophilic compatibility between the drug and vehicle. However, it is not clear why this vehicle shows enhanced absorption compared with the PVA film as both vehicles are hydrophilic. It is possible that absorption of aciclovir from the PVA film formulation is delayed by the time taken to re-hydrate the film and render the drug diffusible in solution. The development of two soluble gel formulations for the delivery of pilocarpine has been reported (Goldberg et al., 1979; Mandell et al., 1979; March et al., 1982), although to date neither drug release mechanisms nor kinetics have been reported. What has been shown is that these gel formulations enhance pilocarpine activity, indicating greater transcorneal penetration, compared to a pilocarpine aqueous solution containing equal drug concentration.
Comparison between the concentration of aciclovir detected in the cornea and AH following application of the 1% gel or the 3% ointment highlights the ability of the aqueous gel formulation to enhance absorption of aciclovir. Application of 30 µl of the 1% gel (unit dose applicator) delivers a 300 µg dose of aciclovir whereas application of 21 µl of the 3% ointment delivers a 570 µg dose to the cornea. Despite applying almost double the dose, no significant differences in corneal absorption were found with the 3% ointment compared to the 1% gel in corneas which were undamaged, chemically or physically damaged. In contrast, vastly greater concentrations of aciclovir were found in the AH with 1% gel when the cornea was chemically or physically damaged. Therefore the total amount of drug detected in corneal and aqueous samples was found to be far greater following application of the 1% gel to eyes with damaged corneas. This suggests that if experimental damage is a reasonably good mimic of HSV-induced damage, then the gel formulation offers a major step forward in enhancing corneal absorption of aciclovir.

The absorption of aciclovir from the gel formulation may be enhanced by the larger applied volume and hydrophilic properties of the formulation, which will cover the 14 mm diameter area of the cornea relatively uniformly, providing a large surface area. Absorption from the ointment formulation may well be hampered as due to the semi-solid properties of the ointment at room temperature (18 - 24°C), achieving a layer of ointment of uniform thickness over the 14 mm diameter area of cornea is difficult. In vivo, this problem is avoided as blinking spreads instilled solutions or...
ointment across the cornea, increasing the surface area of corneal contact (Sieg & Robinson, 1979).

Intracocular penetration of aciclovir in the human and bovine eye

Very little data has been published on penetration of aciclovir in human eyes. Following repeated topical applications of ophthalmic Zovirax ointment to uninfected human eyes prior to cataract surgery, aciclovir accumulated in the aqueous at a concentration of $1.69 \pm 1.13 \mu g/ml$ (Poirier et al., 1982). Since in the present study the limit of detection for aciclovir in the AH was 2 $\mu g/ml$, such a level could not have been measured, we cannot directly compare bovine and human corneal permeability to this drug.

Despite this lack of comparison, from the differences in the anatomy of the bovine and human cornea (table 1), one would expect intraocular drug penetration to occur more readily across the human cornea.

Not surprisingly the level of absorption in the human eye following topical application exceeds that following oral administration (Hung et al., 1984). It is this difference will be much greater between corneal contents following the two different routes of drug administration.
Total drug recovery

Comparisons among the total amounts of drug recovered from the cornea and AH following application of each formulation shows that in the bovine perfused eye model the ointment formulation is not the best vehicle for delivering aciclovir following damage to the eye (Table 18). When the epithelial barrier has been removed, hydrophilic vehicles, such as the PVA film and aqueous gel, appear to greatly enhance the total absorption of this hydrophilic drug. Clinically, this may be of great relevance as the 3% ointment is currently the standard ocular formulation used for delivery of aciclovir to the HSV infected cornea. The use of PVA film or aqueous gel formulations for delivery of hydrophilic drugs offers promising drug delivery systems for ocular administration and the results obtained in this study should be confirmed with an in vivo study. When these formulations are applied in vivo the additional factors of drug dilution in the tear film, blinking, conjunctival drug absorption and drainage via the nasolacrimal duct will influence the level of corneal drug absorption. All the values shown in table 18 are greatly in excess of values you would expect to find in vivo as following application the medicament lies undisturbed on the surface of the cornea. In vivo, less than 5% of most topically applied drugs are absorbed (Järvinen et al., 1995). When this is coupled with the additional factor of enhanced in vivo drug release from ointments due to the shearing action of blinking (Siegel & Robinson, 1979), then the differences in absorption in vivo among the formulations may not be so pronounced.
Despite a concentration dependent increase in the drug concentration in the cornea and AH following application of increasing concentrations of aciclovir film formulations (see figure 23) this relationship is non-linear. As the aciclovir concentration of the film increases the percentage of drug recovered from the cornea and AH generally decreases (see table 18). Therefore a situation of diminishing return arises, where the increases in absorption are not proportional to increases in the applied concentration of the drug, indicating a decrease in the efficiency of drug delivery.
Conclusion (ii)

The bovine perfused eye provides a useful model for the investigation of drug absorption in vitro. Following topical application of different drug formulations the level of drug penetration in the cornea and AH can be measured without the complications associated with in vivo studies. This model is perhaps superior to the isolated corneal preparation normally used to assess corneal drug absorption, as experimental conditions are more physiological, avoiding edge-damage which is likely to occur following excision and clamping of the isolated corneal preparation. The use of the bovine eye again has the advantage that the eyes can be obtained from the local abattoir without the necessity of killing animals for experimental purposes.

This study confirms that experimentally damaging the cornea, effectively removing the barrier properties of the corneal epithelium, increases the concentration of aciclovir in the cornea and AH compared with the undamaged cornea. Physically removing the layers of the epithelium or exposing the cornea to alkali soaked discs of filter paper provides two relatively reproducible methods of inducing experimental damage to the cornea of the bovine perfused eye.

From the present work, the formulations can be ranked in order of corneal and AH absorption of aciclovir.

Ointment < PVA Film < Aqueous Gel
The ointment formulation, used as a standard ocular vehicle for aciclovir, does not appear to be the best vehicle for transcorneal absorption of this drug. PVA film and aqueous gel formulations seem to present promising drug delivery systems for ocular administration of hydrophilic drugs, such as aciclovir. The fact that this is particularly true after experimental damage to the cornea, makes such a formulation especially appropriate in the treatment of established HSV infection.
FUTURE WORK
FUTURE WORK

The effects of cholinergic agonists or prostaglandins on IOP in a variety of species have been well documented in the literature. It is believed that these classes of drugs act to increase the drainage of AH by altering ciliary muscle tone. The lack of any IOP-lowering effects in this model may be a result of the poorly developed ciliary muscle in the bovine eye (Prince et al., 1960). This could be determined by investigating the constrictor effect of pilocarpine on isolated strips of ciliary muscle, or the relaxation effect of the prostaglandins, PGF₂α and PGE₂, on tissue pre-contracted with carbachol.

From the experimental data on IOP, the effect of pilocarpine was shown to alter following the inclusion of BSA in the perfusate, suggesting that BSA may play a role in preventing drug-induced damage to the BAB. The inclusion in the perfusate of an alternative marker of BAB integrity, such as fluorescein, a molecule which does not cross the BAB under normal circumstances, could determine whether pilocarpine disrupts the integrity of the BAB. If the permeability of the barrier increased in response to pilocarpine then fluorescein would be detected in the chamber. Such experiments could also provide information on the effect of the presence of BSA in the perfusate.

There is very little evidence in the literature to suggest that pilocarpine increases the rate of AH formation. However, measurement of any change in AH formation rate (Wilson et al., 1993) in response to pilocarpine would help determine the mechanism.
behind the IOP response to pilocarpine in the bovine perfused eye, as would
determination of facility of outflow using the isolated anterior segment model
(Erickson-Lamy et al., 1991; Wiederholt et al., 1995).

The initial IOP response to apraclonidine is shown to be altered depending on the
route of drug administration. Inclusion of BSA in the perfusate and subsequent
measurement of AH protein content indicates that apraclonidine does not affect the
permeability of the BAB. Intra-arterial injection of fluorescein might confirm that
no change in barrier permeability occurs in response to apraclonidine. Investigation
into the effect of apraclonidine on AH formation might help determine the
mechanism behind the changes in IOP in response to high doses of apraclonidine.

The results show that following intra-arterial injection of apraclonidine, an increase
in vascular perfusion pressure occur concurrently with an increase in IOP. Evidence
in the literature suggests that vasoconstriction occurring in the aqueous and episcleral
veins results in an increased resistance to outflow and increase in IOP. Direct
measurement of episcleral venous pressure and AH outflow facility following intra-
arterial injection of apraclonidine in the bovine perfused eye would determine the
role of these mechanisms in the IOP and perfusion pressure responses.

Monitoring the IOP response to intra-arterial injection of apraclonidine, in the
presence of selective $\alpha_1$- and $\alpha_2$-adrenoceptor antagonists might confirm which
receptor(s) is involved in the IOP response.
In general, the results of experiments which investigated the effects of drugs on the BAB show that sampling AH at the end of each experiment for analysis of protein content does not provide a consistent assessment of the integrity of the BAB. Instead, measurement of drug-induced changes in aqueous flare in the anterior chamber might provide a more effective method of determining permeability changes, whilst also providing information on the time course of barrier-breakdown responses.

A logical extension of the aciclovir absorption work would be to investigate the kinetics of absorption of the various formulations, following the different corneal pre-treatments.

Detailed morphological examination of the two types of experimental corneal damage would provide an opportunity to assess the relationship between corneas damaged experimentally \textit{in vitro} and those which have been infected with HSV.

We have confirmed, by pre-treating the cornea with NaOH, that the weight of excised corneal buttons increases significantly compared to those which are undamaged or physically damaged. Future work involving the assessment of H$_2$O content in the excised corneal samples would probably confirm that the increase in weight associated with NaOH damage was due to swelling of the cornea with H$_2$O from the AH.
For the further development of an alternative formulation of aciclovir, for ophthalmic use, the level of drug absorption in the cornea and AH should be assessed in vivo, perhaps in the rabbit eye.


Bill, A. (1962) The drainage of blood from the uvea and the elimination of aqueous
humour in rabbits. Exp. Eye Res. 1: 200-205.


References


References


References


References


References


References

418.


References


Murakami, M., Sears, M.L., Mead, A. & Yamada, E. (1992) Non-pigmented epithelium is a locus for membranal carbonic anhydrase activity and the Cl/HCO₃⁻...
References


References


218


Schoenwald, R.D. & Hauag, H.-S. (1983) Corneal penetration behaviour of β-


Shahidullah, M. (1994) Mechanisms of action of drugs which alter aqueous humour...


aqueous humour dynamics of the isolated arterially perfused cat eye. Prostaglandins and Medicine, 7: 403-409.


References


