https://theses.gla.ac.uk/

Theses digitisation:

https://www.gla.ac.uk/myglasgow/research/enlighten/theses/digitisation/

This is a digitised version of the original print thesis.

Copyright and moral rights for this work are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This work cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Enlighten: Theses
https://theses.gla.ac.uk/
research-enlighten@glasgow.ac.uk

THE ROLE OF ACETYLCHOLINE IN THE CORNEAL EPITHELIUM OF THE RABBIT

A thesis submitted to the
University of Glasgow
in candidature for the degree of
Doctor of Philosophy
in the
Faculty of Science
by

Catherine E. McKean

Department of Pharmacology,
Glasgow University.

August, 1980.

ProQuest Number: 10760484

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

ProQuest 10760484

Published by ProQuest LLC (2018). Copyright of the Dissertation is held by the Author.

All rights reserved.
This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Thesis
6276
Copy 1.

GLASGOW
UNIVERSITY
LIBRARY

Felix qui potuit rerum cognoscere causas.

Virgil ; Georgics,1,145.

Acknowledgements

I am indebted to Professor J.S. Gillespie for the privilege of working in his department and for his friendly advice and help over the past three years.

I am very grateful to my supervisor, Dr. William S. Wilson, for suggesting the problem and the means of attack; for encouragement and help during the three years and for useful criticism of this text.

Thanks are due to all those who rendered technical assistance; Joanne Groves, Robert Auld, John Thomson, Hannah McCaffery and Trevor Clarke in particular. Nan Johnston and her staff ensured a constant supply of well cared-for animals. Marjory Wright gave me many useful tips on thesis preparation. Josie McDermott was invaluable since she looked after all the glassware and prepared the tea and coffee. Jokes and repartee were provided by Messrs. Auld, McComb and Clark and were much appreciated.

I am thankful for the scientific assistance and discussions of the academic staff, and for their guidance in teaching laboratories. I am especially grateful to Drs. Boyle, Muir and Pollock who also provided a cultural education.

My fellow postgraduate students were ever kind and helpful. Special thanks are due to Angus Crossley for debates on subjects pharmacological, for ideas and advice on anaesthetics and for constant work on my car. Thanks also to Kevin Digges simply for friendship and being around for three years.

I am indebted to Mrs. Anne Mosson who has typed this thesis with precision and elegance.

Lastly, I am indebted to the Medical Research Council who provided financial support during the course of this work.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

SUMMARY

The corneal epithelium forms the smooth anterior surface of the cornea, a structure which is a highly refractive component of the optical system. The corneal epithelium has several unusual properties; it is avascular; it is capable of rapid regeneration; it is a non-keratinised epithelium exposed to the external environment. This epithelium contains a high concentration of acetylcholine (ACh) and high levels of choline acetyltransferase (ChAc) activity, this being the enzyme which is responsible for the synthesis of ACh. There are relatively low levels of cholinesterases (ChE), the enzymes involved in the breakdown of ACh. These components of the cholinergic system do not appear to be entirely associated with the sensory nerves of the cornea. Several theories have been propounded for the function of this ACh, but none has received universal acceptance. This research was an attempt to elucidate possible functions for the ACh of the corneal epithelium of the rabbit.

The first possibility was that the ACh played a role within the epithelium, maybe a role in the regeneration of the tissue after abrasive injury. Removal of the epithelium followed by investigation of the levels of protein and ChAc in the regenerating tissue presented some evidence to suggest that the enzyme ChAc does not play a major role in this process of regrowth.

Thereafter, experiments investigated the postulate that corneal epithelial ACh may act at a site outwith the epithelium. Firstly, the possibility was explored of an anterior release of ACh into the tear film, perhaps to act on one of the

structures bathed by this fluid. It was found that only drugs and stimuli which caused damage to the epithelial cells could increase the release of ACh into the tears. It seemed unlikely that this was a physiological function of epithelial ACh.

Secondly, experiments were designed to investigate posterior release of ACh to act at a site most probably located in the anterior segment of the eye. Levels of cholinesterases in various tissues were measured and it was found that these levels were low in the corneal stroma and the aqueous humour, which the epithelial ACh would probably have to traverse to reach a distant site of action. A second series of experiments traced the tissues to which radio-labelled ACh diffused after it had been injected into the corneal stroma. These results indicated that detectable amounts of ACh could pass through the aqueous humour to reach the iris and ciliary body although only 1.5% of the injected ACh reaches the iris intact and less than this reaches the ciliary body. When ACh was injected into the corneal stroma and its effect on the iris observed, it was found that an injected dose of around 2nmol could produce a significant miosis. This dose of ACh is of the same order as the total amount of endogenous ACh in the corneal epithelium. A total release of the complete store of epithelial ACh to have a physiological role in the iris seemed improbable.

The effect of cholinomimetics on the outflow of aqueous humour is well documented. These drugs, especially pilocarpine, are used in the treatment of glaucoma and probably act by

increasing the outflow of aqueous humour from the anterior chamber. The main outflow system from the rabbit eye is situated in the iridocorneal angle of the anterior chamber. It was found that small doses of ACh (40pmol) injected into the corneal stroma could significantly increase the apparent facility of outflow of aqueous humour. The ACh appeared to act on muscarinic receptors and act through a Ca^{2+}- mediated mechanism. It was potentiated by anticholinesterases. The mechanism of action of ACh on the outflow mechanism is discussed. The possibility that ACh from the corneal epithelium might exert an influence on the outflow of aqueous humour is put forward.

INTRODUCTION

The anatomy of the cornea.

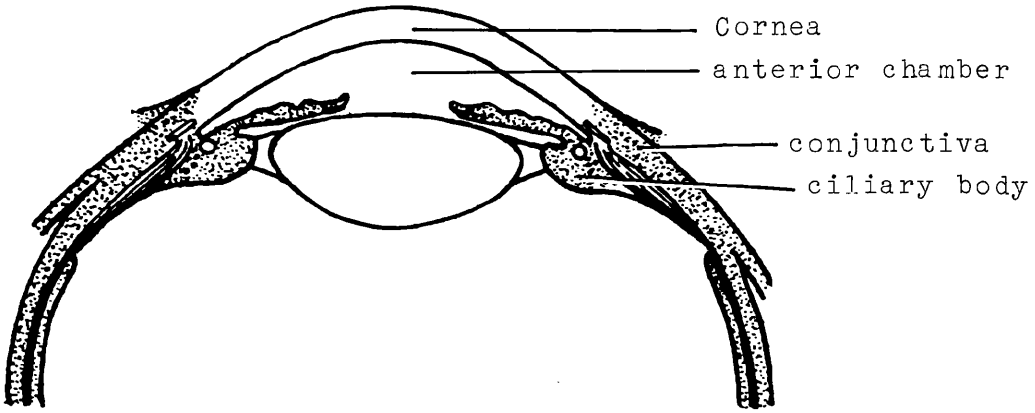
The cornea of the rabbit is tough and semi-rigid and it protects the ocular contents from infection and mechanical trauma. It is completely transparent and avascular with an optically smooth anterior surface and thus forms a highly refractive component of the optical system. The transparency is achieved by an almost perfect geometric lattice structure of the corneal layers and their cellular components (Maurice, 1957).

The cornea is surrounded by the conjunctiva and the junction between these is called the limbus. This is identified as the boundary between the almost transparent conjunctiva, with the white sclera behind it, and the transparent cornea through which the coloured iris may be seen.

The five layers of the cornea are considered separately below and shown in figure 1. The anterior surface is bounded by the epithelium, set on its basement membrane. The posterior surface is bounded by the endothelium, the basement membrane of which is also known as Descemet's layer. Sandwiched between these basement membranes is the stroma which is also known as the substantia propria.

The corneal endothelium is a single layer of uniform, flattened, hexagonal or polygonal cells which are continuous with the cells on the anterior surface of the iris; it is some 3 to 5μ thick. Scanning electron microscope studies have shown a polysaccharide coating on the posterior surface of the endothelium (Schrøder & Sperling, 1977; Sperling & Jacobsen, 1980).

The central region of the cornea derives its nutrients from



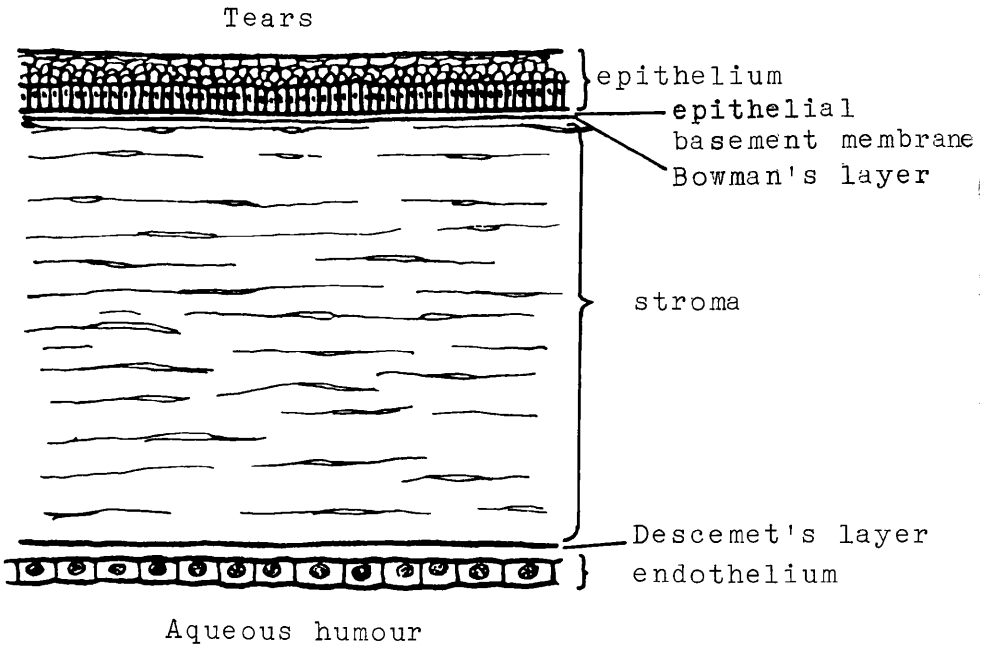


Fig. 1a Cornea : its position in relation to the tissues of the anterior segment of the rabbit eye.

Fig. 1b Cornea : its composition.

the aqueous humour across the highly permeable endothelium (Maurice & Riley, 1970). The peripheral cornea derives some nutrition from blood vessels close to the limbus (Maurice, 1969). Although the centre of the cornea derives its amino acids from the aqueous humour, there is no indication that there are active transport mechanisms for these in the endothelium. Thus the stromal concentrations are dependent entirely on the concentrations in the aqueous humour (Bito, Roberts & Saraf, 1973). The apical junctions of the cells do not form an impervious barrier to macromolecules (Thoft & Friend, 1975; Hirsch, 1978).

The substrate of corneal energy metabolism is glucose and this is transferred across the endothelium from the aqueous humour (Turss, Friend & Dohlman, 1970). Oxygen is obtained mainly from the atmosphere, but it also diffuses into the cornea from the aqueous humour and from the limbal vessels (de Roetth, 1950; Langham, 1951; 1952).

It is thought that active transport of ions across the endothelial cells is the main factor responsible for the regulation of normal stromal hydration, which is necessary for corneal transparency (Harris & Gruber, 1956; Hirsch, 1978). It is thought that bicarbonate ions are translocated from the stroma to the aqueous humour with sodium ions and water moving passively (Hodson & Miller, 1976; Hull, Green, Boyd & Wynn, 1977). The enzyme responsible is thought to be a magnesium-activated ATPase (Hodson, 1977; Riley 1977).

Descemet's layer is the endothelial basement membrane and it is 7 to 8μ thick. It is more firmly attached to the endothelium than to the stroma. It is a homogeneous, collagenous

tissue containing granular elements which are connected by fine fibrils. Descemet's layer divides into two parts at the ciliocorneal junction. One part forms the immediate structure of the iris pillars whilst the other continues as a border of the inner surface of the angle meshwork (p 13).

The stroma, or substantia propria, is at least 0.24mm thick. It consists of collagen fibrils arranged in regular lamellae and embedded in an amorphous ground substance; hence it imparts mechanical strength to the cornea. Some elastic fibres and flat, elongated fibrocytes lie between the lamellae (Maurice, 1960).

The epithelial basement membrane is continuous with the basement membrane of the conjunctiva and has a thickness of 0.4 to 0.6μ. It has been described by Teng (1961) as being composed of two layers : a lipid layer backed by a thick, reticular fibre meshwork. The membrane adheres to the corneal epithelium by means of a tight formation of fine filaments or fibrils.

Wulle & Richter (1978) have given evidence to suggest that the basal membrane is a product of the epithelial cells and, if the corneal stroma is denuded, the regenerating epithelial cells synthesise collagenoid proteins for the rebuilding of the basal membrane. Holly (1978) found that the epithelium does not adhere well to the stroma when the basement membrane is damaged or absent.

Bowman's layer is a membrane immediately below the epithelial basement membrane. It is prominent in primate eyes but not often found in rabbit eyes. It has been identified in electron microscope studies of rabbit eyes by Kayes & Holmberg (1960) as

a modified zone of the anterior stroma. When present, it consists of randomly orientated collagen fibrils with an absence of stromal cells. It is inelastic and has no powers of regeneration (Prince, 1964).

The corneal epitheliumis usually 30 to 40μ thick and consists of one row of columnar basal cells beneath two rows of polygonal and up to six rows of wing-shaped squamous cells which form the anterior surface. Blümcke, Rode & Kudszus (1967) produced evidence for a double lipid membrane forming an outer layer on the surface epithelium in the rabbit cornea; the outer sheet serves to cover the intercellular junctions. This may be the main barrier to penetration of the cornea by substances of low lipid solubility. The epithelium as a whole is certainly a barrier to penetration of the cornea by charged molecules since Thoft & Friend (1975a) have demonstrated that corneal impermeability was reduced upon removal of the epithelium and reappeared simultaneously with the reestablishment of epithelial integrity. The cell membranes of the corneal epithelium are remarkably close and interdigitated, particularly in the middle layers (Pedler, 1962; Jakus, 1964). This alone would produce a considerable barrier to non-lipid soluble materials and would explain why the epithelium is so poorly permeable to electrolytes, glucose, etc. (Maurice, 1969).

Mitosis in the corneal epithelium is, as expected, most prolific in the basal layer of cells and reduces as the cells move upwards (Calmettes, Deodati, Planel & Bec, 1956). The average life span of an epithelial cell is 4 to 7 days, during which time the cell migrates from the basal layer to the superficial

layer to be shed eventually (Hogan, Alvarado & Weddell, 1971; Maudgal & Missotten, 1978). In adult animals, mitosis is least in the periphery of the cornea and increases towards the centre (Kaufman, Gay & Hollaender, 1944). Recently, epithelial cells have been shown to have a diurnal rhythm with respect to mitosis, this being greatest in the evening and least in the morning (Semenova, 1976). Maudgal & Missotten (1978) have demonstrated, using polarisation microscopy, that the superficial epithelial cells of the cornea do not keratinise.

It has been suggested that a primary role of the epithelium may be in preventing the access of tear fluid into the stroma (Riley, 1971). Klyce (1975; 1977) has suggested that the epithelium may play a more positive role in the hydration of the stroma with active transport of Na^+ ions towards the corneal stroma.

Innervation of the Cornea.

There are numerous sensory nerves to the cornea and these are branches of the ophthalmic nerve and in particular, the long ciliary nerves. These form an annular plexus around the limbus and from this plexus fibres enter the stroma in a radial pattern. The nerves then lose their myelin sheaths and ramify throughout the stroma in a delicate network. The terminal filaments form an intricate plexus beneath the corneal epithelium. This is called the subepithelial plexus and from it are given off fine, varicose axons which pass through the anterior limiting membrane to ramify between the epithelial cells. Hoyes & Barber (1976) found axons containing agranular vesicles in the rat cornea and suggested that these were cholinergic neurones. Tervo &

Palkama (1978) observed adrenergic axons in the corneal stroma and both adrenergic and cholinergic axons containing vesicles in the corneal epithelium.

The anatomy of the formation and drainage of aqueous humour.

The aqueous humour was first described by Aristotle (384-322B.C.) and its chemical composition by Jöns Jacob Berzelius in 1808. It is a colourless, aqueous solution which is present in the anterior and posterior chambers of the eye. Since it is a circulating fluid, it can provide nutrition for the lens and cornea whilst removing waste products from these structures.

The fluid is formed continuously by the ciliary body in the posterior chamber and flows through the pupil into the anterior chamber, which has a volume of about 250µl in rabbits and in primates. It flows out of the chamber mainly via a drainage system situated in the iridocorneal angle.

The intraocular pressure is determined largely by the rate of formation and ease of drainage of aqueous humour. Since the intraocular pressure maintains the shape of the anterior chamber, it is important in the performance of the optical function of the eye. If drainage is impaired, for instance, the intraocular pressure may rise to levels which damage the retina and optic nerve. This occurs in glaucoma simplex, a disease of which the primary pathogenesis is unknown.

The normal intraocular pressure of the rabbit is 15 to 23mmHg although this shows seasonal variation. It is highest in winter (mean 19.5mm Hg) and lowest in summer (mean 17.9mm Hg). There

are also diurnal variations and the pressure is highest during the day (Vareilles, Conquet & Le Douarec, 1977).

The chemical composition of aqueous humour has been analysed in detail by Kinsey & Reddy (1964). There are characteristic differences between the freshly secreted aqueous humour in the posterior chamber and the fluid in the anterior chamber. Nevertheless, the composition of both fluids closely resembles the extracellular fluid in peripheral tissues.

The sodium ion and potassium ion content of the anterior aqueous humour are essentially the same as those of plasma (Kinsey & Reddy, 1964). The chloride ion content of the posterior aqueous humour is lower than that of plasma but rises somewhat as the fluid passes through the anterior chamber. This chloride ion deficiency is approximately balanced by an excess of bicarbonate ions, the concentration of this ion in both anterior and posterior aqueous humour being much higher than in plasma. The pH of the fluid in both the anterior and posterior chamber is 0.2 units greater than that of plasma (Kinsey & Reddy, 1964).

The concentration of ascorbic acid is higher in the posterior chamber than in the anterior chamber and these concentrations are many times higher than that in plasma (Kinsey & Reddy, 1964). The glucose content of the aqueous humour is some 10 to 20% lower than that of plasma and this may be due partly to utilization of glucose by the cornea and lens, both avascular structures dependent on the aqueous humour for nutrition (Kinsey & Reddy, 1964).

The anterior aqueous humour contains only 0.02 to 0.06% protein in comparison to over 7% in plasma (Kinsey, 1951).

The concentrations of most amino acids in the aqueous humours is higher than in plasma in rabbits. There appear to be at least three active transport mechanisms (for acidic, basic and neutral compounds) to carry amino acids into the posterior chamber. This is unusual since most other species contain less amino acids in the aqueous humour than in the plasma (Reddy, Rosenberg & Kinsey, 1961).

The formation of aqueous humour.

Aqueous humour is produced by the ciliary processes by two different means :

Active secretion : an active sodium pump is located in the inner layer of the double epithelial lining of the ciliary processes. The enzyme involved in this transport mechanism is a Na - K ATPase (Bonting, Simon & Hawkins, 1961; Bonting, Caravaggio & Hawkins, 1962). The main anion, chloride, either migrates passively with the sodium or it is actively transported at a lesser rate (Cole, 1969). Friedenwald (1955) and Zimmerman, Garg, Vogh & Maren (1976) proposed that bicarbonate formation by carbonic anhydrase in the ciliary processes was a fundamental step in aqueous humour secretion. This process seems linked in some way to the active transport of sodium and the elaboration of the fluid (Maren, 1976).

Cole (1966) proposed that in rabbits, active transport accounts for around 75% of the observed rate of fluid formation and that the normal rate of flow is around 3µl/min.

Ultrafiltration : this is a pressure dependent flow from the plasma to the aqueous which is made possible by 'leakage' between the cells of the non-pigmented inner layer of the ciliary epithelium. Although it has been suggested that ultrafiltration may play a major role in the formation of the aqueous humour (Green & Pederson, 1972; Pederson & Green, 1973), Bill (1973) has found little evidence for a positive contribution by ultrafiltration to aqueous humour formation. Indeed, Bill suggests that ultrafiltration can cause a reabsorption of fluid which has already been secreted.

The subject of aqueous humour formation has been reviewed by Cole (1974; 1977).

The drainage of aqueous humour.

Until just over a century ago, it was believed that the aqueous humour drained through the cornea. Friedrich Schlemm (1830) has been given the credit for the first description of the primary drainage channel in primates. The discovery of Schlemm's canal, and its equivalent in sub-primates, gradually led to the concept that aqueous humour drained through the iridocorneal angle instead of through the cornea (Schwalbe, 1870).

Although the rabbit differs from primates in certain aspects of the anatomy and morphology of the anterior chamber angle, the physiological similarities have often been stressed (see Tripathi, 1971b; Grierson, 1976). The bulk of the work on this subject has been carried out on primates and reference to this work will be made. The tissue of the iridocorneal angle in the rabbit is discussed in detail below.

Conventional drainage of the aqueous humour in the rabbit starts with its entry into sinuses in the chamber angle. It then enters a trabecular meshwork before passing through an endothelial wall into a plexus of drainage channels. Aqueous veins collect the aqueous humour from these channels and convey it to the episcleral venous system.

The limbal zone of the rabbit anterior chamber is characterised by a posterior extension of the chamber angle since the ciliary muscle is poorly developed. (Collins, 1921; Grierson, 1976). In the absence of a substantial ciliary musculature, the patency of the ciliary cleft and the drainage angle is maintained by struts of connective tissue (called pectinate ligaments by Hueck, 1839) which originate in the iris and merge with the sclèro-corneal stroma. These ligaments are lined with endothelium which is continuous with the corneal endothelium. The spaces delineated by the struts are called the spaces of Fontana (see Tripathi, 1974) or the ciliary cleft.

Since the ciliary muscle is insubstantial, the major part of the ciliary body is composed of loose connective tissue. At its anterior limit, the ciliary stroma is continuous with the trabecular lamellae. In the rabbit, a scleral spur is absent and so the outermost trabeculae make contact with the sclera proper (see Grierson, 1976).

The trabecular meshwork in rabbits is shallower and more highly developed than in primates (see Prince, 1964), yet it is possible to apply the same terminology to it as is used in primates (see Tripathi, 1974) since each 'layer' of trabeculae

directly corresponds to tissues in primates. Recent electron microscope studies have shown, however, that there are features of the filtration spaces and lining structures in the rabbit which differ from those in the primate (Orzalesi, Bonomi, Del Fiacco, Fossarello & Carta, 1980).

The trabecular meshwork consists of a series of endothelium-covered collagenous cords and perforated sheets (trabeculae). The endothelial lining of these is a continuation of the corneal endothelium and Descemet's layer. Complete encasement of the trabeculae with endothelium is rare (Grierson, 1976). Berggren (1957) suggested that, since the endothelium seemed functionally identical with that of the cornea, it may be involved in the regulation of hydration of the meshwork tissue and hence the regulation of the size of the apertures in the meshwork.

The innermost trabeculae border the chamber angle and the aqueous humour follows a tortuous path through and between the trabecular structures to reach the angular aqueous plexus. The trabecular meshwork, and also the collecting channels, have rectifying properties which restrict any retrograde flow under conditions of reversal of the normal pressure gradient (see Prince, 1964).

There are three 'layers' of meshwork :

Uveal meshwork : this is closest to the chamber angle and consists of cord-like trabecular cores with wide, open spaces between these cores. This mesh contributes little resistance to the flow of aqueous humour.

Corneoscleral meshwork : this adjoins the uveal meshwork and consists of perforated sheets of trabecular cores with narrow intervening spaces.

Endothelial meshwork : this lies closest to the angular aqueous plexus. It lacks trabecular cores and instead it is composed of endothelial cells surrounded by amorphous and fibrillar material which is probably dispersed in a mucopolysaccharide matrix.

The trabecular meshwork fibres have been shown to be covered with a mucopolysaccharide matrix (Bárány & Scotchbrook, 1954) and the outflow through perfused rabbit eyes is increased by the addition of testicular hyaluronidase to the perfusion medium. Bárány, Berggren & Vrabec (1957) suggested that the endothelial lining of the meshwork produced viscosity-regulating substances to affect the outflow of aqueous humour. Wolf (1968a; 1968b) discovered depolymerising substances in this area which would reduce the viscosity of the matrix and would thus also regulate the outflow. The heaviest concentrations of mucopolysaccharides are found in the extracellular spaces of the endothelial meshwork in rabbits (Grierson, 1976).

The trabecular meshwork is a self-cleaning filter, although large particles are caught in the cilioscleral sinus before they enter the meshwork (Grierson & Lee, 1973). The meshwork then acts as a series of sieves of diminishing gauge and its endothelial lining cells phagocytose and then degrade the entering debris. If the material cannot be digested, the endothelial cells leave the meshwork and penetrate the inner wall of the collecting channels (Rohen & van der Zypen, 1968).

The angular aqueous plexus is the name given to a series of vessels which drain the aqueous humour from the trabecular meshwork (Tripathi, 1971b). These are equivalent to the canal of Schlemm in primate eyes. A further series of vessels, called the aqueous veins (Ascher, 1942), lead from the angular aqueous plexus to the episcleral venous system. These aqueous veins have been demonstrated in rabbit eyes by Ruskell (1961) using neoprene casts.

The vessels of the angular aqueous plexus have a continuous endothelial lining and the cells of this endothelium have the same ultrastructural characteristics as those in the inner wall endothelium lining the canal of Schlemm in primates. The channels themselves are very similar to Schlemm's canal (Kandori & Okamoto, 1960; Tripathi, 1971b; Grierson, 1976).

Recent studies by Sakimoto (1979) using electron-microscopy, have demonstrated smooth muscle cells adjacent to the vessels of the angular aqueous plexus in rabbits. These extend from the limbal region of the cornea to the ciliary body. The muscle cells are usually located on one side of the channel but occasionally completely surround the channel. Sakimoto suggests that this smooth muscle is involved in the regulation of outflow of aqueous humour in rabbit eyes..

As described above, the conventional drainage pathways function as the major exit for aqueous humour from the anterior chamber. These pathways operate in a pressure sensitive manner (see Cole, 1974 for a review). As such, the rate of outflow of fluid is a function of two factors :

i. the small but significant pressure difference between the anterior chamber and the episcleral veins. Measurements of this pressure difference have been made mainly in human eyes and values vary between 5mm Hg (Linnér, 1955; Grant, 1958) and 6.9mm Hg (Podos, Minas & Macri, 1968).

ii. the resistance offered by the structures in the drainage route. In primates, it is thought that much of the resistance to flow is located between the anterior chamber angle and Schlemm's canal since removal of trabecular tissue has shown that some 60 to 80% of the total resistance can be attributed to the trabecular meshwork and the endothelium on the canal's trabecular side (Grant, 1958; 1963; Peterson, Jocson & Sears, 1971). Grant (1958) reached similar conclusions in experiments with rabbits. However, one group of workers (Edwards, Hallman & Perkins, 1967) failed to produce an obvious reduction in outflow resistance by trabeculectomy in monkeys. The remainder of the resistance to flow of aqueous humour is located in the aqueous veins and the episcleral veins.

The relative distribution of resistance through the outflow system is unknown, although Eriksson & Svedbergh (1980) have shown that less than 5% of the total resistance to outflow in the human eye is located in the inner wall endothelium of Schlemm's canal. The corresponding figure in cynomolgus monkeys with normal intraocular pressure is around 10%.

There has been much discussion over the means whereby aqueous humour passes through the inner wall endothelium of the angular aqueous plexus. Several theories have been expounded and these

are summarised briefly below.

Transcellular channels within the inner wall endothelium were described initially by Holmberg (1959). Giant vacuoles have also been seen, and these can have openings towards both the lumen of the canal and the meshwork (Garron, Feeney, Hogan & McEwen, 1958; Speakman, 1959a; 1959b; 1960; Holmberg, 1959; Lieb, 1960). These vacuoles and channels are also present in sub-primate mammals (Tripathi, 1974).

The current view (Inomata, Bill & Smelser, 1972a; Tripathi, 1971b) is that a channel is formed by invagination from the meshwork side. This grows until it reaches the luminal side whereupon micropinocytosis helps in the formation of a membrane-lined 'minipore'. Thus the formation of a transcellular channel is a dynamic process, but the turnover rate is unknown (Cole & Tripathi, 1971). It is probably induced by hydrostatic pressure alone (Tripathi, 1972; 1973), and it is not thought to be dependent on cellular metabolism since the phenomenon has been observed *in vitro* (Johnstone & Grant, 1973) and at $4^{o}C$ (van Buskirk & Grant, 1974).

A measurement of the number of transcellular channels cannot alone give a reasonable estimate of resistance to outflow since they will not all be patent at the same time. Cole & Tripathi (1971) calculated that the normal outflow of aqueous humour is accounted for, even if the life-span of transcellular channels occupies only a fiftieth part of the total life cycle (probably around 100m sec) of the vacuoles. Studies using the scanning electron microscope have shown that many pores are also found in the flat parts of the cell without any preceding invaginations (Segawa, 1968; Inomata *et al.*, 1972a; Lee & Grierson, 1975).

Tripathi (1971a; 1974) has suggested that formation of transcellular channels and the porees themselves account for most of the resistance to the outflow off aqueous humour. This estimate is in conflict with the evidence of Eriksson & Svedbergh (1980) discussed on p16.

Micropinocytosis is an oxygen-dependent, active process by which small vesicles are transporteed across the cells of the inner wall endothelium. This has been advocated as one of the principle modes of drainage of the aaqueous humour (Fine, 1964; Feeney & Wissig, 1966; Rohen, 1969)). However, the subject has been reviewed by Grierson & Lee (19775) who concluded that, under normal intraocular pressures, microppinocytotic vesicles are not involved in the bulk outflow of aqueeous humour.

Intercellular drainage involvess movement of the fluid between the cells of the endotheliumm of the inner wall lining of the vessels of the angular aqueous pplexi and has been suggested as a principal route for drainage (FFeeney & Wissig, 1966; Rohen, 1969; Lutjen-Drecoll & Rohenn, 1970; Shabo, Reese & Gaasterland, 1973) but perfusion stuudies, using ferritin and other tracers to detect the endothellial permeability, have not given any conclusive evidence for thhe passage of fluid through intercellular clefts (Feeney & Wisssig, 1966; Tripathi, 1971a; Inomata et al., 1972a; Raviola, 19774; Svedbergh & Bill, 1976). Studies using scanning electron micrroscopy have also failed to give conclusive evidence for intercellular drainage except in situations where the intraocular preessure is maintained at very high levels for several hours (Svedbeergh, 1974).

Drainage through gross protrusions in the angular aqueous plexus has also been suggested as a route for outflow. These may be described as hernias of the trabecular meshwork into scleral holes (Wegefarth, 1914; Wolff, 1952; Davson, 1956; Shabo & Maxwell, 1972; Johnstone, 1974). Svedbergh (1976) suggested that such hernias are not a principal drainage route since they occupy only 2% of the projected inner wall surface.

In summary, it seems likely that the presence of transcellular channels easily accounts for the bulk drainage of the aqueous humour and little use is made of the alternative drainage mechanisms.

Conventional and non-conventional drainage routes.

The aqueous humour can leave the anterior chamber by several means, the first of which, conventional outflow, has been described in some detail above. This is a fast route for drainage. The second pathway for drainage is called the uveoscleral route (Bill, 1965; 1966b). The aqueous humour enters the trabecular meshwork and the iris root and then flows posteriorly between the bundles of the ciliary muscle into the supraciliary and suprachoroidal spaces. It can then leave the eye via the loose connective tissue around nerves and blood vessels and it can also move through the scleral substance (Inomata et al., 1972b; Inomata & Bill, 1977). Bill (1966a; 1977b) suggested that this route accounts for a negligibly small amount of the total drainage in rabbits. However, Tripathi & Cole (1976) using blue dextran and fluorescein dextran to trace the drainage routes, suggest that as much as 25% of the drainage of aqueous humour in the rabbit is via the uveoscleral route.

Tripathi (1977b) found this result to be in agreement with the anatomy of the anterior chamber angle of the rabbit, the organisation of which is indicative of a higher flow than in primates. It appears that uveoscleral outflow is not sensitive to changes in the intraocular pressure within the physiological range (Bill, 1967).

Tripathi (1974) mentions other routes of aqueous humour drainage : diffusion across the iris vessels; posterior drainage through the vitreous and into the retina and optic nerve; transcorneal flux. The available evidence suggests that only a small proportion of the fluid leaves the anterior chamber via these accessory routes.

The innervation of the tissues in the iridocorneal angle.

The ciliary body.

Myelinated and non-myelinated nerve fibres abound in the ciliary muscle and elsewhere in the ciliary body. The latter are postganglionic fibres derived from the ciliary ganglion where they link with the parasympathetic outflow of the oculomotor nerve but there is considerable evidence to suggest that some of these fibres are sympathetic. The parasympathetic nerves stimulate the fibres of the ciliary muscle to contract but the role of the sympathetic nerves is still unsettled.

The trabecular meshwork.

A great deal of variation exists in reports of the neuroanatomy of the trabecular region. Vrábec (1954) found a rich network of fibres, possibly sensory, ending as free

terminals or loops in the trabecular meshwork. Holland, von Sallmann & Collins (1956) also noted such structures and suggested that, since these fibres derived from the nerve supply to the cornea, they had a sensory function. The fibres gave free endings beneath the endothelium of Schlemm's canal and within the scleral meshwork facing the inner wall of the canal. Later work by Holland et al., (1957) indicated that many of the axons in the trabecular meshwork were parasympathetic neurones whilst a minority derived from the trigeminal and sympathetic pathways. Recent publications produce conflicting evidence since Brancato, Fusaroli, Mallardi & Ravalico (1980) reported both adrenergic and cholinergic axons in the core of the trabecular laminae whereas Gwin, Gelatt & Chiou (1979) found little evidence for cholinergic axons in the meshwork.

Some non-myelinated fibres run within the trabeculae whilst others run on the surface covered by endothelial cells which replace the Schwann cell covering. The endothelial cells have also been seen to envelop structures closely resembling nerve terminals (Feeney, 1962; Chapman & Spelsberg, 1963; Fine, 1964).

The regeneration of the corneal epithelium after abrasive injury.

The normal renewal of corneal epithelium has already been mentioned (pp 5-6). In this section, the events occurring after injury to the epithelium will be discussed.

Friedenwald & Buschke (1944) discovered that small wounds within the corneal epithelium are covered initially by the neighbouring cells of the basal columnar epithelial layer which extend pseudopodia to cover the area in less than three hours.

Larger defects are covered initially by cells from the surrounding epithelium which slide across the denuded area. This movement of corneal epithelial cells was first reported by Peters in 1885 and further described by Friedenwald & Buschke (1944), Maumenee & Scholz (1948) and Hanna & O'Brien (1960). This process starts within an hour of the injury occurring (Leemp, 1976). Kuwabara, Perkins & Cogan (1976) noted that the cells move in an amoeba-like fashion but saw no evidence of the cytoplasmic constituents necessary to support cell movement. This occurs immediately after the wound. All the epithelial cells appear to move as a tide towards the wound whilst

cells at some distance from the wound flatten and proliferate. Gipson & Anderson (1977), however, did see evidence of actin filaments concentrated as parallel bands in the basal regions of the epithelial cells and they suggested that these may play a role in cell migration. There are sufficient cells in a band of 0.5mm within the corneal limbus to cover an abrasion of the rest of the cornea (Heydenreich, 1958). Estimates of the rate of healing vary from $1.0mm^2hr^{-1}$ (Thoft & Friend, 1977) to $1.42mm^2hr^{-1}$ (Moses, Parkison & Schuchardt, 1979) although the latter authors considered that the absence of injury to the basement membrane might contribute to the high rate of movement seen.

If the corneal stroma is denuded completely, the entire corneal epithelium is replaced by conjunctival epithelium. A single layer of cells can be obtained within four to six days of the abrasive injury and within fourteen days the new epithelium is two to three layers deep (Heydenreich, 1958). At this point, the new epithelium still contains goblet cells which are a characteristic of the conjunctival epithelium. Friedenwald (1951) and Maumenee & Scholz (1948) showed that after six weeks, the goblet cells have disappeared and the

epithelium has the appearance of corneal epithelium. However, Burns (1968) suggested that only two weeks are required for the completion of this process. The differences in these reports verifies the finding that there are great differences between experimental animals in their healing reaction to corneal epithelial injuries (Srinivasan, Worgul, Iwamoto & Eakins, 1977; Bolková & Čejková, 1978).

New epithelium, derived from the conjunctiva and growing over a denuded stroma, has a much slower healing rate than the epithelium derived from the cornea itself (Friend & Thoft, 1978). Healing with conjunctival epithelium is more prone to frequent breakdown of the new epithelium with the occasional occurrence of corneal vascularisation. If the stroma is also involved in the wound, the strength of the healing wound is always less when it has been covered by epithelium derived from the conjunctiva than when it is covered by epithelium derived from the cornea (Friend & Thoft, 1978). Complete removal of the corneal epithelium was followed occasionally by a delay of up to 14 days before the healing process began in a study by Srinivasan et al., (1977). Such delayed or incomplete healing patterns did not occur after incomplete removal of corneal epithelium and it is suggested by these authors that the poorer healing after total removal of corneal epithelium is due to a greater degree of inflammation secondary to the more extensive trauma. This inflammation is characterised by numerous polymorphonuclear leucocytes on the surface of the denuded stroma (Srinivasan et al., 1977).

If the basement membrane is removed along with the corneal epithelium, the development of epithelial adhesion to the corneal stroma is much slower than if the corneal epithelium alone is removed (Khodadoust, Silverstein, Kenyon & Dowling, 1968). Indeed, long standing epithelial defects and erosions may be explained by damage to the basement membrane (Goldman, Dohlman & Kravitt, 1969). Newly migrated corneal epithelium does not adhere to the basement membrane until 6 to 7 days afer the monolayer has covered the stroma (Khodadoust, 1967).

Although conjunctival epithelial cells which have migrated across a denuded stroma maintain their histological characteristics for some 2 to 5 weeks (Maumenee & Scholz, 1948; Friedenwald, 1951; Burns, 1968), clinical observations and biochemical studies have shown that functional transformation may take longer (Thoft & Friend, 1977). There are several histological differences between the two types of epithelium. Conjunctival epithelium is thinner than corneal epithelium and its cells are much more loosely packed with fewer desmosomes. Conjunctival epithelium has mucin-secreting goblet cells and a blood supply, both of which corneal epithelium lacks (Kuwabara, 1970). Corneal epithelial cells are distinctive since they contain fewer mitochondria than do other cells (Kinoshita, 1962). Biochemical distinctions between the two epithelia can also be made. The corneal epithelium possesses large amounts of glycogen and glycogen phosphorylase (Thoft & Friend, 1975b; 1977) whilst conjunctival epithelium has little glycogen (Maumenee & Scholz,

1948; Thoft & Friend, 1977). The conjunctival epithelium has high activities of glycolytic enzymes, enzymes of the tricarboxylic acid cycle and enzymes of the respiratory chain but relatively low enzyme activity in the hexose monophosphate pathway (Baum, 1963; Reim & Cattepoel, 1970; Dark, Durrant, McGinty & Shortland, 1974). In contrast, the corneal epithelium possesses high levels of hexose monophosphate shunt activity and lactate dehydrogenase and a relatively inactive tricarboxylic acid cycle (Kinoshita, Masurat & Helfant, 1955).

After complete removal of corneal epithelium, acid phosphatase and alkaline phosphatase activities have been measured in the regenerating epithelial layer (Bolková & Čejková, 1978). Acid phosphatase activity was low at the beginning and grew steadily until it has reached normal corneal epithelial levels by the 28th day of regrowth. In contrast, alkaline phosphatase showed a delay in growth during the repair process and was still not at normal levels after 28 days of regrowth.

Migrating cells have a high requirement for glycogen and the sliding of cells is prevented by glycolytic and sulphydryl enzyme inhibitors (Kuwabara et al., 1976). Migrating cells also have high levels of lactic dehydrogenase (Kuwabara et al., 1976).

Mouse epithelial growth factor(mEGF).

Cohen (1962) isolated a substance from the submaxillary gland of the male mouse. This was subsequently found to be a polypeptide (Taylor, Cohen & Mitchell, 1970) which produced hyperplasia of the corneal epithelium (Savage & Cohen, 1973; Ho, Davies, Elliot & Cohen, 1974). Savage & Cohen (1973) reported that

this hyperplasia only occurred when the corneal epithelium had been removed from part or all of the stroma and regrowth was occurring. Hyperplasia stopped after six days of regrowth and subsequently the epithelial thickness returned to normal in eight days. The healing process was thus accelerated by the application of mEGF; it was also altered in pattern since a multilayer formed over the wound initially instead of the normal monolayer (Frati, Daniele, Delogu & Covelli, 1972).

The integrity of the stroma is necessary to allow mEGF to act on the epithelial cells (Daniele, Frati, Fiore & Santoni, 1979) and a typical mEGF response will only occur in organ culture where the stroma is present but not in epithelial cell culture where stroma is absent (Gospodarowicz, Mescher, Brown & Birdwell, 1977).

Recent work (Frati, D'Armiento, Gulletta, Verna & Covelli, 1977) has prompted the proposal that mEGF stimulates protein synthesis with a secondary effect on the cyclic adenosine monophosphate system.

An interesting detail in the work of Cohen (1962) and Cohen, Carpenter & Lembach (1975) is that the injection of milligram quantities of mEGF into newborn mice causes precocious opening of the eyelids.

The subject of mEGF has recently been reviewed by Carpenter (1978).

Acetylcholine, choline acetyltransferase and cholinesterase in the rabbit corneal epithelium.

Several tissues, which have no motor or autonomic innervation, have been shown to contain one or more components of the cholinergic system. These include erythrocytes, spermatozoa, the human placenta and the corneal epithelium of many mammals.

The rabbit cornea contains a high concentration of acetylcholine (ACh) (von Brücke, Hellauer & Umrath, 1949; Williams & Cooper, 1965; Fitzgerald & Cooper, 1971). This concentration exceeds even that found in ganglion and brain (MacIntosh, 1941) although it must be noted that this comparison does not take into account the amount of connective tissue present in homogenates of these tissues and absent from the corneal epithelium. High levels of the enzyme choline acetyltransferase (ChAc) have also been found in the rabbit corneal epithelium (van Alphen, 1957; Williams & Cooper, 1965; Mindel & Mittag, 1976; 1977). These levels of ChAc are equivalent to those found in the most highly active forms of cholinergic nerves (Gnädinger, Heimann & Markstein, 1973). This is the enzyme which is responsible for the synthesis of ACh.

$$CH_3CSCoA + HOCH_2CH_2{}^{+}N(CH_3)_3 \xrightleftharpoons{ChAc} CH_3COCH_2CH_2{}^{+}N(CH_3)_3 + HSCoA$$

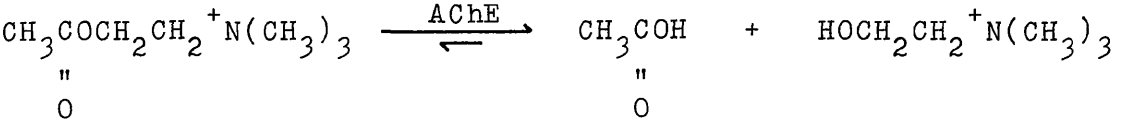
Acetyl CoA — Choline — Acetylcholine

There are marked inter-species, inter-animal and intra-animal variations to be found in the ChAc levels in the corneal epithelium. High amounts of the enzyme are found in the rabbit, bovine and human cornea but very little found in the cornea of the cat. There can be great variations within one specie;

over fifty-fold differences occurring in the ChAc content of rabbit corneal epithelium (van Alphen, 1957; Howard & Wilson, 1972; Howard, Wilson & Dunn, 1973; Mindel, Mittag & Green, 1973; Mindel & Mittag, 1976). In any one animal, the estimated variation between left and right eyes vary from less than 1% (Stevenson, 1974) to 11% (Mindel & Mittag, 1977). In any one rabbit eye, the centre of the cornea has the highest levels of ChAc whilst the superior and temporal areas of the cornea contain the least (Mindel & Mittag, 1977).

Rabbit corneal stroma has negligible amounts of ChAc and ACh (Williams & Cooper, 1965; Gnädinger et al., 1967; Mindel et al., 1973). Very little Ach has been found in the corneal endothelium of the rabbit (Williams & Cooper, 1965).

Acetylcholinesterase (AChE) is the enzyme involved in the breakdown of ACh.



Acetylcholine Acetic acid Choline

It is found in low amounts relative to that found in other tissues (Williams & Cooper, 1965; Gnädinger, Walz, Hahn & Grün, 1967; Laties, 1969; Howard et.al., 1973). Gnadinger et al., (1973) proposed that the low AChE/ChAc ratio which they found in the epithelium explains the high ACh content of this tissue.

The possibility of a neuronal function of the ACh, ChAc and ChE found in the corneal epithelium.

Histochemical and electron microscopy techniques have shown many sensory nerve endings in the corneal epithelium and

have described the histology of this tissue in great detail (Zander & Weddell, 1951; Lele & Weddell, 1959; Pedler, 1962; Robertson & Winkelman, 1970; Hogan et al., 1971; Pei & Rhodin 1971). None of these authors have reported the presence of autonomic effector nerve endings in the epithelium and Laties & Jacobowitz (1964), looking specifically for such structures, could not find any. Ligand binding studies by Olsen & Neufeld (1979) corroborate the earlier evidence for a lack of autonomic nerve endings in the epithelium by showing an absence of muscarinic and nicotinic receptors in this tissue. The only contrary evidence to the above is the claim of Tervo & Palkama (1978) that possible cholinergic neurones containing vesicles exist in the corneal epithelium.

Denervation of the cornea has been carried out by several groups of workers in order to investigate the fall in the epithelial content of ACh, ChAc and AChE. Destruction of the V^{th} nerve resulted in a 35 to 50% drop in ACh levels (von Brücke et al., 1949; Williams & Cooper, 1965). A perilimbal denervation only reduced AChE levels to 50% of the controls (Petersen, Lee & Donn, 1965). The controls for these experiments showed that denervation was associated with loss of corneal sensation. However, Fitzgerald & Cooper (1971) reported that local denervation of the rabbit cornea resulted in an 87 to 100% loss of epithelial ACh content, which was associated with a loss of corneal sensation. The most recent work in this field has shown that a 'denervation' of the eye by retrobulbar injection of ethanol, produced a reduction in ChAc levels in the iris-ciliary body but not in the corneal epithelium (Mindel & Mittag, 1977).

The pattern of development of ChAc and AChE in the cornea of neonatal rabbits has been reported to differ considerably from the patterns of development in the rabbit central nervous system (Howard et al., 1973; Hebb, 1956; McCaman & Aprison, 1964). In neonate rabbits the bulk of ACh and ChAc in the corneal epithelium is absent until about the time that the eyelids open, at which time the presence of corneal sensation can be shown. After the eyelids open, ChAc activity shows a steady development for a further seven weeks.

Further evidence against the claim that corneal ChAc activity is mainly associated with nerves was presented by Gnadinger et al., (1973): cultured corneal epithelial cells, obviously outwith the influence of nerves, possess about 50% of the ChAc activity measurable in whole corneal epithelium. A significant part of the corneal epithelial ACh and ChAc content does not appear to be associated with the sensory neurones of the cornea.

The localisation of AChE, ChAc and ACh within the corneal epithelium.

The AChE which is found in the corneal epithelium has been shown not to be directly associated with nerve endings. It is associated with the epithelial cell surfaces and within the epithelial cells (Howard, Zadunaisky & Dunn, 1975). In the cat cornea, AChE is located mainly in the surface layers of the epithelium (Mittag, Harris, Cohn, Galin & Ehrenpreis, 1970).

Location of epithelial ChAc has been difficult since a specific and sensitive histochemical method to detect its presence has yet to be found (see Burt & Silver, 1973, for a review).

ChAc may be a soluble enzyme (see the review by Rossier, 1977) but its exact intracellular location has yet to be established.

Little is known of the location of epithelial ACh but Murahata & Wilson (personal communication) suggest that it is located largely in the epithelial cell cytoplasm.

Uptake of choline in the corneal epithelium.

Choline is one of the compounds which is necessary for the synthesis of ACh. It is a quaternary ammonium compound and because of its charge it does not diffuse freely through membranes. Two carrier systems for the transport of choline into nerve terminals have been described in the literature. The first system has a low affinity for choline (Marchbanks, 1968; Potter, 1968; Diamond & Kenmedy, 1969) and it seems to be a component of all cell membranes (Diamond & Milfay, 1972). The second carrier system has been described as a high affinity choline transport system (Yamamura & Snyder, 1973; Haga & Noda, 1973; Guyenet, Lefresne, Rossier, Beaujouan & Glowinski, 1973). This latter system has been shown to be dependent on the presence of sodium ions (Simon & Kuhar, 1976) and it is specifically associated with cholinergic nerve endings (Kuhar, Sethy, Roth & Aghajanian, 1973; Sorimachi & Katsaoka, 1975).

Paterson & Wilson (1977) have characterised the choline uptake system of the corneal epithelium as low affinity, not dependent on sodium ions and relatively insensitive to inhibition by hemicholinium-3, a drug which blocks the high affinity uptake system. Thus, despite the large capacity of the epithelium to

synthesise ACh, it lacks the high affinity choline uptake mechanism which is associated with cholinergic neurones. This is further evidence for the lack of association of corneal ACh with nerves. The choline uptake into corneal epithelium was similar to that found in the human placenta, a non-innervated tissue rich in ACh and ChAc, which also possesses only a low affinity choline uptake mechanism.

Possible functions of acetylcholine in the corneal epithelium.

The ACh of the corneal epithelium appears to be largely unassociated with neuronal function and there appears to be no cholinergic innervation of this tissue. A variety of functions for the bulk of the ACh have been proposed and each proposal will be discussed briefly below.

1. Light stimulation of the eye.

Since ACh has also been found in the aqueous humour of the anterior chamber, Engelhart (1931) proposed that the ACh content of the aqueous humour may be related to the amount of light entering the eye. Rabbits deprived of light for some hours did not have any ACh in the aqueous humour yet the ACh content rose in the aqueous humour of one eye exposed to light thereafter. These results could not be confirmed, however, by Fitzgerald & Cooper (1971).

2. Integrity of the corneal epithelium.

The presence of ACh may be a requirement for the maintenance of the integrity of the tissue. Early work by von Brücke et al., (1949) using lesions of the V^{th} nerve (either viral or surgical) reported an ensuing breakdown of the corneal epithelium. A

dramatic regeneration of the epitheelium then followed after topical administration of ACh. TIhis was compatible with an earlier theory of von Brücke (1938)) that the ACh exerted a trophic influence upon the epitheliial cells.

Until recently, no other work had been reported in this field until Mindel & Mittag (1978) started to work with rabbits subjected to blepharorrhaphy. Thesse authors found that in a number of animals subjected to lid closure, epithelial defects were observed upon reopening their eyes. It was found that by the 8th day after lid closure, by wwhich time many of the original epithelial cells will have been repplaced, the ChAc activity was reduced considerably in the corneall epithelium but not in the iris, ciliary body or retina. The recovery of the original enzyme levels required more than foour weeks, so blepharorrhaphy affected subsequent cell generationms. Epithelial defects are found in 50% of the eyes but it is unlikeely that the low ChAc levels are due to corneal epithelial defects since low ChAc levels were also seen in intact epithelia..

3. Sensory role.

ACh has been implicated as a possible sensory mediator at the chemoreceptors of the carotid body (Schweitzer & Wright, 1938; Liljestrand & Zotterman, 1954; Eyzaguirre & Koyano, 1965; Eyzaguirre & Zapata, 1968); at thermal receptors (Zotterman, 1953); at taste fibre endings (Landgren, Liljestrand & Zotterman, 1954); at cutaneous mechanoreceptors (Harvey, Lilienthal & Talbot, 1941; Diamond, 1959); and in the auditory system (Comis & Davies, 1969).

Hellauer (1950) studied various different species and found an approximate correlation between corneal ACh content and corneal sensitivity. Van Alphen (1957) found that topical atropine did not alter corneal sensitivity and Oer (1961) had rather variable results with the effect of topical atropine and choline esters on corneal sensation. In 1971, Fitzgerald & Cooper revived the idea that the ACh of the corneal epithelium in rabbits was involved in sensory mediation since a perilimbal denervation of the cornea reduced the epithelial ACh by 87 to 100% and this was accompanied by a complete loss of corneal sensation. These authors also found that intraocular injection of hemicholinium-3 reduced the corneal ACh levels by 60% and abolished corneal sensation. On the other hand, Stevenson & Wilson (1974) found that similar injections of hemicholinium-3 had no effect on the epithelial ACh content and did not abolish the corneal reflex. These authors also used intraocular injection of triethylcholine, which is known to cause depletion of ACh from tissues, and trans-4-((1-naphthylvinyl) pyridine hydrochloride (NVP) which is a specific ChAc inhibitor, to produce some depletion of ACh from the corneal epithelium. However, this depletion had no effect on corneal sensation.

Howard & Wilson (1972) demonstrated that in neonatal rabbits the corneal reflex can be elicited before the appearance of the bulk of ChAc and ACh in the epithelium, thus indicating that sensation does not depend on mature levels of ACh. Boberg-Ans (1955) found that corneal sensitivity was greatest at the centre of the cornea, the site shown to contain the greatest quantity of epithelial ChAc (Mindel & Mittag, 1977). Although this very

simple coincidence exists, the latter authors failed to find a correlation between corneal ChAc activity and the loss of corneal sensation which follows retrobulbar injection of ethanol.

4. Ion transport.

There has been much investigation into the mechanisms involved in the control of corneal hydration. An electrical potential across the corneal epithelium has been shown to be responsible for an inward transport of sodium ions (Donn, Maurice & Mills, 1959a; 1959b; Green, 1965; 1966; 1967a; 1967b; 1968; Hodson, 1974). Green (1969) has proposed that this epithelial sodium 'pump' may contribute to the maintenance of the normal swelling properties of the mucopolysaccharide in the stroma by supplying sufficient sodium ions to saturate the anionic sites on the chondroitin and keratan sulphates in the stroma (Otori, 1967). This would prevent swelling of the mucopolysaccharides as a result of mutual repulsion of like charges (Hedbys, 1961). A second process, an endothelial salt 'pump' (Hodson, 1974) may regulate the movement of water and sodium ions out of the stroma into the aqueous humour. There is general agreement that the endothelium is more important than the epithelium in controlling stromal hydration (Mishima & Kudo, 1967; Maurice, 1972; Hodson, 1974; 1977; Hirsch, 1978). Indeed, in some species, the epithelium can be replaced by an impermeable contact lens without adversely affecting the stroma; the rabbit cornea, however, did show increased hydration following this procedure (Doane & Dohlman, 1970).

Gnädinger et al., (1973) suggested that the epithelial ACh content may be associated with the maintenance of this electrical potential across the corneal epithelium. Stevenson & Wilson (1975) proposed that since ACh influences sodium permeability in many other tissues, the endogenous ACh of the corneal epithelium may modify sodium movements across this tissue and hence play a role in controlling corneal thickness and transparency. They found that high concentrations of ACh applied to the isolated eye could decrease the movement of $^{22}Na^{+}$ across the epithelial barrier. The use of NVP to deplete corneal ACh increased the movement of $^{22}Na^{+}$ and this could be reversed by application of ACh. On the other hand, a number of inconsistencies existed in the effects of physostigmine and atropine on this system.

Mindel, Szilagyi, Zadunaisky, Mittag & Orellana (1979) reported that prolonged blepharorrhaphy in rabbits produced not only a major depletion of epithelial ChAc and ACh but also a small but significant increase in corneal hydration, and decreases in the resting potential and short-circuit currents. It was tempting to explain these observations in terms of an effect of epithelial ACh on active sodium transport by the epithelium. However, it was found that application of ACh, pilocarpine or physostigmine to such eyes failed to restore the resting electrical potentials and short-circuit currents of the epithelium to normal. Moreover, although the ChAc activity of the epithelium after lid closure was closely related to the ACh content of the tissue, it could not be related to either the resting potential or the short-circuit current in individual eyes.

Recovery of these electrical phenommena to normal after lid closure occurred sooner than recoveery of ChAc and ACh.

5. Possible role in regulation of outflow of aqueous humour.

Cholinergic agonists, in partiicular pilocarpine, have long been used to reduce intraocular pressure in disease states like glaucoma simplex. This effectt is achieved by reducing the resistance to outflow of the aqueous humour from the anterior chamber. This is more commmonly expressed as an increase in the facility of outflow. Much ccontroversy exists over the mechanism of action of cholinergic agonists on outflow. The proximity of the ACh stores in the corneal epithelium to the trabecular region which controls thee outflow, introduced the possibility that ACh from this sourcce could influence the outflow of aqueous humour. Such a ssuggestion has not appeared in the literature.

OBJECT OF RESEARRCH

In view of the fact that the rabbit corneal epithelium possesses high levels of ACh which do not appear to be entirely neuronal in function nor in location, this research attempted to elucidate a possible function for this ACh and its associated enzymes. The corneal epithelium has several unique properties, one of which is its capability for rapid regeneration after injury. The possibility that the high levels of ACh were linked to regeneration of this tissue after abrasive injury was investigated.

There is a possibility that the corneal epithelial ACh has a role to play outwith the tissue itself. If this were the case, it may move either laterally, anteriorly or posteriorly. The ACh might be released into the tear film and then act upon any of the structures bathed in the tear fluid. This postulate was investigated. Alternatively, the hormone may more posteriorly into the aqueous humour in order to act upon any of the structures bathed by this fluid. Several experiments were carried out to explore the feasibility of such movement. Firstly, the levels of ChE in the tissues of the anterior chamber were measured in order to estimate the possibility of complete destruction of the ACh before it could reach a possible site of action. Secondly, we determined the sites to which radiolabelled ACh, injected into the corneal stroma, had diffused. Thirdly, the iris was used as an *in vivo* test tissue to allow investigation of whether exogenous ACh was capable of diffusing from the cornea to affect tissues at a distance. Fourthly, in view of the well-known effect of cholinergic agonists on the outflow of aqueous humour, it seemed logical to examine the effect of intracorneally injected ACh on this parameter.

METHODS

Measurement of protein levels and ChAc activity in the corneal epithelium after regeneration of the tissue.

Removal of the corneal epithelium.

Male and female Dutch rabbits (1.2 to 1.6kg) were anaesthetised with halothane (3.5% v/v) in O_2:N_2O (1:3 v/v).

In experiments in which a 100% removal of the corneal epithelium was required, the entire surface was scraped up to the boundary between the corneal and conjunctival epithelia (the limbus). When a 95% removal of epithelium was required, the cornea was scraped to within 1mm inside the limbus. In all cases, the epithelium was removed using a spoon-shaped scalpel (4mm diameter, Storz).

The rabbits were treated immediately and then 4hr after the operation with one drop of Xylocaine (lignocaine hydrochloride, 4% w/v) eye drops in each eye.

The epithelium from each eye was homogenised in 200µl of buffer (50mM Tris in 150mM sodium chloride) in a ground-glass homogeniser.

The homogenates were stored at -40°C until analysis.

After removal of the epithelia, the animals were allowed to recover from anaesthesia and the epithelia permitted to regenerate for various time intervals between 2 and 28 days. After the allotted time, the animals were killed with halothane - N_2O and the regenerated epithelium removed from both eyes, then homogenised and frozen as previously described.

The homogenates from the initial scraping acted as controls for the homogenates of regenerated epithelium and both were

assayed simultaneously for choline acetyltransferase (ChAc) and their protein levels determined (see p 42).

Mouse Epithelial Growth Factor (mEGF) treatment.

Bilateral 95% biopsy of the corneal epithelia was carried out as described above with local anaesthetic application immediately and 3.5 hr after the operation. In each rabbit so operated, the left eye was treated topically with 25μl sterile saline (0.9% w/v Minims and the right eye with 25μl of a solution (0.4mg/ml) of mEGF in sterile saline (0.9% w/v). The saline and mEGF applications were made at 9.00 a.m., 1.00 p.m., 5.00 p.m., and 9.00 p.m. for 4 days, or, in a second experiment, for 5 days.

The animal was killed with halothane - N_2O and the corneal epithelia removed (95%) as described previously. The two homogenates (control and mEGF treated) were assayed for ChAc activity and protein as described below.

Protein Determination (Lowry, Rosebrough, Farr & Randall, 1951).

Aliquots of homogenate containing an estimated 10 to 50μg of protein were made up to 100μl volume with N sodium hydroxide. 1ml of solution C (see appendix p164) was added to each sample and mixed.

After 15 min, 100μl of N Folin-Ciocalteu reagent was added to each sample. The solutions were mixed immediately and allowed to stand at room temperature for 30 min before the measurement of their extinction at 750nm on a Cecil grating spectrophotometer (Model CE303). Distilled water was used

as the reference.

A standard curve was prepared for each batch of samples by assaying a series of bovine serum albumin samples (0 to 50μg) made up in distilled water and diluted to 100μl with N sodium hydroxide.

ChAc Determination (Schrier & Shuster, 1967; as modified by Wilson & Cooper, 1972).

The enzyme ChAc catalyses a reaction between choline and acetylcoenzyme A (AcCoA) to form ACh

$$\text{Choline} + \text{AcCoA} \xrightarrow[37^{o}C]{\text{ChAc}} \text{ACh} + \text{CoA}$$

If endogenous AcCoA is removed from a tissue homogenate and replaced with radioactively labelled AcCoA, the end product is radiolabelled ACh which can be separated from the radiolabelled AcCoA by the use of an anion exchange resin. The resin can exchange formate ions from within its matrix for anions passing through the column i.e. AcCoA, whilst allowing cations, i.e. ACh, to pass through the column unbound. Hence the amount of product formed by the endogenous ChAc in the homogenate can be determined. This is a direct measure of the amount of ChAc in the tissue.

Aliquots of tissue homogenate were made up to 100μl with buffer (50mM Tris base in 150mM sodium chloride). Then 90μl of this buffer mixture, to which choline chloride (5mM), sodium ethylene-diaminetetra acetate (5mM) and neostigmine methylsulphate (50μm) (expressed as final concentrations) had been added, was mixed with every sample. The mixture was incubated

at 37°C for 15 min to permit metabolism of any AcCoA present in the tissue homogenate.

[1- ^{14}C] AcCoA (10µl, 0.84 nmol, 50nCi) was added to each sample and the incubation continued for 20 min. The reaction was halted by addition of an excess of ice-cold ACh (40µl of 0.72M) and then placing the test-tube in ice. Each reaction mix was put on a separate column (5 x 0.8cm) of the anion-exchange resin AG 1 X 8 (200 to 400 mesh, formate form) and the [1- ^{14}C] ACh formed during the reaction was eluted into a counting vial by 6 x 0.4ml distilled water washes. Toluene-Triton X scintillant mixture (8ml) was added. The ^{14}C emission was counted for 5 min in a Packard Tri-Carb Liquid Scintillation Spectrometer (Model 3390).

Each homogenate was assayed in duplicate. Blanks i.e. with no addition of homogenate, were also run to account for ^{14}C-labelled AcCoA which failed to bind to the resin.

Efflux of [^{3}H] Choline and [1- ^{3}H] ACh from the cornea into the tear fluid.

In an effort to discover whether the endogenous ACh of the corneal epithelium can be released anteriorly into the tear film and whether this release can be stimulated by any chemical or physical means, experiments were conducted in which labelled choline was injected into the corneal stroma and subsequent efflux of radioactively labelled ACh into the tear fluid was monitored.

Male and female Dutch rabbits (1.2 to 1.6kg) were anaesthetised with halothane (3.5% v/v) in O_2 : N_2O (1 : 3 v/v) and given diazepam (10mg) intravenously. The fine needle tip of a 10µl microsyringe was inserted tangentially through the corneal stroma for a distance of at least 4mm until the tip lay within 1 to 2mm of the centre of the cornea. This area lies under the central corneal epithelium which is believed to contain the highest levels of endogenous ACh in the whole of the cornea (Wilson, unpublished observations). The needle insertion permitted intra-corneal injection of [^{3}H] choline chloride (1µl containing 1µCi, 4nmol) in distilled water, at time zero. This procedure is known to result in the labelling of a substantial part of the epithelial ACh store : the ratio of [1- ^{3}H] ACh to [^{3}H] Choline after the injection is in the range 0.5 to 2.5 after 60 min, and this ratio is considerably higher (3 to 8), if ChE inhibitors are present (Wilson, unpublished data). Thus, sampling of the tear film at 5 min intervals was begun an hour after the injection was made. Three methods for collecting the tear fluid were attempted :

1. an attempt at cannulation of the entire nasolacrimal duct was made to allow sampling of the tear fluid at the nasal end. This was unsuccessful due to the long and tortuous nature of the canal.

2. a shorter cannulation was made, bringing the cannula through the skin after it had travelled some 6 to 7 mm down the lacrimal duct. The cannulation was made with Portex 800/100/200/100 polythene tubing which fitted tightly inside the duct. This method was unsuccessful since the cannulae frequently became blocked with mucus, making routine sampling difficult.

3. the last method employed was blockage of the lacrimal duct with a 1cm length of the Portex tubing filled with wax. This prevented loss of tear fluid down the duct. Samples of fluid were removed from the conjunctival sac using 4mm wide strips of Whatman No. 40 filter paper. The filter paper was left in the sac for 45 sec and the amount of fluid (approximately 10μl) removed was measured by weight difference before and after sampling. This last method was chosen for the investigation of [1- ^{3}H] ACh release.

Control collections were taken by letting 10μl of saline (0.9% w/v) flow over the surface of the cornea immediately before collection by filter paper. In test situations, 10μl of the drug solution was allowed to flow over the corneal surface 5 min before sampling in order to give sufficient time for penetration of the drug to its site of action. In most cases, this volume, reduced in size by evaporation, but supplemented by fresh tears formed, was sufficient to allow sampling by

filter paper. It was always sufficiently small to stay totally within the conjunctival sac and so none was lost by overflow. Occasionally, the volume of liquid in the conjunctival sac was insufficient to permit sampling with ease and it was supplemented by 10μl of saline (0.9% w/v) washed over the cornea immediately before sampling..

The various stimuli used were :

Hypertonic saline (1.8% w/v) which would double the tonicity of the tear film and this could be a stimulus for the release of ACh.

Distilled Water which would reduce the osmotic strength of the tear film and act as a stimulus for ACh release.

Iso osmotic sucrose (0.25M) would remove by dilution most of the extracellular Na^+ ions present in the tear film bathing the epithelium, without altering the osmolarity of the tear fluid; some link between Na^+ movement and the corneal content of ACh has been postulated (Stevenson & Wilson, 1975).

Calcium chloride (2.5mM) in saline (0.9% w/v) was used since Ca^{2+} ions are involved in the transmission of nerve impulses and might act as a stimulus for ACh release if this process was initiated by a neuronal mechanism.

Potassium chloride (50mM) in saline (100mM) was applied since K^+ ions, like Ca^{2+} ions, are involved in the transmission of nerve impulses.

Oxybuprocaine hydrochloride (0.4% w//v, Minims) is a local anaesthetic and would block impulse traffic in corneal sensory

nerves; an association between corneal ACh and sensory mediation has been put forward by Fitzgerald & Cooper (1971).

Amethocaine hydrochloride (1% w/v, Minims) is another local anaesthetic.

Tetrodotoxin (10^{-4}g/ml) in saline at pH 4.0 with the control being citrate at pH 4.8 in saline ((0.9% w/v), specifically blocks Na^+ channels in the membranes of excitable cells and, in this instance, will have an effect similar to that of the local anaesthetics.

Sodium ethacrynate (2mM) in saline (0.9% w/v) has an effect on Na^+ movement through the epithelium which is similar to the effect of ACh on Na^+ movement across the corneal epithelium (Stevenson & Wilson, 1975).

Atropine sulphate (100μM; 10mM; 25mM) in saline (0.9% w/v) has been reported to increase ACh release from the cerebral cortex (Aquilonius, Lundholm & Winbladh, 1972).

Neostigmine methylsulphate (100μM; 10mM) in saline (0.9% w/v) is an anticholinesterase and may prevent hydrolysis by cholinesterases of any ACh released. Anticholinesterases applied topically are known to increase the ACh content of the epithelium (Wilson, unpublished observation).

Compound 48/80 (0.64 mg/ml) in saline (0.9% w/v) is known to release radioactivity from the corneal epithelium loaded *in vitro* with [^{3}H] -choline (Wilson, unpublished observation).

Nicotine (0.77μM; 5.8μM) in saline (0.9% w/v) has been shown to release ACh from the human placenta and other non-neuronal tissues which contain ACh (Rowell & Sastry, 1977).

Oxotremorine (10μM; 1mM) in saline (0.9% w/v) has been shown to have an action similar to nicotine in rat brain (Holmstedt, 1967).

Physical stimulation of the corneal sensory mechanisms was achieved by formation of dryspot using a fan and benzalkonium chloride (0.01% w/v), 10μl) which is known to produce dryspots (Wilson, Duncan & Jay, 1975) or by mechanical stimulation. This was carried out by touching the cornea six times per minute with the tip of a 4cm length of surgical silk (size 0) alone, and then in the presence of benoxinate (0.4% w/v, 10μl, Minims).

Deprivation of light (during the experiment; for 24hr prior to the experiment; for 72hr prior to the experiment) might affect the anterior release of ACh since it has been shown that blepharorrhaphy suppresses corneal epithelial ChAc activity (Mindel & Mittag, 1978).

The filter papers containing each sample were inserted into counting vials which contained 1ml of distilled water and left standing for 60min to allow elution of the radioactive material from the paper into the water before addition of 8ml of toluene-Triton X scintillant. Control experiments showed that the quenching due to the filter paper was constant. The ^{3}H emission was counted for 10 min in the Packard Liquid Scintillation Spectrometer.

In order to collect sufficient radioactive material for thin layer chromatography (T.L.C.), three consecutive samples were collected on the same piece of filter paper to obtain a "pre-test" control. The paper was dried in a stream of warm air in the intervals between collections. The same procedure yielded a single strip of paper for the "test" sample and another strip for the "post-test" control.

Since it was necessary to protect the ACh (in the samples for T.L.C.) from cholinesterases in the tears, neostigmine methylsulphate (30μM) was added to the saline (0.9% w/v) which was used for control washes and to make up the drug solutions for the test washes. This provision did not apply to the experiment which involved neostigmine as the test drug.

Once the filter papers contained the control or test fluids, they were dried and the [^{3}H] choline or [1-^{3}H] ACh eluted from the paper by allowing a N formic acid : acetone (15 : 85 v/v) mixture to run by capillary action down the paper into a collection tube. The paper was soaked for a further 40 min in the eluate. Then the eluate was evaporated to dryness in a vacuum drier and reconstituted with 10μl of distilled water for T.L.C. as described below.

Each filter paper, after withdrawal from the eluate, was dried, placed in a counting vial containing 1ml distilled water and allowed to stand for 60 min before 8ml of toluene-Triton X scintillant was added. The ^{3}H emission was then counted for 10 min to allow estimation of the residual [^{3}H] choline and [1- ^{3}H] ACh which had not been eluted from the filter paper.

Thin layer chromatography.

The method used was a modification of that described by Hasson-Voloch & Simas (1969). Kodak-Eastman plastic backed plates (20 x 20cm, Code 13253 without fluorescent indicator) were used throughout the project and these were heated (100°C, 15 min) to desiccate. The mobile phase consisted of glacial acetic acid: butan-2-one (1:15 v/v). The "cold carriers" applied to the T.L.C. plate before application of each sample were 2µl of choline chloride (100mM) and 2µl of ACh Cl (100mM). A minimum of five samples and a maximum of eight samples were run on each plate.

The reconstituted sample (8µl from the 10µl) was spotted onto the plate in aliquots not larger than 0.5µl which were dried before further additions.

The T.L.C. plate was set in the tank containing the mobile phase and the solvent front was allowed to rise to 14cm before the sheet was removed and dried in a stream of warm air. The plate was then sprayed evenly with either a modified Draggendorf's reagent or iodoplatinate reagent (see appendix, p 163) for identification of the ACh (pink spot, R_f value 0.6) and choline (purplish-brown spot, R_f value 0.46).

The zones of colour for ACh and choline were outlined in pencil and the colour driven off the whole plate by warm air. The surface of the plate was divided into rectangles of approximately equal area so that the quenching caused by alumina in each sample would be approximately equal. The chromatogram derived from each sample was divided into five rectangles.

	Approximate R_f value
Origin area	-0.1 to 0.1
Pre-choline spot area	0.1 to 0.3
Choline spot area	0.3 to 0.5
ACh spot area	0.5 to 0.7
Solvent front area	0.7 to 0.9

Each area was scraped from the T.L.C. plate into a scintillation counting vial containing 1ml of distilled water. After 2hr, 12ml of toluene-Triton X scintillant was added to allow the ^{3}H emission to be counted for 100 min on the Packard Tricarb.

Assay of cholinesterases in tissues of the anterior segment of the eye.

Rabbits were killed with a halothane -N_2O mixture. The corneal epithelia were completely removed up to the limbus (see p 40) and the epithelia of the left and right eyes homogenised together in 200µl potassium phosphate buffer (0.1M, pH 8.0) containing 0.5% (v/v) Triton X-100 (henceforth referred to as phosphate-Triton). This was then kept on ice until the other homogenates were prepared.

The aqueous humour was removed by paracentesis, mixed with 100µl phosphate-Triton and placed on ice. Air was injected into the anterior chamber to support the cornea and the central area of the cornea delimeated with a 1cm diameter trephine.

Then each eye was enucleated and incised from the posterior aspect. The irides were excised along with the ciliary bodies and frozen in liquid nitrogen. The pooled frozen tissue from both eyes was pulverised with a loosely fitting stainless steel pestle in a stainless steel centrifuge tube, and homogenised in 0.5ml phosphate-Triton.

Lastly, the corneas were removed by cutting around the trephine mark, frozen in liquid nitrogen and pulverised. Thereafter, the tissue from both eyes was homogenised in 1ml of phosphate-Triton. The stainless steel centrifuge tube containing powdered frozen tissue was first plunged into a beaker of wet ice, thereby bringing the temperature to $0^{\circ}C$ so as to prevent freezing of the buffer solution. The

homogenisation was carried out with a loosely fitting Perspex pestle rotating at about 1000 rev/min.

Blood samples removed from each animal were centrifuged (1000g, 10 min, 4°C) and 0.5ml of the supernatant plasma was mixed with 100µl of phosphate-Triton.

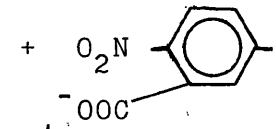
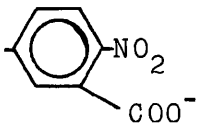
Cholinesterase (ChE) activity was measured by the method of Ellman, Courtney, Andres & Featherstone, (1961). This spectrophotometric assay measures enzyme activity by following the increase in intensity of yelloww colour produced from thiocholine when it reacts with the 5,5 dithiobis-2-nitrobenzoate (DTNB) ion

$$H_2O + (CH_3)_3{}^+NCH_2CH_2SCOCH_3 \xrightleftharpoons{ChE} (CH_3)_3{}^+N\,CH_2CH_2S^-$$

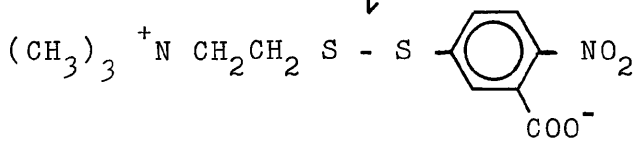
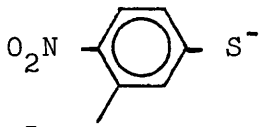
Acetylthiocholine — thiocholine

$$+ \quad CH_3COO^- + 2H^+$$

acetate

$(CH_3)_3{}^+NCH_2CH_2S^-$ +  DTNB 

1

 + 

5' thio 2 nitrobenzoic acid

yellow

The second reaction is sufficiently rapid to prevent it being the rate limiting step.

DTNB solution (100µl, see appendix p164) was mixed with acetylthiocholine chloride solution (300µl, see appendix p 164) in a cuvette (1ml capacity) and 100µl of the tissue homogenate

was added. The extinction (E) of the reaction mix was examined at a wavelength of 412nm at 25°C using a Unicam recording spectrophotometer.

As a standard, pure acetylcholinesterase (AChE, 1μmolar unit per μl) from the electric eel (Electrophorus electricus) was used, 20μl being mixed with 20ml of potassium phosphate buffer (pH 8.0).

After measurement of the E_{412} of each sample for 5 min, a specific antagonist of AChE, BW 284 C51, was added (100μl of 50μM) and the E_{412} read for a further 2 to 3 min. Neostigmine methylsulphate (10μl of 0.5mM) was then added as a non-specific anticholinesterase and readings continued for a further 2 to 3 min. Hence total ChE and AChE concentrations could be determined. Protein assays of the tissue samples were carried out as described above by the method of Lowry et al., (1951) (pp 41-42).

Recovery of [1-^{3}H] ACh and [^{3}H] choline from the tissues of the anterior segment after an intracorneal injection of [1-^{3}H] ACh.

Male and female Dutch rabbits were anaesthetised with halothane 3.5% (v/v) in O_2:N_2O (1:3 v/v) and [1-^{3}H] ACh Cl (1μl, 1μCi, 4nmol) injected intracorneally (see p 44), at time zero. The animals were killed with halothane - N_2O as close to 5 min after the injection as possible and the following tissues removed as described previously (p 52) : the aqueous humour, irides and corneal buttons. The ciliary body was removed as a ring of pigmented tissue and the greyish ciliary muscle underlying the processes was also removed.

The tissue were treated as follows; separate homogenates being made for tissue from each eye :

Aqueous humour : 0.2ml of aqueous humour was added to 0.5ml of a N formic acid : acetone (1:15 v/v) mixture. These samples were allowed to stand on ice for 2 hr in sealed tubes.

Irides : these were frozen in liquid nitrogen and pulverised (as described on p 52) before being homogenised in 0.5ml of N formic acid : acetone (1:15 v/v) mixture. These were allowed to stand on ice for 30 min in sealed tubes

Ciliary bodies : as for irides

Corneal buttons : as for irides

All the samples were centrifuged (1000g, - 4^{o}C, 5 min) and the supernatant removed and evaporated to dryness on a vacuum drier. The residues were dissolved in 10μl of distilled water and 8μl of the resultant solution was used in T.L.C analysis

using the method described previously (pp50-51), the reagent for identification of the ACh and choline was iodoplatinate.

The radioactivity in the tissue residue after the centrifugation was estimated by dissolving the tissue entirely in 0.5ml of 10 N KOH at 60°C. Some of this solution (0.1ml) was transferred to 1ml of distilled water and 8ml toluene-Triton X scintillant. The ^{3}H emission was counted for 10 min on a Packard Tricarb.

The effect of intracorneal injection of ACh on pupil diameter.

Male and female Dutch rabbits (1.2 to 1.6kg) were anaesthetised with halothane (3.5% w/v) in O_2:N_2O (1:3 v/v) and set in a restrainer box in a dark room. Measurements of pupil diameter were made using a modified Hamblin slit lamp microscope with a graticule set in the eyepiece.

Once constant control readings of pupil diameter had been obtained, ACh Cl solution was applied to the eye, either by dropping small volumes (up to 0.5μl) onto a 4mm diameter circle of bared stroma in the centre of the cornea (the corneal epithelium was removed as described previously, p 40) or by injecting into the corneal stroma small volumes (0.5μl of varying concentrations) using the method described previously (p 44). Thereafter, measurements of pupil diameter were made at frequent intervals over the first 30 min after the injection in order to quantify any miosis which occurred.

In several experiments, atropine sulphate (1% w/v) in saline (0.9% w/v) was applied topically (10μl) to confirm antagonism of the ACh-induced effect.

The effect of intracorneal injection of ACh on the apparent facility of outflow from the anterior chamber.

Male and female Dutch rabbits (1.2 to 1.6kg) were anaesthetised with urethane (25% w/v in H_2O) until the corneal reflex disappeared. The animals were laid prone in a restrainer box designed to hold the head steady. In later experiments, a tracheal cannula was inserted and the animals laid supine in the box.

A 26-gauge needle (5/8 inch, Becton-Dickinson with the hub removed) which was connected to a 10µl microsyringe by fine silicone rubber tubing (0.5mm bore, 0.25mm wall thickness) was inserted tangentially through the stroma for at least 4mm until the needle tip lay within 1 to 2mm of the diametral centre of the cornea. This microsyringe was used to inject small volumes (less than 1.0µl) of ACh Cl solution into the corneal stroma.

A similar needle was inserted parallel to the plane of the iris into the anterior chamber of both eyes. This needle ran within the stroma for 2 to 3 mm before entering the chamber so that it was leakproof and held securely in position as far from the other structures in the chamber as possible. This needle was connected by silicone rubber tubing (as above) to a Statham-Gould (P23 1D) pressure transducer and a Grass Polygraph recorder (Model 7) via a double 3-way stopcock assembly. Drugs could be introduced into the system at the stopcock.

A polythene funnel reservoir was also connected by silicone rubber tubing to the intraocular needle via the stopcocks. The reservoir was hung on the arm of a Gallenkamp torsion

balance (full scale deflection 1000mg) which stood upon a rack of adjustable height. This rack allowed the reservoir to be quickly adjusted to a height which exerted a pressure of 4mm Hg greater than the resting intraocular pressure of the eye.

The reservoir, tubing and pressure transducer were fitted with an aqueous humour substitute of the following composition. Later experiments employed the second formula.

Formula 1 (after Bárány, 1964)	mM	Formula 2	mM
NaCl	140.0	NaCl	110.0
KCl	5.0	KCl	3.0
$CaCl_2$	1.0	$CaCl_2$	1.4
$MgCl_2.6H_2O$	0.3	$MgCl_2.6H_2O$	0.5
Na_2HPO_4	0.6	Na_2HPO_4	0.9
glucose	5.5	$NaHCO_3$	30.0
ascorbic acid	1.0	glucose	6.0
heparin	20.0 units/ml	ascorbic acid	1.0
		heparin	20.0 units/ml

both were aerated with $O_2:CO_2$ 95:5v/v and had a final pH of 7.4.

The needles for intraocular use were filled with heparin (1000 units/ml). As soon as this needle had been inserted, the stopcock was opened and the undiluted heparin run into the eye to prevent formation of fibrin clots.

In order to measure the apparent facility of outflow, the stopcock was opened and within 20 sec the pressure of the aqueous humour substitute adjusted to 4mm Hg above the resting intraocular pressure by reference to the Polygraph recorder. The

stopcock remained open for a period of 2 min, during which the aqueous humour substitute flowed from the reservoir into the anterior chamber. The weight loss from the reservoir was used to calculate the apparent facility of outflow of aqueous humour from the anterior chamber. (The density of the aqueous humour substitute was assumed to be 1g/ml).

This procedure was repeated once every 8 to 12 min. The interval allowed between facility determinations was sufficient for the intraocular pressure to return to the pre-test value.

After reproducible control readings of apparent facility of outflow were obtained, ACh was injected intracorneally and 10min allowed for its diffusion from the injection site before the apparent facility of outflow was again determined. The injection and readings were repeated twice and then control determinations were carried out until these returned to the level of previous controls.

In order to determine the effect of various drugs on the ACh-induced response, drugs were applied topically to the corneal surface or introduced as a bolus into the perfusate or, latterly, added to the entire perfusate. The drugs used were :

Drug	Method of application
Neostigmine methylsulphate	topical
Ecothiopate iodide	topical
Atropine sulphate	topical or perfusion
Hyoscine hydrobromide	topical
Lignocaine hydrochloride	topical
(+) - tubocurarine	bolus perfusion
Pancuronium bromide	perfusion
Hexamethonium bromide	bolus perfusion
Mecamylamine hydrochloride	perfusion
Phenyoxybenzamine hydrochloride	perfusion
Choline chloride	perfusion
Verapamil hydrochloride	perfusion

During experiments examining the involvement of Ca^{2+} in the ACh - induced effect, the aqueous humour substitute was modified by the omission of $CaCl_2$ and $MgCl_2$ and by the addition of 5mM EDTA to remove Ca^{2+} from the endogenous aqueous humour. Other experiments investigating the role of Ca^{2+} involved intracorneal injection, as described above for ACh, of the calcium ionophore, Lilly A23187.

The apparent facility of outflow was also measured after injections of saline (0.9% w/v) or choline chloride (400 pmol) intracorneally in eyes which had already shown a response to ACh. Simultaneous intracorneal injections of saline (0.9% w/v) and ACh into left and right eyes were also made to obtain control and test effects.

RESULTS

Measurements of protein levels and ChAc activity in the corneal epithelium after regeneration of the tissue.

Complete removal of the corneal epithelium (100%).

Measurements of protein levels (μg) and ChAc activity (nmol ACh formed. mg protein^{-1} hr^{-1}) were made in the regenerated tissue and comparisons made with the original epithelium. The results for both protein and ChAc activity are expressed as :

$$\frac{\text{activity of regenerated tissue sample}}{\text{activity of original tissue sample (day 0)}} \times \frac{100}{1}$$

Protein levels were calculated by reference to a standard curve produced by known quantities of bovine serum albumin.

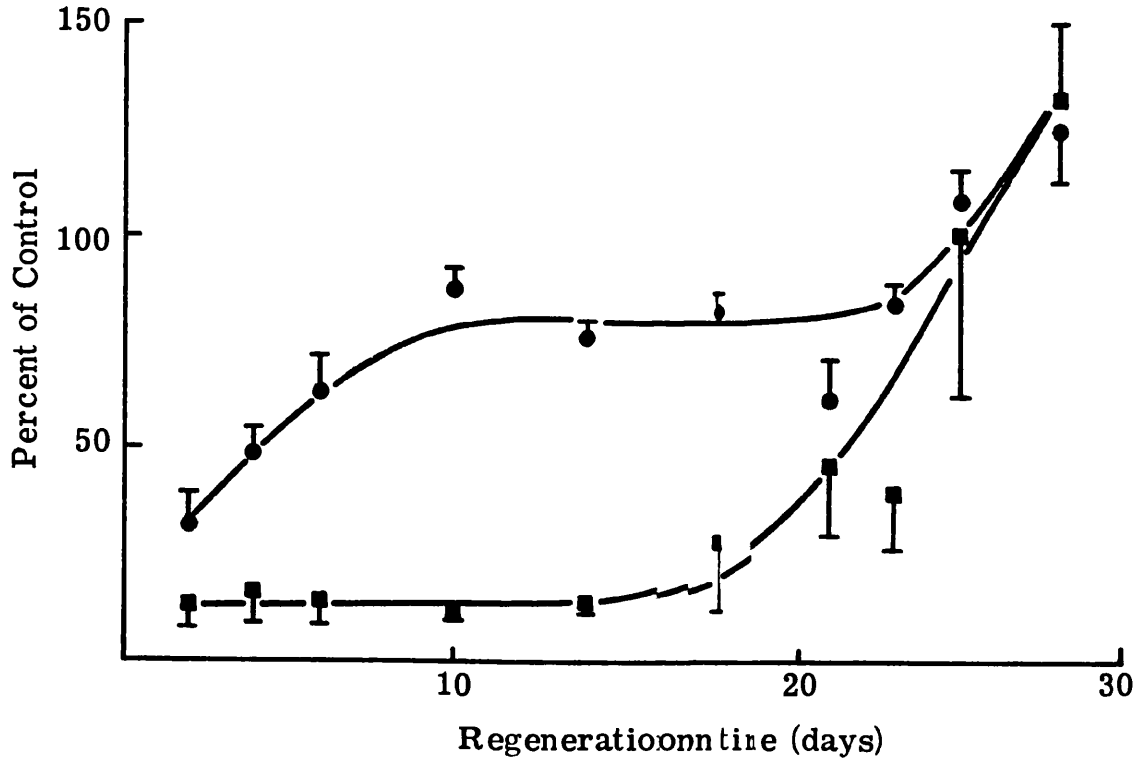
ChAc activity was calculated by the following formula :

$$\frac{\text{net c.p.m.}}{\text{net c.p.m. for 10}\mu\text{l } [1\text{-}^{14}\text{C}] \text{ AcCoA}} \times \frac{\text{nmol AcCoA used} \times 3}{\text{mg protein per eye}} \times \frac{200}{\text{sample volume } (\mu\text{l}) \text{ used}}$$

The values for the blank samples were subtracted from both the control and regenerated tissue values before the calculations of ChAc activity were made.

The results are shown in fig.2 and related to the time course for the reappearance of protein and ChAc activity. Each point represents the mean $\pm$ standard error of the mean (S.E.M.)

Protein levels rose steeply in the first 10 days of regrowth and this period was followed by a plateau phase until day 21. There then ensued a further rise in protein content of the new tissue.



● Protein level

■ ChAc activity

Fig. 2. Complete removal of corneal epithelium (100%). Regeneration of protein levels and ChAc activity.

The ChAc activity remained low (12 to 15% of control) in the regenerating tissue until around the 14th day of regrowth. Enzyme activity then rose rapidly to reach control values by day 21, and by the 28th day, the ChAc activity had exceeded control values.

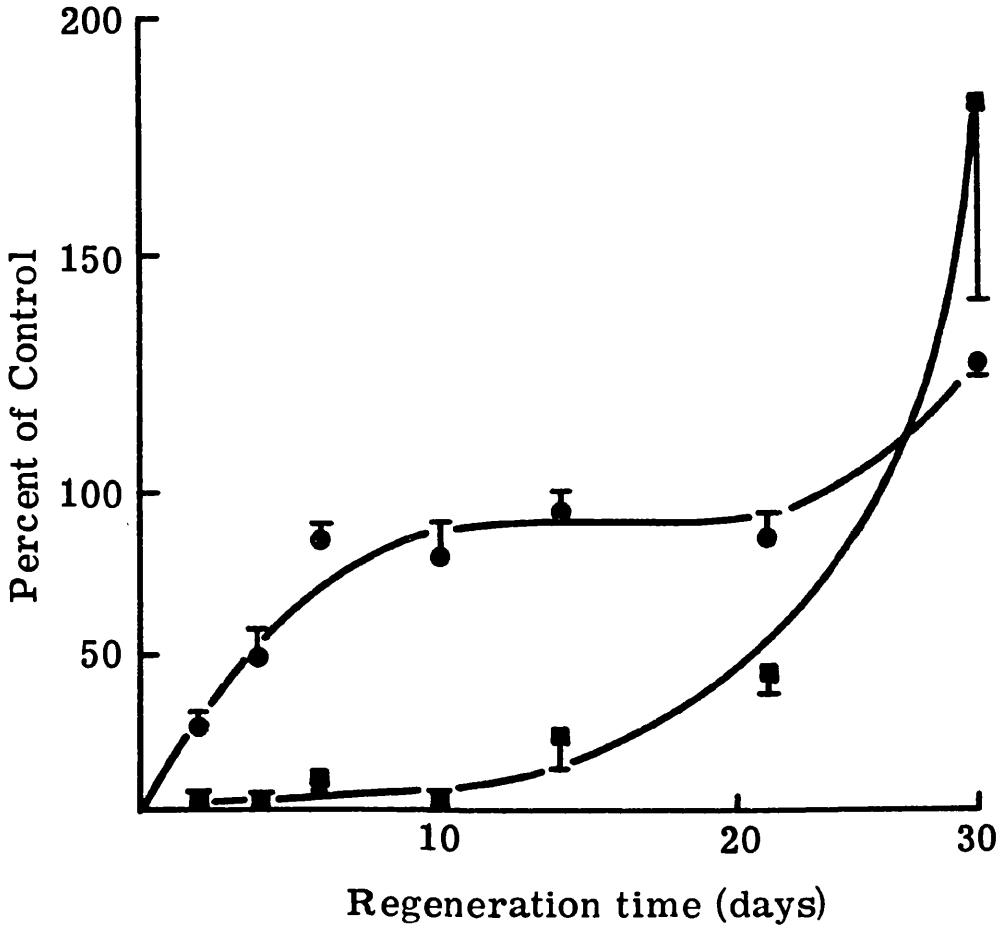
Incomplete removal of the corneal epithelium (95%).

The results have been expressed in the same manner as above, and shown in fig. 3. The trends in these results are similar to those seen upon complete removal of the epithelium.

Protein levels showed two rapid rises interrupted by a plateau phase in regrowth. The ChAc activity in the regenerating tissue remained low for the first 10 to 14 days and then rose rapidly until the level of activity surpassed control values by the 28th day of regrowth.

mEGF treatment.

Four rabbits were treated in one eye four times daily for four days with mEGF; the other eye was treated with saline and acted as the control. Protein levels and ChAc activity have been expressed in the same manner as above (p62), calculated as a percentage of day 0 control. The results are shown in table 1 .



● Protein levels

■ ChAc activity

Fig 3. Incomplete removal of corneal epithelium (95%). Regeneration of protein levels and ChAc activity.

Table1:

		Content of regenerated tissue : % of day 0 control. mean ± S.E.M.	
		ChAc	Protein
4 day treatment n = 4	saline	5.4 ± 1.2	71.8 ± 7.2
	mEGF	11.3 ± 3.1	71.9 ± 4.8
5 day treatment n = 3	saline	10.1 ± 2.0	35.6 ± 1.8
	mEGF	40.9 ± 3.0	60.2 ± 4.9

The second experiment, the results of which are also expressed in table 1 , involved five days of treatment instead of four.

The first experiment, involving four days of treatment, showed no significant difference (paired t test, Wilcoxon test) between the saline-treated and the mEGF-treated eyes either in protein levels or in ChAc activities. In the second experiment, five days of treatment, the protein levels in saline-treated and mEGF-treated eyes were significantly different (paired t test, $0.02 > \underline{P} > 0.01$). The ChAc activities in the two groups were not significantly different (paired t test).

Efflux of [^{3}H]choline and [1-^{3}H] ACh from the cornea into the tear fluid.

After 60 min equilibration time, the control counts which appeared in the tear fluid were almost constant and reproduceable. However, to ensure that exponential decay of control readings is eliminated, test readings were superimposed upon the curve generated by the control readings. The results were analysed as follows :

i. logarithmic transformation of control and test readings which consisted of three 'pre-test', three test and three 'post-test' readings.

ii. linear regression analysis carried out using the 'pre-test' and 'post-test' controls. This produced theoretical values for the controls (c) at times when the tests (T) were being carried out.

iii. $\ln T - \ln c = D$

iv. $\frac{\Sigma D}{n}$ where n is number of test values

v. $\bar{D} \pm$ S.E.M. were then calculated.

The analysed results are presented in table 2 . The statistical significance of the results is derived from paired t tests.

Table2:

Test drug or situation	Number of tests	Effect on release	Degree of significance
Amethocaine 1%	12	↑	0.001 > P
Oxybuprocaine 0.4%	16	↑	0.01 > P > 0.001
Atropine 10mM	12	↑	P = 0.05
Atropine 25mM	12	↑	0.02 > P > 0.01
Dry spot formation + benzalkonium chloride	16	↑	0.05 > P > 0.02
Sodium ethacrynate 2mM	24	↑	0.02 > P > 0.01
Mechanical stimulation	12	↑	0.01 > P > 0.001
Mechanical stimulation + oxybuprocaine 0.4%	16	↑	0.001 > P

The tests which did not produce a significant effect on release of radioactivity into the tear film are shown in table 3 .

Table 3:

Test drug or situation	Number of tests
Dry spot formation	16
Distilled water	21
1.8% w/v saline	24
2.5mM $CaCl_2$	12
50mM KCl	12
iso osmotic sucrose	24
tetrodotoxin 31.3nM^{-4}	12
atropine 100μM	12
neostigmine 100μM	12
neostigmine 10mM	12
compound 48/80 0.64mg/ml	12
deprivation of light during experiment	12
deprivation of light 24 hr prior to experiment	12
deprivation of light 72 hr prior to experiment	12
oxotremorine 10μM	12
oxotremorine 1mM	12

The effects of some drugs were examined further using T.L.C. separation of the tear sample. This permitted the

measurement of three parameters

i activity of $[^3H]$ ACh in drug-treated and control samples.

ii activity of $[^3H]$ choline in drug-treated and control samples.

iii ratio of ACh : choline in each sample.

Differences in these activities and ratios were tested for significance using a paired t test. The results are shown in table 4 .

Table 4 : Activity of [1-^{3}H] ACh and [^{3}H] choline in drug-treated and control samples of tear fluid after intracorneal injection of [^{3}H] choline. Ratio of [1-^{3}H] ACh : [^{3}H] choline in each sample.

Drug applied	Number of tests	[1-^{3}H] ACh in drug treated and control samples *			[^{3}H] choline in drug treated control samples *			Ratio of ACh : choline in each sample		
		Mean ± S.E.M.		Degree of Significance	Mean ± S.E.M.		Degree of Significance	Mean ± S.E.M.		Degree of Significanc
		Drug	Control		Drug	Control		Drug	Control	
Atropine 25mM	6	180.5 ± 45.7	32.2 ± 16.4	0.1 > P > 0.05	127.7 ± 23.3	17.9 ± 15.7	0.1 > P > 0.05	1.29± 0.42	0.81± 0.16	0.2 > P > 0.
Amethocaine 1%	8	584.7 ± 148.8	28.8 ± 15.2	0.02 > P > 0.01	556.0 ± 83.6	135.6 ± 82.4	0.05 > P > 0.02	1.01± 0.12	0.92± 0.49	N.S.
Oxybuprocaine 0.4%	6	282.5 ± 97.6	102.3 ± 28.6	0.1 > P > 0.05	488.2 ± 156.4	163.5 ± 57.5	0.1 > P > 0.05	0.63± 0.05	0.88± 0.16	0.2 > P > 0.
Tetrodotoxin 31.3 nM	6	23.2 ± 7.9	41.6 ± 17.8	N.S.	62.5 ± 24.9	52.6 ± 20.5	N.S.	0.92± 0.31	0.90± 0.19	N.S.
Controls	6	19.4 ± 15.2	8.6 ± 5.2	N.S.	47.1 ± 10.9	56.5 ± 15.2	N.S.	0.94± 0.26	0.98± 0.27	N.S.

* : Units are c.p.m. corrected for background

N.S. : not significant

Measurement of cholinesterases in tissues of the anterior segment of the eye.

The continuous recording of the spectrophotometric reading gave a plot of E_{412nm} against time. The best straight line was drawn through the plot, and the slope, $Emin^{-1}$, calculated. The extinction coefficient of the yellow anion is 13.6×10^3 and so the following equation could be used :

$$\text{rate of reaction} = \frac{Emin^{-1}}{13.6 \times 10^3}$$

The units for rate of reaction are mmole.μg protein^{-1}min^{-1}.

The use of BW 284 C51, a specific inhibitor of acetylcholinesterase, enabled the amount of AChE to be calculated. The total ChE and the AChE in each tissue are presented in table 5 .

Table 5 : Estimation of ChE and AChEactivity in tissues of the anterior segment of the eye, calculated per µg protein.

	ChE activity in pmole.μg protein^{-1} min^{-1} (mean $\pm$ S.E.M.)				
	Plasma	Aqueous humour	Iris	Corneal epithelium	Corneal stroma
total ChE	0.198 ± 0.024 n = 5	0.62 ± 0.15 n = 7	0.17 ± 0.041 n = 4	0.085 ± 0.008 n = 5	$0.7 \times 10^{-5} \pm 0.43 \times 10^{-}$ n = 4
AChE	0.136 ± 0.015 n = 3	0.66 ± 0.19 n = 4	0.079 ± 0.013 n = 3	0.076 ± 0.012 n = 5	-

The ChE present in the aqueous humour appears to be entirely AChE. The amount of AChE in relation to total ChE in the other tissues varies. Aqueous humour contains a considerably greater specific activity of ChE than does plasma.

The relevance of ChE measurement to the fate of ACh diffusing from the cornea is best seen when the tissue activities are expressed as ACh hydrolysed per whole tissue, as in table 6 .

Table6:

	ChE activity in pmole.whole tissue^{-1} min^{-1}			
	Aqueous humour	Iris	Corneal epithelium	Corneal stroma
total ChE	1.24	0.53	0.17	0.70

Recovery of $[1\text{-}^3H]$ ACh and $[^3H]$ choline from the tissues of the anterior segment after an intracorneal injection of $[1\text{-}^3H]$ ACh.

Dissection of each tissue and assay for content of radioactivity permitted the calculation of the total count in each tissue expressed as a percentage of the overall counts in the tissues of the anterior segment.

Separation by T.L.C. of the radioactive components in each tissue permitted the calculation of the amount of intact ACh reaching each tissue as a percentage of the total count in that tissue. This assumes that there is no resynthesis of $[1\text{-}^3H]$ ACh from its breakdown product in the tissue.

The $[1\text{-}^3H]$ ACh injected, 4nmole, was approximately four times the amount of endogenous ACh in the corneal epithelium.

The results are expressed in table 7 .

Table7:

Tissue	$\frac{\text{Count(tissue)}}{\text{Count (eye)}} \times 100$ Mean $\pm$ S.E.M. n=16	$\frac{\text{ACh count(tissue)}}{\text{Total count(tissue)}} \times 100$ Mean $\pm$ S.E.M. n=15
Cornea	55.6 $\pm$ 3.1	58.1 $\pm$ 1.9
Aqueous humour	27.4 $\pm$ 3.4	33.6 $\pm$ 3.0
Iris	11.8 $\pm$ 1.5	14.4 $\pm$ 1.4
Ciliary body	5.0 $\pm$ 0.8	23.0 $\pm$ 2.2

A considerable proportion of ACh injected diffused unchanged into the aqueous humour and appreciable amounts were detectable in the iris and ciliary body. The amount of radioactivity in each tissue fell with the distance from the site of injection. The amount of intact ACh reaching each tissue also fell with distance from the cornea.

The effect of an intracorneal injection of ACh on the pupil diameter.

The diameter of the pupil was measured at 1 min intervals after the intracorneal injection of ACh and miosis followed until a maximum had been observed.. The reading was taken at this point, before relaxation of the iris muscle began. The results are expressed graphically in fig 4 . Log dose of ACh injected is plotted on the ordinate. Iris contraction is plotted on the abscissa and this was calculated by the following equation :

$$\frac{\text{Pupil diameter (control) - pupil diameter (test)}}{\text{Pupil diameter (control)}} \times 100$$

Mean values $\pm$ S.E.M. are shown.

There appeared to be two populations of animals, differing in their sensitivity to ACh injected into the corneal stroma. These two groups have been plotted separately on the graph.

If ACh was replaced by normal saline, no change in pupil diameter occurred. When atropine sulphate 1% eye drops were applied simultaneously with the injection of ACh, no miosis occurred.

The time course of the effect of intracorneally injected ACh was observed, and the maximum miosis was usually attained around 10 min after the injection of ACh had been made.

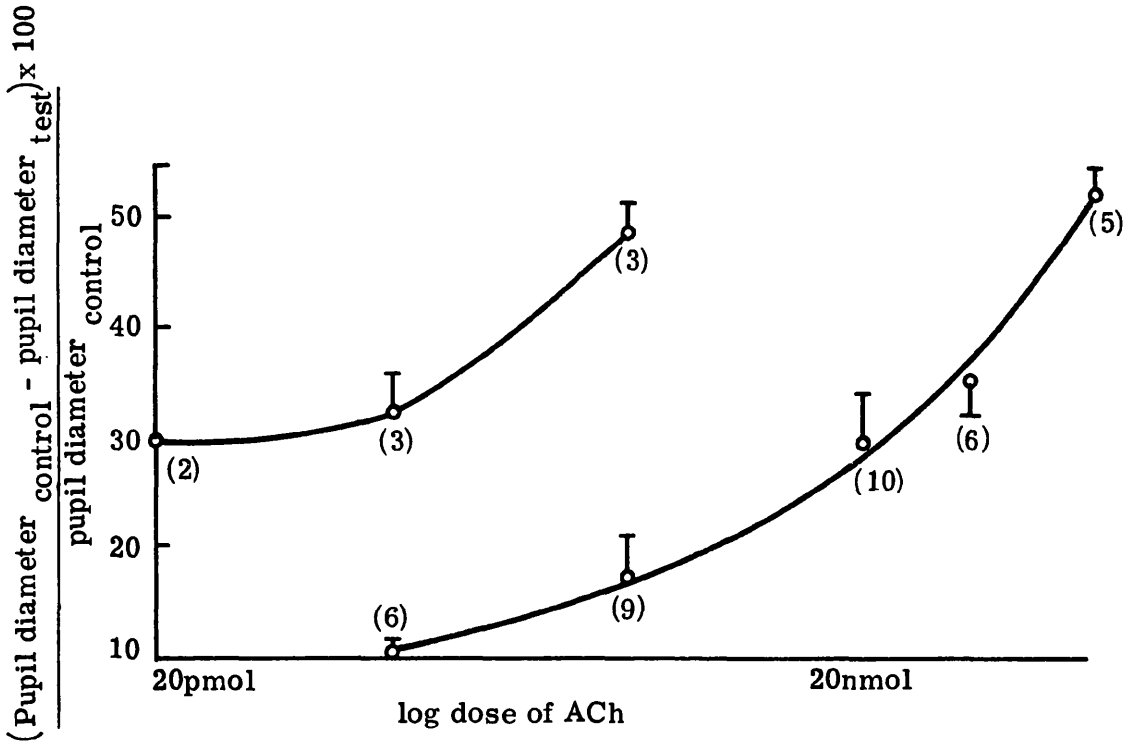


Fig. 4. Log dose response curve : effect of an intracorneal injection of ACh on pupil diameter. The graph represents the response in two populations of animal.

The effect of intracorneal injection of ACh on the apparent facility of outflow of aqueous humour from the anterior chamber.

The parameter measured in these experiments was the apparent facility of outflow.

$$\text{Apparent facility of outflow} = \frac{\text{volume of fluid moving into anterior chamber from reservoir}}{\text{pressure applied x time stopcock was open}}$$

$$= \frac{\text{volume of fluid}}{\text{4mm Hg x 2 min}}$$

Hence, the units of facility of outflow are μl.mmHg.$^{-1}$min^{-1}

A typical polygraph trace of the pressure within the anterior chamber and readings for the apparent facility of outflow are shown in fig 5 . The measurement of apparent facility of outflow occurred during the 2 min period when the stopcock was open and the pressure within the chamber was raised by 4mm Hg. After constant control readings had been gained, ACh was injected and 10 min allowed for its diffusion to a site of action, since this was the time required for ACh diffusion to the iris in earlier experiments.

The effect of ACh was reproduceable and dose-dependent, as shown in fig 6 . The effect of ACh was not mimicked by intracorneal injection of saline (0.9% w/v) or by choline chloride (400pmole) in eyes which had already shown a response to ACh (40pmole). The control values remained constant during the 2 to 4 hr of the experiment. A similar response to ACh applied topically could be elicited if a small area of the corneal epithelium was first abraded.

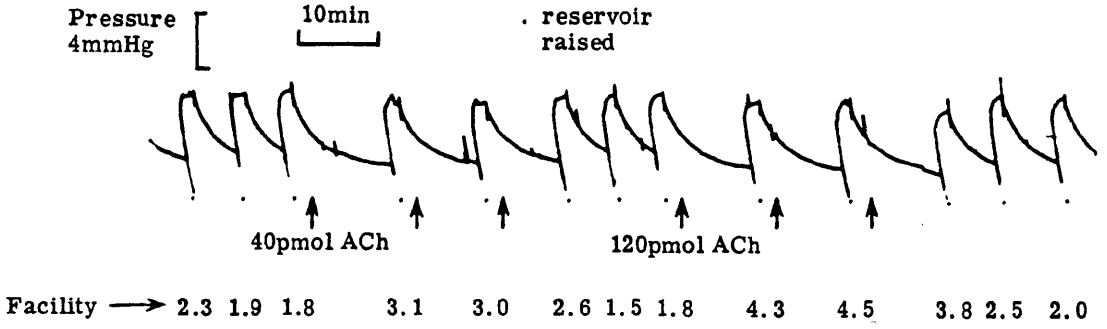
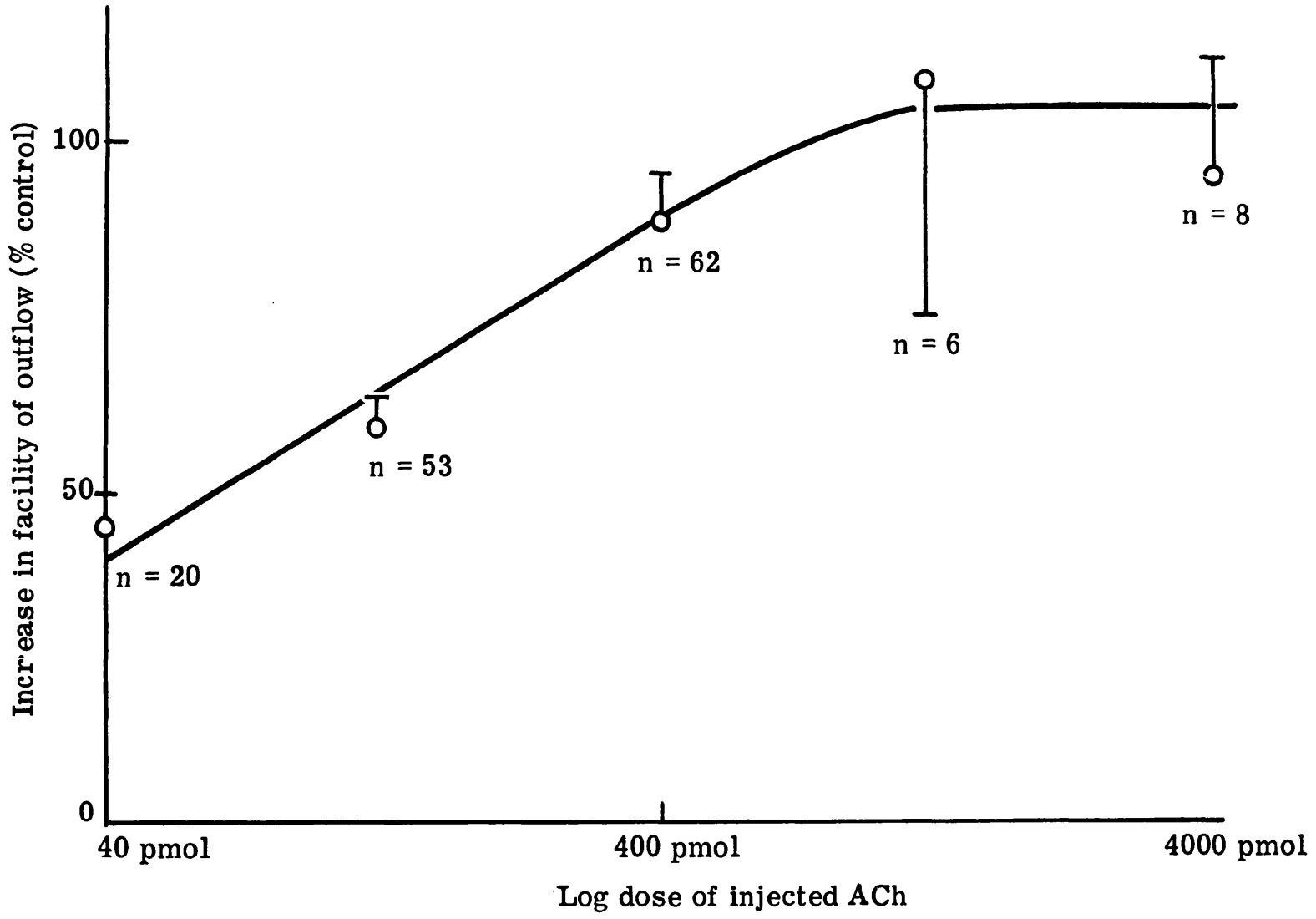


Fig. 5. Polygraph record of the pressure within the anterior chamber and readings for the apparent facility of outflow of aqueous humour. Effect of ACh on the apparent facility of outflow.

Fig. 6. Log dose response curve : effect of an intracorneal injection of ACh on the apparent facility of outflow of aqueous humour.


Increase in facility of outflow (% control)
100
50
0
n = 20
n = 53
n = 62
n = 6
n = 8
40 pmol
400 pmol
4000 pmol
Log dose of injected ACh

The effect of ACh was potentiated when neostigmine methylsulphate (3% w/v) was applied topically simultaneously with the injection of ACh. The results of these experiments are shown in the histogram, fig 7 . Firstly, a dose of 40pmol ACh produced a significant (0.01>P>0.001) increase in the apparent facility of outflow when compared to control values by Student's t test. Application of neostigmine significantly increased that effect. A similar result was obtained when ecothiopate iodide (0.25% w/v) was applied topically instead of neostigmine, fig 8 . The effect of injected ACh was also prolomged by both of these ChE inhibitors

Table8:

Drug	Dose & route of administration	ACh control (Mean ± S.E.M)	ACh + Drug control (Mean ± S.E.M)	Significance of difference
Neostigmine	45nmol topical	11.59 ± 0.11 n = 6	2.07 ± 0.15 n = 6	0.05>P>0.02
Ecothiopate	3.3n mol topical	11.58 ± 0.18 n = 4	1.98 ± 0.28 n = 4	0.05>P>0.02

A variety of drugs were administered in an attempt to block the response to ACh. Many of these failed to affect the ACh-induced increase in apparent facility and these are shown in table 9 .

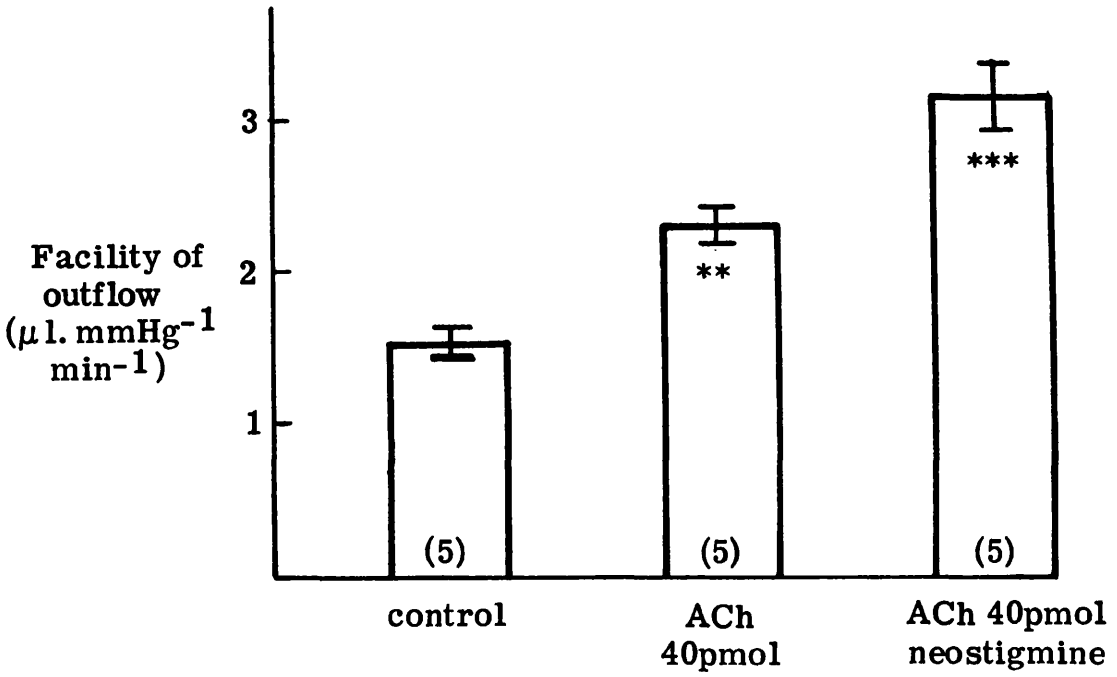


Fig. 7. Histogram : effect of ACh on the apparent facility of outflow of aqueous humour. Effect of neostigmine (3% w/v) on the response to ACh.

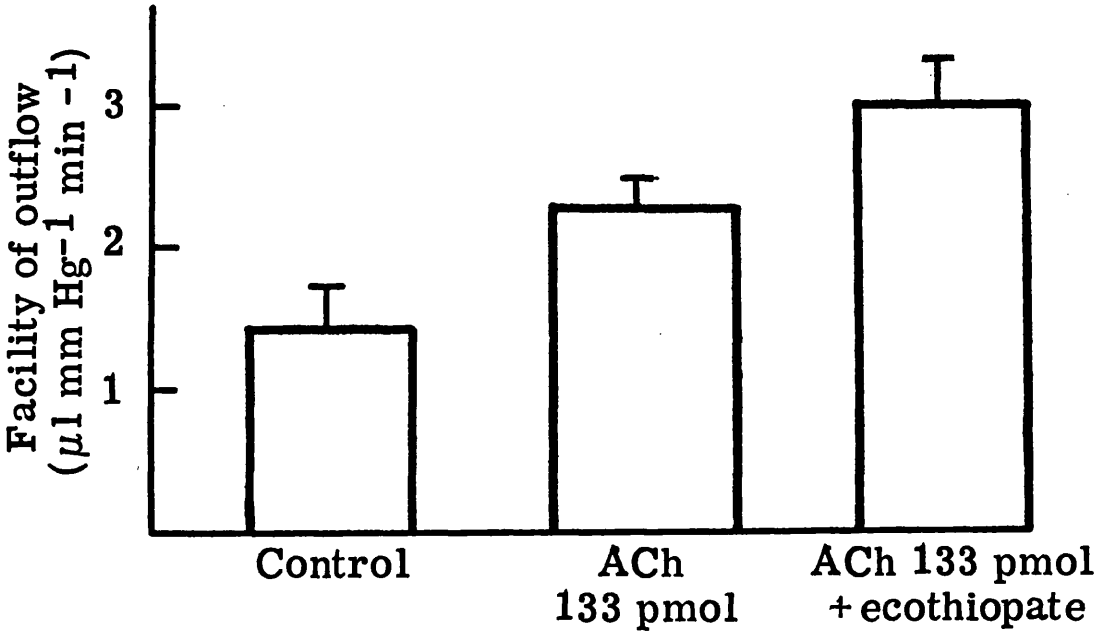


Fig. 8. Histogram : effect of ecothiopate (0.25% w/v) on the response to ACh in the apparent facility of outflow.

Table 9 : Drugs which had no significant effect on the ACh-induced increase in the apparent facility of outflow.

Drug	Dose & route of administration	ACh control (mean ± S.E.M.)	ACh + Drug control (Mean ± S.E.M.)	Significance of difference
(+)- tubocurarine	13nmol perfused	1.86 ± 0.08 n = 4	1.87 ± 0.05 n = 4	N.S.
Pancuronium	200nmol perfused	1.51 ± 0.11 n = 7	1.60 ± 0.06 n = 7	N.S.
Lignocaine	1.38μmol topical	1.72 ± 0.10 n = 5	1.53 ± 0.12 n = 5	N.S.
Hexamethonium	625nmol perfused	1.75 ± 0.17 n = 4	2.09 ± 0.15 n = 4	N.S.
Mecamylamine	100nmol perfused	1.75 ± 0.16 n = 4	2.10 ± 0.22 n = 4	N.S.
Phenoxybenzamine	300nmol topical	1.41 ± 0.10 n = 4	1.41 ± 0.04 n = 4	N.S.

N.S. = No Significant difference

Muscarinic and nicotinic antagonists were also used and these produced blockade of the ACh effect when given intravenously in high dose but not when applied topically, as shown in table 10 .

Table 10 :

Drug	Dose & route of administration	ACh / control (Mean ± S.E.M.)	ACh + Drug / control (Mean ± S.E.M)	Significance of difference
Hyoscine	60nmol topical	1.53 ± 0.07 n = 4	1.78 ± 0.14 n = 4	N.S.
Atropine	140nmol topical	1.94 ± 0.13 n = 12	1.95 ± 0.12 n = 12	N.S.
Atropine	0.19mmol.kg^{-1} intravenous	1.61 ± 0.06 n = 5	1.03 ± 0.02 n = 5	0.01>P>0.001

The ACh-induced increase in apparent facility of outflow did not occur when the artificial aqueous humour was replaced with a Ca^{2+} and Mg^{2+}-free artificial aqueous humour containing EDTA. The results for these experiments are shown in histogram form, fig 9 , and in table 11 .

Table 11 :

Drug	Route of administration	ACh / control (Mean ± S.E.M)	ACh+drug / control (Mean ± S.E.M)	Significance of difference
Ca^{2+},Mg^{2+}-free artificial aqueous humour + 5mM EDTA	perfused	1.69 ± 0.12 n = 11	0.95 ± 0.05 n = 11	P<0.001

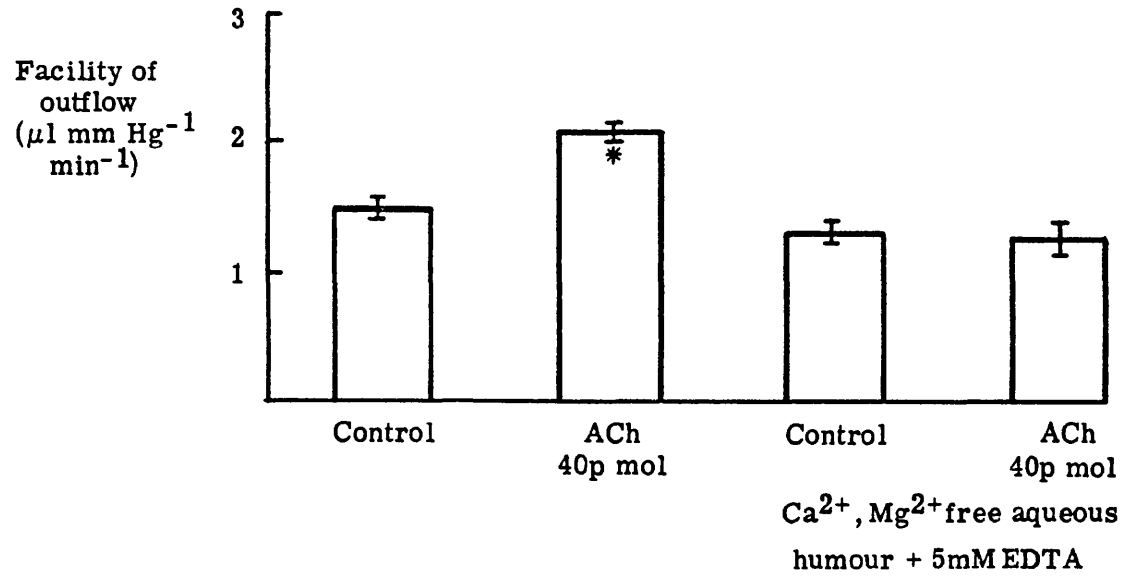


Fig. 9. Histogram : effect of a Ca^{2+} and Mg^{2+} free artificial aqueous humour containing EDTA (5mM) on the response to ACh in the apparent facility of outflow.

Since it appeared that one of these cations may be involved in the mechanism through which ACh had its effect, the Ca^{2+} ionophore, Lilly A23187, was injected in place of ACh in eyes which had already responded to ACh. It was found that reproduceable, dose-dependent increases in the apparent facility of outflow could be elicited by Lilly A23187 (fig 10). Log dose of ionophore is plotted on the ordinate whilst the percentage increase in apparent facility of outflow is plotted on the abscissa.

Verapamil, a drug which blocks the uptake of Ca^{2+} into smooth muscle and nerve was infused into the anterior chamber to ascertain its effects on the ACh-induced increase in the apparent facility of outflow. The results are shown in table 12 .

Table 12 :

Drug	Dose & route of administration	$\frac{\text{ACh}}{\text{control}}$ (Mean $\pm$ S.E.M)	$\frac{\text{ACh+drug}}{\text{control}}$ (Mean $\pm$ S.E.M)	Significance of difference
Verapamil	1.25mg/ml perfusion	1.78 $\pm$ 0.16 n = 4	0.96 $\pm$ 0.09 n = 4	0.05>P>0.02

These experiments suggest that ACh injected into the corneal stroma produced an increase in the apparent facility of outflow by acting on a muscarinic receptor and through a mechanism which is dependent on Ca^{2+}. The effect of ACh can be potentiated and prolonged by drugs which block ACh breakdown by cholinesterases and hence it is an effect specific to the ester. This is supported by the fact that intracorneal

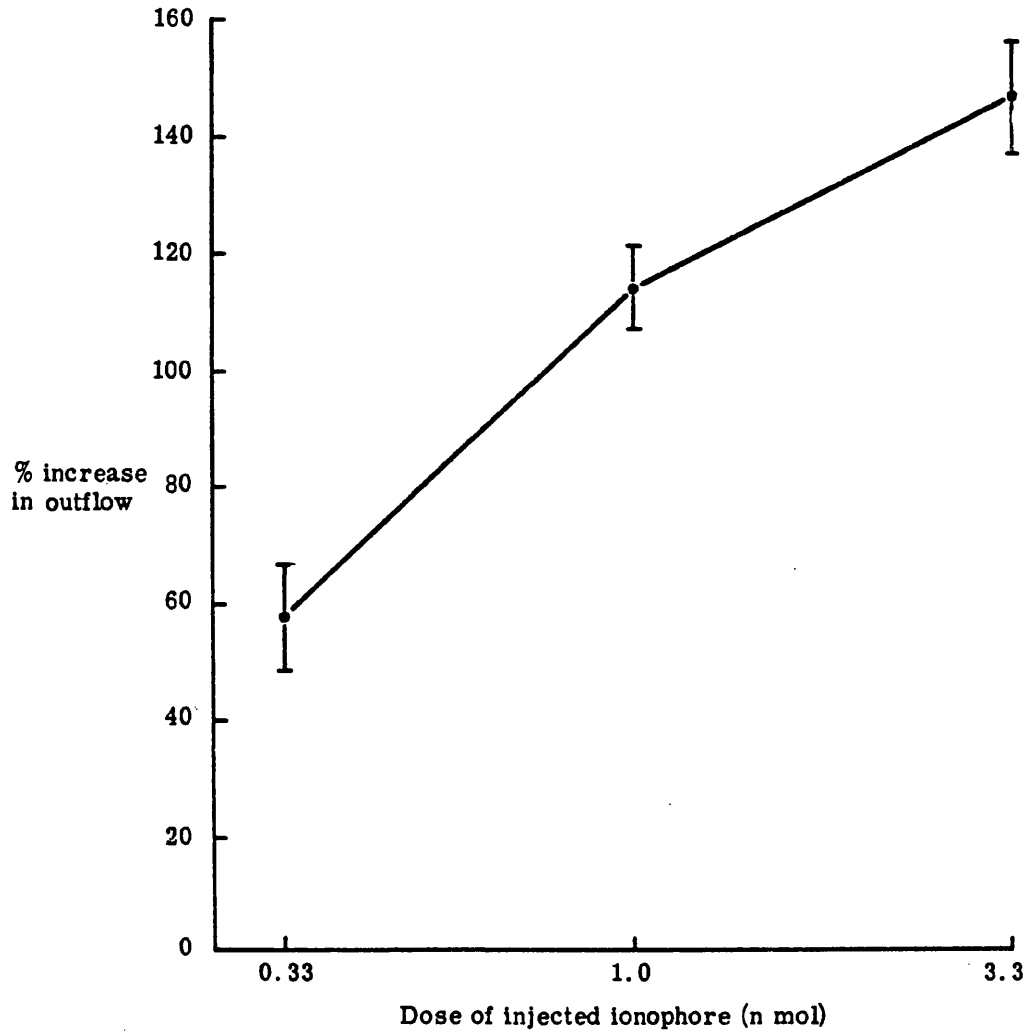


Fig. 10. Log dose response curve : effect of an intracorneal injection of Lilly A23187 on the apparent facility of outflow of aqueous humour.

injection of choline could not produce a similar effect.

Since anticholinesterases applied without simultaneous injection of ACh, did not affect the apparent facility of outflow, it would seem that there is no continual release of endogenous epithelial ACh to regulate the facility of outflow under the conditions of these experiments.

The apparent facility of outflow as measured by the method described here is not solely a measure of outflow via the conventional drainage route. It is composed of four main factors:

i conventional drainage

ii scleral compliance

iii uveoscleral drainage

iv pseudofacility

An attempt was made to ascertain the contribution of scleral compliance to the measurement of apparent facility of outflow since this was likely to be the component most liable to distort the estimation of true facility of outflow by the present technique. This was done by determining the fluid lost from the reservoir at 30 sec intervals during the normal 2 min perfusion period. Preliminary experiments showed that most of the compliance occurred in the system within the first 30 sec of perfusion. The 2 min perfusion period was divided, on this basis, into a 30 sec reading followed by a 90 sec reading, to examine the effect of ACh on compliance. The contribution of outflow to the initial 30 sec reading was calculated by dividing the 90 sec value by 3 and subtracting the value obtained from the initial 30 sec value.

The results are shown in table 13 .

Table 13 : Units : µl/mm Hg/min

	Control (Mean ± S.E.M)	ACh (Mean ± S.E.M)	Significance of difference (paired t test)
Initial 30 sec corrected for outflow	0.99 ± 0.06	1.4 ± 0.08	0.001 > P
Remaining 90 sec	0.59 ± 0.06	1.12 ± 0.09	0.001 > P

Paired t test statistics show that ACh significantly increases the scleral compliance as estimated here and facility of outflow which is composed of true facility of outflow by conventional drainage, by uveoscleral drainage and pseudofacility.

DISCUSSION

Measurements of protein levels and ChAc activity in corneal epithelium after regeneration of the tissue.

Rationale.

As discussed previously, the high levels of ACh and ChAc in the corneal epithelium may be related to its potential for rapid regeneration after injury. Cavanagh (1975) suggested that a role in control of the rate of epithelial mitosis may be one function of this non-neuronal ACh store. Topically applied pilocarpine (a cholinomimetic agent) and ecothiopate (an anticholinesterase) have been shown to cause mild desquamation of the corneal epithelium (Pfister & Burstein, 1976). However, Fogle & Neufeld (1979), have not clinically observed any obvious epithelial lesion during topical use of such cholinergic drugs. The effect is therefore not gross and has, indeed, only been detected by microscopy.

It was the intention of this study to remove part or all of the corneal epithelium and to allow either corneal or conjunctival epithelium to grow over the surface of the denuded stroma. Measurement of ChAc activity in the regenerating tissue at different intervals would give an indication of the rate of recovery of enzyme levels and consequently, it was thought, of ACh levels. If recovery was rapid, the cholinergic components might have been involved in the process of regeneration. However, Mindel et al., (1979) reported some time after our work was completed, that only a portion of ChAc activity is required to maintain corneal ACh levels and that considerable amounts of ACh may be present even when

ChAc activity is low. This is in agreement with *in vitro* work on brain ChAc activity (Krell & Goldberg, 1975; Cheney, LeFevre & Racagni, 1975). A measurement of epithelial ChAc activity does not appear to be a good index of the levels of ACh in the tissue.

Method.

The method chosen for assay of ChAc levels was a radioisotope assay developed by Schrier & Shuster (1967), and modified by Wilson & Cooper (1972). This method attempts to achieve maximal linear enzymic activity throughout the experiment by the following means. Firstly, any breakdown of the radioactive product, [1-^{14}C] ACh, by the enzyme ChE is reduced to a minimum by the use of Tris buffer which inhibits ChE activity (see Pavlič, 1967) and by the addition of the anticholinesterase, neostigmine, to the reaction mix. Secondly, an optimal incubation time of 20 min was chosen since ChAc activity tends to fall with longer incubation times when a preformed substrate (as opposed to a continually synthesised)substrate is used (Schrier & Shuster, 1967).

Protein levels in the regenerating corneal epithelium.

The total protein level increases rapidly in the developing epithelium in a biphasic manner whether the new tissue is of corneal or conjunctival origin. The intervening plateau period lasts some ten days. Although there are several reports in the literature on enzyme levels within regenerating corneal epithelia, as discussed previously, none have analysed

the protein levels per se and this permits no comparisons or gives any information as to why such a cessation of total protein formation should occur during a period of rapid cellular growth.

After complete removal of the corneal epithelium, delays in healing of up to 14 days, or breakdown of the new tissue have often been observed (Srinivasan, Worgul, Iwamoto & Eakins, 1977; Thoft & Friend, 1977; Moses, Parkison & Schuchardt, 1979), but healing was much easier when the regenerating epithelium was of corneal origin. However, although such events might correlate with the plateau phase in regrowth shown in this study, the two phenomena are probably not linked since the plateau phase in protein regeneration occurs whether the new epithelium is of corneal or conjunctival origin.

It has been shown that newly regenerated epithelium is loosely attached to the basement membrane for approximately one week after abrasion (Khodadoust, 1967). If the basement membrane is removed along with the epithelium during abrasion, formation of the new basement membrane begins some 6 to 8 days later (Blümcke, Rode & Niedorf, 1969). The basement membrane appears to be the product of cytoplasmic secretion by the epithelial cells, probably involving synthesis of collagen by the cornea (Leuenberger & Gnädinger, 1972). It may be that the abrasion carried out in the experiments described here caused damage to the basement membrane and the observed apparent cessation of protein synthesis is a reflection of a period during which the epithelial cells were effecting repairs in, and attaching themselves to the basement membrane.

The data obtained in this study on protein levels in the first 10 days confirms earlier observations (Maumenee & Scholz, 1948; Friedenwald, 1951) that both the corneal and conjunctival epithelia are capable of rapid regeneration, since approximately 65% of the original epithelial protein content is regained within 6 days of the abrasion.

The second rise in protein synthesis carries the levels above the original control values, perhaps indicating a hyperplasia of the epithelium. Other reports have not indicated that such an event occurs.

ChAc activity in the regenerating epithelium.

There is wide inter-animal variation in the amount of ChAc activity in the normal corneal epithelium. Mindel & Mittag (1977), using the same method as used here, found this value for ChAc to be 20.6 $\pm$ 13.6 nmole ACh formed.mg protein.$^{-1}$ hr^{-1} (mean $\pm$ S.E.M., n = 21). The value for ChAc activity reported by Howard *et al*. (1973) was 54.6 $\pm$ 18.4 nmole ACh formed. mg protein.$^{-1}$ hr^{-1} using the same method. There is also a wide variation in the data presented here.

The ChAc activity remained low for almost 14 days before it rapidly rose to exceed control values by the 28th day, whether the regenerating epithelium was of corneal or conjunctival origin. Several groups of workers have investigated the levels of other enzymes in regenerating epithelia, both of corneal and conjunctival origin. A similar pattern of events to that seen for ChAc, has been observed for acid phosphatase

levels which required almost 28 days to return to control values after complete denudation of the stroma (Bolková & Čejková, 1978); alkaline phosphatase levels were slower to recover and had not reached control values even by the 28th day of regrowth.

Several authors have suggested that there are differences in enzyme levels in the new epithelium which are dependent on the source of this regenerating tissue. This did not appear to be the case in this study and this may be so because the peripheral corneal epithelium contains very little ChAc (Mindel & Mittag, 1977) and is thus more similar to the conjunctival epithelium than to the epithelium of the central cornea. The conjunctival epithelium contains very little ChAc (Mindel & Mittag, 1976).

Another situation in which the cornea is devoid of ChAc activity occurs when lid closure persists for more than 8 days. When the lids are opened, reappearance of normal levels of ChAc takes more than 30 days.

In the experiments presented here, ChAc activity is seen to increase abruptly around the 14th day of regrowth and this occurs after complete and 95% removal of the corneal epithelium. This corresponds to the earlier stages of the histological transformation which occurs between 2 and 5 weeks after complete denudation of the stroma (Maumenee & Sholz, 1948; Friedenwald, 1951; Burns, 1968) and to the earlier part of the biochemical transformation reported by Thoft & Friend (1975).

There have been reports that human limbal epithelium varies

histologically from both corneal and upper tarsal conjunctival epithelium (Greiner, Covington & Allansmith, 1979) and that this transition zone may act as a generative tissue for corneal epithelial cells (Davanger & Evensen, 1971). This would suggest that our research only studied the regeneration of the peripheral corneal epithelium and of the limbal epithelium, the biochemistry of the latter being unknown. Further experiments, involving removal of a ring of conjunctival epithelium along with the corneal epithelium, are required to ensure that the cells which migrate are ones for which biochemical data is available.

The results of this study represent preliminary observations. However, they do suggest that the enzyme ChAc is not involved in the migratory stage after removal of the epithelium, since it is only present in very low amounts at this time. It is probably not involved in initiation of transformation of the cells since levels of activity only rise mid-way through this phase. ChAc activity then rises rapidly and the possibility that it is involved in the second phase of growth cannot be eliminated by these results.

Little can be said on the role of ACh during this regeneration process since it has been shown that ACh may be present in considerable quantity even when ChAc activity is low (Mindel et al., 1979).

The effect of mouse epithelial growth factor (mEGF).

Frati, Daniele, Delogu & Covelli (1972) demonstrated that one drop of a 2mg/ml solution of mEGF, applied four hourly to a partly denuded stroma, significantly reduced the time required for regeneration. The tissue had reached the normal depth of cells within 50 hr of injury, at which time the control was still at the monolayer stage. Ho, Davis, Elliott & Cohen (1974) used a 0.05mg/ml solution and found that it significantly increased the rate of healing and a forty fold increase in dose had no greater effect on healing.

The mEGF available for the experiments here was in very limited supply and only 50% pure. If the 'one drop' as used by Frati et al., (1972) and Ho et al., (1974) is assumed to be 50 µl, then the dose applied in our experiments was 1/20th that of Frati et al., and twice that of Ho et al. The dose of mEGF was, therefore, in the right dose range but there was barely enough to allow its use in sufficient animals for statistical analysis.

The general trend of these results suggests that mEGF hastens the transformation stage and hence the reappearance of ChAc activity, as might be predicted from its effects on epithelial proliferation.

Further investigation.

Howard, et al., (1973) have shown that the ChAc levels in neonate rabbits was very low until around the time that their eyes opened. Then ChAc activity began to rise until reaching

adult levels around 56 days after birth. Cohen, Carpenter & Lembach (1975) and Gregory (1975) noted that the injection of mg quantities of mEGF caused precocious opening of the eyes in newborn mice. It would be of interest to measure the ChAc activity in the epithelia of rabbits treated in this fashion and to compare the results with those of Howard *et al*. (1973).

Efflux of [^{3}H] choline and [1-^{3}H] ACh from the cornea into the tear fluid.

Rationale.

It may be postulated that the ACh of the corneal epithelium may not have an action within the epithelium itself, and that it may act at a site distant to its site of storage. One such possibility would involve an anterior release of ACh into the tear film. The hormone would then be able to act on any of the tissues bathed by the tears, for example, the goblet cells of the conjunctiva where ACh might regulate the secretion of mucus, or the secretions from the Meibomian glands of the eyelids which might be controlled by ACh released from the corneal epithelium.

Method

This postulate was investigated by labelling the endogenous ACh of the corneal epithelium with ^{3}H. This was attempted by injecting the labelled precursor [^{3}H] choline into the corneal stroma. The [^{3}H] choline had to be presented to the basement membrane side of the epithelium since Paterson & Wilson (1977) had demonstrated that bathing the anterior surface with [^{3}H] choline resulted in no accumulation of radioactivity in the epithelium, whereas bathing the stroma resulted in ^{3}H appearing in the epithelium. It has been shown that one hour is required for substantial uptake of this choline and incorporation into the epithelial ACh stores (Paterson & Wilson, 1977). This was the reason for leaving one hour after the injection before the sampling was started.

The bolus of labelled material was placed in the stroma centrally with respect to the limbus since Mindel & Mittag (1977) had observed that the centre of the epithelium contains the highest ChAc activity. Hence the [^{3}H] choline would be converted to [1-^{3}H] ACh most easily from this injection site.

Results.

After the equilibration period, the release of radioactivity into the tears was measured and a variety of possible stimuli were used to provoke release. The rationale for each stimulus is mentioned in the methods section (pp 46-48). The stimuli fell into distinct categories : mechanical stimuli, local anaesthetics drugs acting on cholinergic mechanisms, changes in concentration of ions in the tear film and drugs producing such changes, deprivation of light. Local anaesthetics and atropine caused an increased release of [1-^{3}H] ACh and [^{3}H] choline and atropine and oxybuprocaine increased the ratio of ACh:choline in the tears at a marginal level of significance.

It is well known that atropine has many effects other than acting at muscarinic receptors. One such effect, seen at high concentrations of atropine, is a local anaesthetic action. Olsen & Neufeld (1979), using ligand binding studies, have demonstrated that the cornea contains no muscarinic or nicotinic receptors. If we assume that the effect of the drugs applied is confined to some action within the cornea, the atropine applied may be acting via a mechanism other than its anticholinergic action. It is possibly acting as a local anaesthetic since amethocaine and oxybuprocaine produced a similar response.

Since tetrodotoxin had no effect on the release of ^{3}H into the tears, either the dose of tetrodotoxin was insufficient or the tetrodotoxin and local anaesthetics were acting at different sites. The first point can only be refuted or proved by further experiments using larger doses of tetrodotoxin. Tetrodotoxin specifically blocks sodium conductance in membranes of nerve and skeletal muscle. Local anaesthetics affect both the sodium and the potassium permeability in all potentially excitable membranes including those of nerve, skeletal muscle, smooth muscle and secretory cells, not all of which are dependent on sodium ions for conductance of the action potential (see Bowman & Rand, 1980). It is possible that the local anaesthetics and atropine are acting on some excitable membrane which is not dependent on sodium ions for conductance of action potentials. However, a stabilisation of excitable membranes in the cornea might be expected to prevent release of ACh and this is not compatible with the observed tendency to increase release of labelled ACh and choline.

Local anaesthetics applied for prolonged periods are known to cause epithelial damage by inhibiting epithelial respiration, glucose metabolism and mitosis (Sheldon, 1971; Augsburger & Hill, 1972; Henrotte & Weekers, 1972; Adenis, Merle, Dangoumau, Robin & Nicot, 1978; Henkes & Waubke, 1978). Recent work suggests that inhibition of mitosis and disruption of intercellular spaces in the epithelium can occur after a single application of local anaesthetic (Harnisch, Hoffmann & Dumitrescu, 1975; Brewitt & Honegger, 1978). Several applications of local anaesthetic can disrupt the plasma membrane and cytoplasm of

several layers of epithelial cells (Brewitt & Honegger, 1978). This may be the effect observed here since disruption of epithelial plasma membranes would probably release the cellular [1-^{3}H] ACh and [^{3}H] choline into the tear film. The observation that mechanical stimulation of the cornea and the formation of dryspots, which is known to damage the epithelial layer, also increase the release of radioactivity into the tear film supports the hypothesis that atropine and the local anaesthetics produced their effect by causing damage to the epithelial cells. In conclusion, it appears that no true release of epithelial ACh is occurring since a low 'background' ratio of ACh:choline would be expected to rise if any form of specific ACh release occurs and this did not happen.

Measurement of cholinesterases in the tissues of the anterior segment of the eye.

Rationale.

In any hypothesis involving a posterior movement of ACh from the corneal epithelium to a distant site of action, it is necessary to know the likelihood of ACh breakdown by cholinesterases.

Results.

The levels of ChE in the iris and corneal epithelium were found to be close to ChE levels in plasma, but in stroma the ChE level is much lower than in plasma. ChE activity in aqueous humour appeared to be higher than that of plasma but since the plasma is much richer in protein than is the aqueous humour, then the values of ChE in the aqueous humour are, in reality, much lower than those in plasma. This supports the hypothesis that ACh of the corneal epithelium could diffuse away from its storage site without undergoing total breakdown by ChE.

It is interesting to note that the aqueous humour, which is produced from plasma, has received an enrichment of ChEs relative to other plasma proteins, although ChEs do not have low molecular weights.

It appears that most of the ChE in the aqueous humour and corneal epithelium is AChE, whereas in the iris, AChE is less than 50% of the total ChE.

Recovery of [1-^{3}H] ACh and [^{3}H] choline from the tissues of the anterior segment after an intracorneal injection of [1-^{3}H] ACh.

Rationale.

The ACh of the corneal epithelium might diffuse posteriorly to a distant site of action within the anterior segment of the eye. These experiments were an attempt to mimic the natural situation by using radiolabelled ACh injected into the corneal stroma and tracing the sites to which it diffused.

Results.

The results indicate that the amount of radioactivity reaching each tissue falls with the distance of the tissue from the site of injection and that more than 50% of the [1-^{3}H] ACh remains within the stroma. Only 5% of the [1-^{3}H] ACh reaches the ciliary body and this small quantity is explained by the distance from the injection site.

The amount of intact [1-^{3}H] ACh reaching each tissue falls almost in relation to the distance from the injection site and this might be related to the amount of ChE encountered by the [1-^{3}H] ACh as it diffused from the corneal stroma. Due to the time taken to dissect tissues, the recovery of unhydrolysed ACh from these tissues may be underestimated.

The effect of an intracorneal injection of ACh on the pupil diameter.

Rationale.

Previous experiments had indicated that small quantities of ACh can diffuse from the corneal stroma to reach tissues at some distance from the cornea. The amount of ChE in the anterior chamber is low and there is not a total breakdown of the diffusing ACh. The iris is an ideal tissue for investigating the postulate that the endogenous ACh of the corneal epithelium may act on a tissue within the anterior chamber. This is because the iris is situated on the posterior aspect of the anterior chamber and is several mm distant from the site of the ACh injection. The smooth muscle of the constrictor pupillae, innervated by parasympathetic fibres from the ciliary ganglion, contracts (miosis) to ACh *in vitro* and to topically applied muscarinic agonists, if these penetrate the corneal epithelium.

Method.

The radioactive technique employed to investigate possible anterior release of epithelial ACh, was not appropriate for investigation of posterior release since loading the epithelial stores with $[^{3}H]$ choline and monitoring diffusion from the stromal side would make it difficult to detect small quantities of labelled ACh released posteriorly.

Hence a bolus of ACh was injected into the corneal stroma with particular care over insertion of the needles since proptosis of the eyeball may cause contusions which are known

to cause release of prostaglandins into the aqueous humour (Ambache, Kavanagh & Whiting, 1965; see Eakins, 1977). Prostaglandins can cause miosis among other events (Duke-Elder & Duke-Elder, 1931; Davson & Quilliam, 1947) which is obviously undesirable in an experiment measuring pupil diameter.

Results.

This experiment suggests that small quantities of ACh can diffuse from the corneal stroma posteriorly to have a pronounced effect on the iris. The ACh has to cross a distance of at least 2.9mm, the depth of the anterior chamber (Sorsby, Stone, Leary & Sheridan, 1960). Since the effect is antagonised by atropine, the ACh is probably acting on muscarinic receptors.

The mean dose of ACh which had a detectable effect (15%) in the majority of rabbits was 2nmole, although we know from previous experiments that only 1.6% of this ACh reaches the iris intact. This amount of ACh, 2nmole, is of the same order as the total amount of endogenous ACh in the epithelium which is approximately 1 nmole (Howard et al., 1973; Mindel et al., 1979).

In a few animals, the doses of ACh required to produce miosis were uncharacteristically low. This result may be attributed to inter-animal variation.

If there is a mechanism for the release of endogenous ACh across the epithelial basement membrane into the stroma, there is a possibility that this hormone is acting at a site within the anterior chamber. It is, however, unlikely that the iris itself is the target tissue for epithelial ACh since this

tissue is well innervated by both sides of the autonomic nervous system. There seems little requirement for further control by hormonal ACh. It is also unlikely that a high percentage of the epithelial store of ACh would be released at once to act upon the iris.

Effect of intracorneal injection of ACh on the apparent facility of outflow of aqueous humour.

Rationale.

Experiments described here have shown that ACh injected into the corneal stroma can diffuse intact to distant sites of action in a concentration sufficient to have a physiological action. In the light of the knowledge that parasympathomimetic drugs can reduce the intraocular pressure, as well as causing miosis, it was thought possible that corneal epithelial ACh might act to influence the outflow of aqueous humour.

Method.

The method chosen to determine the apparent facility of outflow was a constant pressure method as opposed to a constant rate method. The former method was developed by Becker & Constant (1956) and chosen by Bárány (1962) for his experiments on primates. The constant rate method for determination of facility of outflow (Sears, 1960) has several disadvantages, the main one being the long time (20 min in rabbits) taken for the eye to adjust from one steady state to another. This time is reduced greatly when a constant pressure method is used. Either method requires a steady state of intraocular pressure, of blood flow through the eye, of secretion of aqueous humour, and of flow into the eye of artificial aqueous humour.

In the constant pressure method, two predetermined pressures are chosen and in the present case these were the resting

intraocular pressure and a pressure 4mm Hg above the resting intraocular pressure. The perfusion rate was measured at the artificial pressure. The apparent facility of outflow, C, could be calculated from Bárány's formula (1964) :

$$C = \frac{F_2 - F_1}{p_2 - p_1}$$

C = Apparent facility of outflow.

F_1 F_2 = Rates of flow, where F_1 is assumed to be zero.

p_1 p_2 = pressures applied where $p_2 - p_1$ = 4mm Hg.

Several assumptions have to be made. The eye is assumed to be in a steady state and the globe to be elastic. In the ideal situation flow between the reservoir and the eye can be recorded instantaneously, and the infusion system, including the needle in the chamber, has zero resistance; then the pressure can be changed instantaneously from p_1 to p_2 and an immediate volume change due to elasticity recorded. This is followed by an immediate change to a new steady rate of flow, F_2. In practice, delays due to the apparatus are less than 5 sec. and changes due to elasticity are largely completed within 30 sec.

Various factors e.g. heart beat of the animal, eye movements etc. cause unsteadiness in the rate of flow and this makes it necessary to average the flow over a certain interval of time to obtain a reliable value. Bárány (1964) used an interval of 2.5 to 3min at each pressure applied in monkeys to obtain a good value for the flow from the reservoir into the eye.

The apparent facility of outflow as measured by a constant pressure method is a property of the structures of the chamber angle and these structures may be affected by the state of contraction of the iris. The apparent facility of outflow is dependent on the inverse resistance of all the outflow channels down to that part of the extraocular venous system where the venous pressure is independent of aqueous humour flow. A third component in the determination of apparent facility of outflow is the pressure dependence of formation or resorption of aqueous humour. This pressure dependence is known as pseudofacility (Bárány, 1963). The higher the pressure is within the anterior chamber, the more the formation of aqueous humour is suppressed and this appears as if it were an increase in the facility of outflow. Bárány (1963) estimated the value for pseudofacility in rabbits to be 9%. Bill & Bárány (1966) used the term 'gross facility of outflow' for the measurement of facility by changes in pressure and this is equivalent to the term 'apparent facility of outflow' used in the present work. Bill (1977a) pointed out that additions to the gross facility can also be made by slow changes in the intraocular blood volume and the amount of fluid in the ocular tissues.

A fifth factor which might affect the values for facility of outflow, is the value for the resting intraocular pressure. However, Bárány (1964) found no pressure dependence of the facility of outflow and Bill & Bárány (1966) and Brubaker & Worthen (1973) have observed linear relationships between pressure and flow from a reservoir into the eye in the ranges of 0 to 7mm Hg below and 0 to 9mm Hg above the resting

intraocular pressure.

A small increase in pressure (4mm Hg) was chosen for these experiments because the artificial value for intraocular pressure fell within the range within which a linear relationship between pressure and flow had been determined. It was also chosen because it was known that high pressures maintained in the anterior chamber can lead to a reduction in the resistance to outflow. A high flow through the meshwork has been claimed to wash out the ground substance and might disrupt the inner wall of Schemm's canal to create new, wide communications between the anterior chamber and the lumen of the canal (Svedbergh, 1974; Grierson & Lee, 1975). Grierson, Lee & Abraham (1979) have suggested that the manipulation required to determine the facility of outflow has a harmful effect on the morphology of the outflow tissues and renders them useless for further determinations. On the other hand, Neufeld (1978) reported no morphological changes after successful use of vervet monkeys for repeated determinations of facility of outflow. In the experiments reported here, controls taken at the beginning and end of the experiment are comparable which suggests that damage causing an increase, or decrease, in facility of outflow did not occur.

Insertion of the needles : this required some care since the rabbit eye tolerates very little trauma during this procedure (Bill, 1975a). Ambache et al.,(1965) showed that trauma to the rabbit iris caused a prolonged increase in intraocular pressure, miosis and vasodilatation. In the present work,

intraocular pressure recorded immediately after needle insertion rarely exceeded 22mm Hg and usually settled to a constant value of 14 to 18mm Hg within 15 min. This suggests that the trauma caused was minimal and that widespread release of prostaglandins and other substances (see Eakins, 1977) did not occur to any extent.

Aqueous humour substitute : the formula chosen was that of Bárány (1964) since simple, unbuffered saline (0.9% w/v) regularly caused a decline in resistance to outflow on prolonged infusion into the anterior chamber (Bárány, 1962). Glucose was required to provide nutrition for lens and cornea. Ca^{2+} ions, a requirement in all balanced salt solutions, had an additional role in maintaining the integrity of the corneal endothelial junctional complexes (Kaye, Mishima, Cole & Kaye, 1968) since we were particularly concerned to maintain a physiologically normal cornea. In later experiments, bicarbonate ions were added to the formula since Hodson (1974) had shown that the active pump in the corneal endothelium, which strives to maintain the hydration of the cornea, has a reduced activity in the absence of bicarbonate ions from the aqueous humour. In both formulae, heparin was added to the solution since it reduces the possibility of fibrin clotting in the aqueous humour, in the outflow mechanism or in the intraocular needle.

Anaesthesia : the majority of animals were anaesthetised with urethane which produces a prolonged, stable anaesthesia in rabbits. It has been suggested that urethane possesses an anticholinesterase activity (see Bowman & Rand, 1980), but in the experiments described here, both neostigmine and ecothiopate were able to potentiate the effect of ACh. Urethane anaesthesia has a tendency to reduce blood pressure and hence the intraocular pressure. The intraocular pressures in rabbits anaesthetised in this manner never fell below physiological limits.

The fact that almost 50% of rabbits were unresponsive to ACh, prompted a change in the type of anaesthetic used. A few experiments were carried out using halothane-N_2O-O_2 anaesthesia and some with a mixture of methohexitone, haloperidol, phenobarbitone and ketamine. However, no response to ACh could be elicited from rabbits anaesthetised in these ways and it was concluded that the type of anaesthetic used was not involved in the failure of the animals to respond to ACh.

The responsiveness of the rabbits was almost cyclical in pattern and responses were obtained consistently for periods lasting several months. These periods were broken by periods of equal length in which no responses were elicited. Seasonal variation, however, did not totally explain the phenomenon since responses were not elicited at the same time in each year. Genetic variation was also unable to explain the phenomenon since all the rabbits were obtained from one supplier who maintained only one strain of the species.

The effect of intracorneal injection of ACh.

The primary observation made was that small doses around 40pmole of ACh injected intracorneally could increase significantly the apparent facility of outflow of aqueous humour. This ACh-induced increase was blocked by large doses of atropine administered intravenously and hence appeared to be an action mediated through muscarinic receptors. Although the effect of ACh was blocked by atropine given intravenously, it was not affected by atropine administered topically in doses sufficient to produce a profound mydriasis. This was an apparent paradox. It may be that high doses of intravenous atropine were required since some 25 to 65% of strains of rabbit possess the enzyme atropinesterase in their serum (Duke-Elder, 1962). It is not yet known whether atropinesterase is present within the ocular tissues - indeed there is some debate as to whether atropinesterase is found in any tissues (Margolis & Feigelson, 1963; Stormont & Suzuki, 1970). Thus, we were unable to ascertain if the inability of topical atropine to block the effect of ACh was due to atropinesterase in the tissues of the anterior chamber. However, since topical atropine produced a mydriasis we might conclude that it was not completely hydrolysed. It would also appear that the site of action of ACh to increase the apparent facility of outflow is a site distant from the iris. This is corroborated by the finding that intravenous atropine, able to block the response to ACh, did not produce mydriasis.

An alternative explanation of this paradox was suggested by the report that melanin in the iris can bind atropine

(Lyons & Krohn, 1973; Patil, 1974). Perhaps the pattern of atropine binding to melanin varies according to whether it is administered intravenously or topically.

There are a variety of possible studies still to be undertaken. Obvious approaches involve the use of albino rabbits and rabbits without serum atropinesterase. Another method would be to use anticholinergic drugs which are resistant to atropinesterase.

Further investigation of the ACh-induced increase in facility of outflow explored the role of Ca^{2+} ions in the mechanism. The Ca^{2+} content of the aqueous humour was reduced by replacing the artificial aqueous humour with a Ca^{2+}-free fluid containing EDTA, to chelate Ca^{2+} ions in the natural aqueous humour. Since Mg^{2+} ions are also chelated by EDTA, they were also removed from the artificial medium. Other experiments involved the use of the Ca^{2+} ionophore, Lilly A23187, which forms stable complexes with divalent cations rendering them lipid soluble and able to pass through membranes. Ca^{2+} ions from the extracellular fluid are the main ions carrying the inward current during the rising phase of the action potential in smooth muscle. They are also important in membrane excitability. Verapamil, a drug which blocks the transport of Ca^{2+} ions across the plasma membrane of smooth muscle cells, was also used in a few experiments. The results of these three experiments suggested that ACh is acting via a Ca^{2+}-dependent mechanism.

There are two disadvantages of the use of EDTA and a Ca^{2+}-

free medium in experiments such as these. Firstly, EDTA removes Mg^{2+} ions as well as Ca^{2+} ions. In their action on membranes, Mg^{2+} ions have a similar but less powerful effect than Ca^{2+} ions. In their action on transmitter release from cholinergic and adrenergic neurones and at other sites of action, Mg^{2+} and Ca^{2+} ions are antagonistic to each other. Secondly, perfusion of the anterior chamber with a Ca^{2+}-free medium *in vitro* has been shown to break up the intercellular junctions in the corneal endothelium (Kaye, Mishima, Cole & Kaye, 1968; Kaye, Hoefle & Donn, 1973). This may have affected the release of ACh from the stromal injection site but it was not apparent since control values were maintained during perfusion of Ca^{2+}-free medium into the anterior chamber. In contrast, Bill (1977a) found that agents which chelated Ca^{2+} nearly abolished the resistance to outflow of aqueous humour through the conventional drainage route. Again, the control values in our experiments did not rise significantly during each experiment using EDTA.

In the study presented here, 40pmole of ACh injected into the stroma produced a significant increase in the outflow of aqueous humour. This is the equivalent of a small proportion of the store of endogenous ACh (1000pmole)in the corneal epithelium, and it might be suggested that the physiological release of 4% of the stored ACh is a possibility. Since the application of anticholinesterases alone, without simultaneous injection of ACh, did not produce an increase in the outflow of aqueous humour, it would appear that this release, should it occur, does not act continually. There may be some stimulus

for the release of endogenous ACh, which is not revealed under the conditions of these experiments i.e. normal intraocular pressure, urethane anaesthesia etc.

The possible sites of action of ACh to influence the apparent facility of outflow.

There are several tissues on which cholinomimetic drugs might act to improve the facility of outflow. Most of the research on this problem has employed pilocarpine, instead of ACh, since the former is used clinically in the treatment of glaucoma. These possible sites of action are discussed below :

Iris : miotic drugs are known to reduce the resistance to outflow. Contraction of the constrictor pupillae muscle may pull the iris tissue away from the iridocorneal angle thus enhancing the passage of aqueous humour into the drainage system. It is unlikely that the ACh injected intracorneally in these experiments acts on the iris to increase the apparent facility of outflow. Previous experiments have shown that the doses of intracorneal ACh required to produce miosis are much higher than those required to affect the outflow of aqueous humour.

Ciliary muscle : Armaly & Burian (1958), working with primates, found that contraction of the ciliary muscle induced by voluntary accommodation or by cholinergic drugs causes an acute increase in the facility of outflow of aqueous humour. Shaffer (1961) found that the facility of outflow is less improved by voluntary accommodation than by the same amount of pilocarpine-induced accommodation. Contraction of the ciliary muscle may cause a tighter packing of the muscle bundles and make the

muscle thicker in the limbus region (Bárány & Rohen, 1965; Rohen, Lütjen & Bárány, 1967). This thickening would cause a separation of the lamellae in the uveal meshwork and probably reduce the resistance to outflow in this area. (see Bill 1975a). The volume of the interstitial spaces is likely to be reduced upon contraction of the muscle and this would result in a reduced interstitial flow.

In rabbits, this action of cholinomimetics may not be as marked as in primates since the ciliary muscle of the rabbit is less well developed (Grierson, 1976).

Trabecular meshwork and angular aqueous plexus : Auricchio & Diotavelli (1959a; 1959b) suggested that pilocarpine or high levels of ACh may have a direct effect on the outflow channels to improve the facility of outflow. Pilocarpine is known to have a histamine-like action (Burn, 1957) which could make the endothelium of the canal 'leaky'. Atropine in high concentrations has been shown to block this effect (Zweifach, 1961; Majno & Palade, 1961). There is a possibility that the ACh administered in our experiments exerted a histamine-like action on the endothelial lining of the angular aqueous plexus, if it could reach this site in sufficiently high concentration.

Microcirculation and blood flow of the anterior segment : Since it has been shown in primates that topical pilocarpine can double the rate of blood flow in the iris, ciliary processes and ciliary muscle, this drug may modify the intraocular pressure and resistance to outflow by modulating the blood flow in such

tissues (Alm, Bill & Young, 1973). This is a possible mode of action for the injected ACh to increase outflow. Pilocarpine applied topically has been shown to cause a brief dilatation of the episcleral blood vessels (Krakau, 1976). This could cause a transient increase in the outflow of aqueous humour from the anterior chamber. The ACh applied in our experiments may act in such a manner but this is unlikely due to the distance from the site of injection to the episcleral veins.

Formation of aqueous humour by the ciliary epithelium : When gross facility of outflow is measured, any reduction in formation of aqueous humour appears as an increase in outflow. It has been shown in primates that pilocarpine can reduce the rate of formation of aqueous humour. (Wålinder & Bill, 1969). There is some controversy on the effects of parasympathomimetics on aqueous humour formation in rabbits. Uusitalo (1972) found that parasympathomimetics did not affect the formation of aqueous humour *in vivo.* Green & Padgett (1979) saw a small increase in formation of aqueous humour caused by pilocarpine. These latter authors concluded that pilocarpine produced a true increase in facility of outflow because it did not increase pseudofacility yet reduced the intraocular pressure.

Attempts to define the link between accommodation and the effects of drugs upon the facility of outflow have not been conclusive. Ballintine (1961) reported that pilocarpine affected the resistance to outflow at doses which had little effect on accommodation. Bárány (1962; 1966a; 1966b) concluded that pilocarpine had a direct effect in improving

the facility of outflow because an intracameral injection of pilocarpine reduced the resistance to outflow but had little effect on accommodation. It was thought originally, that this additional effect might be on the endothelium of Schlemm's canal in primates (Bárány, 1962; 1966a).

Bárány (1962) postulated two distinct mechanisms of action for pilocarpine in increasing the facility of outflow. Firstly, a parasympathomimetic action on the ciliary muscle, as described above, and this action was rapidly blocked by atropine. A second mechanism was only slowly reversed by atropine, over 30 min being required to produce a complete blockade. This suggested to Bárány that smooth muscle was not involved in this second mechanism. Holmberg & Bárány (1966) found changes in the endothelium of the canal of Schlemm caused by pilocarpine but they were unable to assess their functional importance.

Bárány later rejected the hypothesis that pilocarpine acted on the outflow channels since intravenous pilocarpine reduced the resistance to outflow so quickly after injection that it seemed likely that most of its effect was on a site other than the inner wall of Schlemm's canal (Bárány, 1967). After intravenous injection, pilocarpine does not reach appreciable concentrations in the aqueous humour for some time. The drug must reach the ciliary muscle to have an effect well before the concentration in the aqueous humour is high enough to produce an effect on the endothelial cells lining the outflow channels. In contrast, the ACh administered in our experiments is much more likely to reach the outflow structures than to

diffuse the further distance to the ciliary muscle.

Recent work by Kaufman & Bárány (1976a; 1976b) and Kaufman & Lütjen-Drecoll (1975) has employed surgical disinsertion and retrodisplacement of the ciliary muscle from the scleral spur in cynomolgus monkeys. These experiments have shown that the effect of pilocarpine in these animals is mediated by a contraction of the ciliary muscle. Kaufman & Bárány (1976b) explain the results of Bárány (1962) where atropine was unable to completely reverse the effects of pilocarpine. They suggest that a mechanical hysteresis of the meshwork occurs; contraction of the ciliary muscle forces a rapid structural change on the meshwork but muscle relaxation alone cannot reverse this change. The elasticity of the meshwork is involved in reversing the changes produced within it an this process may be slow. Again, this mechanism is less likely to pertain in rabbits which have a poor ciliary musculature.

Grierson, Lee & Abraham (1978) investigated the use of pilocarpine in human eyes and found that it distended the endothelial meshwork by an action on the ciliary muscle and also doubled the incidence of giant vacuoles in the endothelium of Schlemm's canal. However, in a later publication (Grierson et al., 1979) it was suggested that there was little evidence that the increased vacuolation was the result of a direct action of pilocarpine on the endothelium. The phenomenon was more likely to be a consequence of the increased passage of fluid through the drainage system caused by the action of pilocarpine on the ciliary muscle.

In conclusion, there are many conflicting pieces of evidence and hypotheses in the literature on the effect of parasympathomimetics on the facility of outflow. Most authors, considering evidence obtained from work on primates, now conclude that the increase in facility of outflow produced by pilocarpine is due to its action on the ciliary muscle. This conclusion does not necessarily pertain in sub-primate species. Most authors do not completely discard the hypothesis that parasympathomimetics may have a direct action on the outflow channels in primates and this mode of action may be of importance in rabbits. Sakimoto (1979) demonstrated the presence of smooth muscle cells adjacent to the aqueous drainage channels in rabbits and such cells have not been reported to be present near primate outflow channels. It could be speculated that parasympathetic drugs act on such cells to regulate the outflow of aqueous humour in the rabbit. Since it appears that intracorneally injected ACh affects both scleral compliance and outflow, it may be that an action of the hormone on these smooth muscle cells alters the shape of the anterior chamber. This might allow the accommodation of more fluid, in addition to increasing the facility with which it drains from the chamber.

Experiments reported here have shown that only a small proportion of ACh injected into the corneal stroma can diffuse as far as the ciliary muscle, whilst a high proportion of this ACh remained in the stroma. It was also found that the level of ChE in the aqueous humour is considerably higher than that in the stroma. In view of these findings, and the decided

effect of intracorneally injected ACh on the facility of outflow, it may be suggested that ACh diffuses to a site of action within the outflow mechanism via the stroma.

REFERENCES

The journal abbreviations used in this thesis are those of the Journal of Physiology. The abbreviations for journals which do not appear in the Journal of Physiology "Suggestions to Authors", 1966 have been obtained from :

List of Journals indexed in Index Medicus, 1980.
Bethesda, Maryland : National Institute of Health
Publication No. 80-267.

All references from J. Physiol and Nature which are cited in this thesis are from the London editions of the journals.

ADENIS, J.P., MERLE, L., DANGOUMAU, J., ROBIN, A. & NICOT, G. (1978). Problèmes posés par l'utilisation des collyres et pommades anesthésiques en ophtalmologie. Thérapie. 33, 791-795.

ALM, A., BILL, A. & YOUNG, F.A. (1973). The effects of pilocarpine and neostigmine on the blood flow through the anterior uvea in monkeys. A study with radioactively labelled microspheres. Exp. Eye Res. 15, 31-36.

van ALPHEN, G.W.H.M. (1957). Acetylcholine synthesis in the corneal epithelium. Archs. Ophthal., N.Y. 58, 449-451.

AMBACHE, N., KAVANAGH, L. & WHITING, J. (1965). Effect of mechanical stimulation on rabbits' eyes : Release of active substance in anterior chamber perfusates. J. Physiol. 176, 378-408.

AQUILONIUS, S.-M., LUNDHOLM, B. & WINBLADH, B. (1972). Effects of some anticholinergic drugs on cortical acetylcholine release and motor activity in rats. Eur. J. Pharmacol. 20, 224-230.

ARMALY, M.F. & BURIAN, H.M. (1958). Changes in the tonogram during accommodation. Archs. Ophthal., N.Y. 60, 60-69.

ASCHER, K.W. (1942). Aqueous veins. Am. J. Ophthal. 25, 31-38.

AUGSBURGER, A.R. & HILL, R.M. (1972). Corneal anaesthetics and epithelial oxygen flux. Archs. Ophthal., N.Y. 88, 305-307.

AURICCHIO, G. & DIOTALLEVI, M. (1959a). La resistenza al deflusso in occhi di coniglio dopo prolungato trattamento con diisopropilfluorofosfato. Ann. Ottalmol. e. Clin. Oculi. 85, 493-496.

AURICCHIO, G. & DIOTALLEVI, M. (1959b). Ulteriori ricerche sull' influenza esercitata dal DFP sulla resistenza al deflusso in occhi di coniglio. Ann. Ottalmol. e. Clin. Ocul. 85, 567-570.

BALLINTINE, E.J. (1961). In : Newell, F.W., Glaucoma. Transactions of the Fifth Conference pp 246-247. New York : Josiah Macy Jr. Foundation.

BÁRÁNY, E.H. (1962). The mode of action of pilocarpine on outflow resistance in the eye of a primate (Cercopithecus ethiops). Invest. Ophthalmol. 1, 712-727.

BÁRÁNY, E.H. (1963). A mathematical formulation of intraocular pressure as dependent on secretion, ultrafiltration, bulk outflow and osmotic reabsorption of fluid. Invest. Ophthalmol. 2, 584-590.

BÁRÁNY, E.H. (1964). Simultaneous measurement of changing intraocular pressure and outflow facility in the vervet monkey by constant pressure infusion. Invest. Ophthalmol. 3, 135-143.

BÁRÁNY, E.H. (1966a). The mode of action of miotics on outflow resistance. A study of pilocarpine in the vervet monkey Cercopithecus ethiops. Trans. Ophthalmol. Soc. U.K. 86, 539-578.

BÁRÁNY, E.H. (1966b). Dissociation of accommodation effects from outflow effects of pilocarpine. In: Paterson, G., Miller, S.J.H. & Paterson, G.D. Drug Mechanisms in Glaucoma pp 275-282. London: J. & A. Churchill Ltd.

BÁRÁNY, E.H. (1967). The immediate effect on outflow resistance of intravenous pilocarpine in the vervet monkey, Cercopithecus ethiops. Invest. Ophthalmol. 6, 373-380.

BÁRÁNY, E., BERGGREN, L. & VRABEC, F. (1957). The mucinous layer covering the corneal endothelium in the owl Strix Aluco. Br. J. Ophthal. 41, 25-30.

BÁRÁNY, E.H. & ROHEN, J.W. (1965). Localized contraction and relaxation within the ciliary muscle of the vervet monkey, Cercopithecus ethiops. In : Rohen, J.W. The structure of the eye II Symposium. pp 287-311. Stuttgart : Schattauer-Verlag.

BÁRÁNY, E.H. & SCOTCHBROOK, S. (1954). Influence of testicular hyaluronidase on the resistance to flow through the angle of the anterior chamber. Acta physiol. scand. 30, 240-248.

BAUM, J.L. (1963). A histochemical study of corneal respiratory enzymes. Archs Ophthal., N.Y. 70, 59-68.

BECKER, B. & CONSTANT, M.A. (1956). The facility of aqueous outflow : A comparison of tonography and perfusion measurements in vivo and in vitro. Archs Ophthal., N.Y. 55, 305-312.

BERGGREN, L. (1957). A histochemical study of the anterior segment of the eye in gold id (Idus Idus L.), chicken, rabbit and man with special regard to the angle of the anterior chamber. Acta Soc. Med. Upsal. 62, 157-175.

BILL, A. (1965). The aqueous humor drainage mechanism in the cynomolgus monkey (Macaca irus) with evidence for unconventional routes. Invest. Ophthalmol. 4, 911-919.

BILL, A. (1966a). The routes for bulk drainage of aqueous humour in rabbits with and without cyclodialysis. Documenta Ophth. 20, 157-169.

BILL, A. (1966b). Conventional and uveoscleral drainage of aqueous humour in the cynomolgus monkey (Macaca irus) at normal and high intraocular pressures. Exp. Eye Res. 5, 45-54.

BILL, A. (1967). Further studies on the influence of the intraocular pressure on aqueous humor dynamics in cynomolgus monkeys Invest. Ophthalmol. 6, 364-372.

BILL, A. (1973). The role of ciliary blood flow and ultrafiltration in aqueous humor formation. Exp. Eye Res. 16, 287-298.

BILL, A. (1975a). Blood circulation and fluid dynamics in the eye. Physiol. Rev. 55, 383-417.

BILL, A. (1975b). The drainage of aqueous humour. Invest. Ophthalmol. 14, 1-3.

BILL, A. (1977a). Basic physiology of the drainage of aqueous humor. Exp. Eye Res. 25 (Suppl),291-304.

BILL, A. (1977b). A reply to R. Tripathi : Uveoscleral drainage of aqueous humor Exp. Eye Res. 25 (Suppl.), 309-310.

BILL, A. & BÁRÁNY, E.H. (1966). Gross facility, facility of conventional routes and pseudofacility of aqueous humor outflow in the cynomolgus monkey: The reduction in aqueous humor formation rate caused by moderate increments in intraocular pressure. Archs Ophthal., N.Y. 75, 665-673.

BITO, L.Z., ROBERTS, J.C. & SARAF, S. (1973). Maintenance of normal corneal thickness in the cold in vivo (hibernation) as opposed to in vitro. J. Physiol. 231, 71-86.

BLÜMCKE, S., RODE, J. & NIEDORF, H.R. (1969). Formation of the basement membrane during regeneration of the corneal epithelium. Z. Zellforsch. mikrosk. Anat. 93, 84-92.

BLÜMCKE, S., RODE, J. & KUDSZUS, G. (1967). Feinstrukturelle Veränderungen des Corneaepithels in der Gewebekultur. I. Die Lipoidschicht an der epitheloberfläche. Z. Zellforsch. mikrosk. Anat. 82, 589-603.

BOBERG-ANS, J. (1955). Experience in clinical examination of corneal sensitivity. Corneal sensitivity and the naso-lacrimal reflex after retrobulbar anaesthesia. Br. J. Ophthal. 39, 705-726.

BOLKOVÁ, A. & ČEJKOVÁ, J. (1978). Biochemical and histochemical response to a complete epithelial denudation of the rabbit cornea. Alkaline and acid phosphatase. Albrecht v. Graefes Arch. Ophthal. 209, 137-143.

BONTING, S.L., CARAVAGGIO, L. & HAWKINS, N.M. (1962). Studies on sodium-potassium-activated adenosinetriphosphatase. IV Correlation with cation transport sensitive to cardiac glycosides. Archs Biochem. Biophys. 98, 413-419.

BONTING, S.L., SIMON, K.A. & HAWKINS, N.M. (1961). Studies on sodium-potassium-activated adenosinetriphosphatase I. Quantitative distribution in several tissues of the cat. Archs. Biochem. Biophys. 95, 416-423.

BOWMAN, W.C. & RAND, M.J. (1980). Textbook of Pharmacology 2nd edn. Oxford; London; Edinburgh : Blackwell Scientific Publications.

BRANCATO, R., FUSAROLI, P., MALLARDI, F. & RAVALICO, G. (1980). Histochemical and ultrastructural study on the adrenergic innervation of the aqueous outflow system. Abstracts of the European Glaucoma Society.

BREWITT, H. & HONEGGER, H. (1978). The influence of local anaesthetics on corneal epithelium. A scanning electron microscopic study. Klin. Monatsbl. Augenheilkd. 173, 347-354.

BRUBAKER, R.F. & WORTHEN, D.M. (1973). The filtration coefficient of the intraocular vasculature as measured by low-pressure perfusion in a primate eye. Invest. Ophthalmol. 12, 321-326.

von BRÜCKE, H. (1938). Zur Kenntnis der Hornhautschädigung nach Trigeminus verletzung. Klin. Wschr. 17, 420-422.

von BRÜCKE, H., HELLAUER, H.F., & UMRATH, K. (1949). Azetylcholin-und Aneuringehalt der Hornhaut und seine Beziehungen zur Nervenversorgung. Ophthalmologica Basel. 117, 19-35.

BURN, J.H. (1957). The principles of therapeutics pp 56-57. Oxford : Blackwell Scientific Publications.

BURNS, R.P. (1968). Meesman's Corneal Dystrophy. Trans. Am. Ophthalmol. Soc. 66, 530-635.

BURT, A.M. & SILVER, A. (1973). Histochemistry of choline-acetyltransferase : A critical analysis. Brain Res. 62, 509-516.

van BUSKIRK, E.M. & GRANT, W.M. (1974). Influence of temperature and the question of involvement of cellular metabolism in aqueous outflow. Am. J. Ophthal. 77, 565-572.

CALMETTES, DEODATI, PLANEL & BEC. (1956). Étude histologique et histochimique de l'épithélium antérieur de la cornée et de ses basales. Arch. Ophthal. Paris. 16, 481-506.

CARPENTER, G. (1978) The regulation of cell proliferation : advances in the biology and mechanism of action of epidermal growth factor. J. invest. Derm. 71, 283-287.

CAVANAGH, H.D. (1975). Herpetic ocular disease: therapy of persistent epithelial defects. Int. Ophthalmol. Clin. 15, 67-88.

CHAPMAN, G.B. & SPELSBERG, W.W. (1963). The occurrence of myelinated and unmyelinated nerves in the iris angle of man and rhesus monkey. Exp. Eye Res. 2, 130-133.

CHENEY, D.L., LE FEVRE, H.F. & RACAGNI, G. (1975). Choline acetyltransferase activity and mass fragmentographic measurement of acetylcholine in specific nuclei and tracts of rat brain. Neuropharm. 14, 801-809.

COHEN, S. (1962). Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. J. biol. Chem. 237, 1555-1562.

COHEN, S., CARPENTER, G. & LEMBACH, K.J. (1975). Interaction of epidermal growth factor (EGF) with cultured fibroblasts Adv. Metab. Disord. 8, 265-284.

COLE, D.F. (1966). Aqueous humour formation. Documenta Ophthal. 21, 116-238.

COLE, D.F. (1969). Evidence for active transport of chloride in ciliary epithelium of the rabbit. Exp. Eye Res. 8, 5-15.

COLE, D.F. (1974). Comparative aspects of the intraocular fluids In: The Eye Vol. 5 ed Davson, H. New York; London: Academic Press.

COLE, D.F. (1977). Secretion of the aqueous humour. Exp. Eye Res. 25 (Suppl.), 161-176.

COLE, D.F. & TRIPATHI, R.C. (1971). Theoretical considerations on the mechanism of the aqueous outflow. Exp. Eye Res. 12, 25-32.

COLLINS, E.T. (1921). Changes in the visual organs correlated with the adoption of arboreal life and with the assumption of the erect posture. Trans. Ophthal. Soc. U.K. 41, 10-90.

COMIS, S.D. & DAVIES, W.E. (1969). Acetylcholine as a transmitter in the cat auditory system. J. Neurochem. 16, 423-429.

DANIELE, S., FRATI, L., FIORE, C. & SANTONI, G. (1979). The effect of the epidermal growth factor (EGF) on the corneal epithelium in humans. Albrecht v. Graefes Arch. Ophthal. 210, 159-165.

DARK, A.J., DURRANT, T.E., McGINTY, F. & SHORTLAND, J.R. (1974). Tarsal conjunctiva of the upper eyelid. Am. J. Ophthal. 77, 555-564.

DAVANGER, M. & EVENSEN, A. (1971). Role of the pericorneal papillary structure in renewal of corneal epithelium. Nature. 229, 560-561.

DAVSON, H. (1956). In: Physiology of the ocular and cerebrospinal fluids. London: J. & A. Churchill, Ltd.

DIAMOND, I. & KENNEDY, E.P. (1969). Carrier-mediated transport of choline into synaptic nerve endings. J. biol. Chem. 244, 3258-3263.

DIAMOND, I. & MILFAY, D. (1972). Uptake of [^{3}H-methyl] choline by microsomal, synaptosomal, mitochondrial and synaptic vesicle fractions of rat brain. The effects of hemicholinium. J. Neurochem. 19, 1899-1909.

DIAMOND, J. (1959). The effects of injecting acetylcholine into normal and regenerating nerves. J. Physiol. 145, 611-629.

DOANE, M.G. & DOHLMAN, C.H. (1970). Physiological response of the cornea to an artificial epithelium. Exp. Eye Res. 9, 158-164.

DONN, A., MAURICE, D.M. & MILLS, N.L. (1959a). Studies on the living cornea in vitro. I. Method and physiologic methods. Archs Ophthal. N.Y. 62, 741-747.

DONN, A., MAURICE, D.M. & MILLS, N.L. (1959b). Studies on the living cornea in vitro II. The active transport of sodium across the epithelium. Archs Ophthal., N.Y. 62, 748-757.

DUKE-ELDER, S. (1962). System of Ophthalmology Vol. 7. London: Henry Kimpton.

DUKE-ELDER, P.M. & DUKE-ELDER, W.S. (1931). The vascular responses of the eye. Proc. R. Soc. B. 109, 19-28.

EAKINS, K.E. (1977). Prostaglandin and non-prostaglandin mediated breakdown of the blood-aqueous barrier. Exp. Eye Res. 25 (Suppl.), 483-498.

EDELHAUSER, H.F., VAN HORN, D.L., MILLER, P. & PEDERSON, H.J. (1976). Effect of thiol-oxidation of glutathione with diamide on corneal endothelial function, junctional complexes and microfilaments. J. Cell Biol. 68, 567-578.

EDWARDS, J., HALLMAN, V.L. & PERKINS, E.S. (1967). Perfusion studies on the monkey eye. Exp. Eye Res. 6, 316-326.

ELLMAN, G.L., COURTNEY, K.D., ANDRES, V. & FEATHERSTONE, R.M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmac. 7, 88-95.

ENGELHART, E. (1931). Der humorale wirkungs mechanismus der oculomotoriusreizung. Pflügers Arch. ges. Physiol. 227, 220-234.

ERIKSSON, A. & SVEDBERGH, B. (1980). Transcellular aqueous humor outflow : A theoretical and experimental study. Albrecht v. Graefes Arch. Ophthal. 212, 187-197.

EYZAGUIRRE, C. & KOYANO, H. (1965). Effects of some pharmacological agents on chemoreceptor discharges. J. Physiol. 178, 410-437.

EYZAGUIRRE, C. & ZAPATA, P. (1968). Pharmacology of pH effects on carotid body chemoreceptors in vitro. J. Physiol. 195, 557-588.

FEENEY, L. (1962). Ultrastructure of the nerves in the human trabecular region. Invest. Ophthalmol. 1, 462-473.

FEENEY, L. & WISSIG, S. (1966). Outflow studies using an electron dense tracer. Trans. Am. Acad. Ophthalmol. Otolaryngol. 70, 791-798.

FINE, B. (1964). Observations on the drainage angle in man and rhesus monkey : A concept of the pathogenesis of chronic simple glaucoma. A light and electron microscopic study. Invest. Ophthalmol. 3, 609-646.

FITZGERALD, G.G., & COOPER, J.R. (1971). Acetylcholine as a possible sensory mediator in rabbit corneal epithelium. Biochem. Pharmacol. 20, 2741-2748.

FOGLE, J.A. & NEUFELD, A.H. (1979). The adrenergic and cholinergic corneal epithelium. (Editorial) Invest. Opthalmol. Vis. Sci. 18, 1212-1215.

FRATI, L., D'ARMIENTO, M., GULLETTA, E., VERNA, R. & COVELLI, I. (1977). The control of epidermis proliferation by epidermal growth factor (EGF). Relationship with cyclic nucleotides systems. Pharmac. Res. Comm. 9, 815-822.

FRATI, L., DANIELE, S., DELOGU, A. & COVELLI, I. (1972). Selective binding of the epidermal growth factor and its specific effects on the epithelial cells of the cornea. Exp. Eye Res. 14, 135-141.

FRIEDENWALD, J.S. (1951). Growth pressure and metaplasia of conjunctival and corneal epithelium. Documenta Ophth. 5-6, 184-192.

FRIEDENWALD, J.S. (1955). Carbonic anhydrase inhibition and aqueous flow. Am. J. Ophthal. 39(ii), 59-64.

FRIEDENWALD, J.S. & BUSCHKE, W. (1944). Mitotic and wound healing activities of the corneal epithelium. Archs Ophthal. N.Y. 32, 410-413.

FRIEND, J. & THOFT, R.A. (1978). Functional competence of regenerating ocular surface epithelium. Invest. Ophthalmol. Vis. Sci. 17, 134-139.

GARRON, L.K., FEENEY, M.L., HOGAN, M.J. & McEWEN, W.K. (1958). Electron microscopic studies of the human eye. 1. Preliminary investigations of the trabeculas. Am. J. Ophthal. 46,(ii), 27-35.

GIPSON, I.K. & ANDERSON, R.A. (1977). Actin filaments in normal and migrating corneal epithelial cells. Invest. Ophthalmol. Vis. Sci. 16, 161-166.

GNÄDINGER, M.C., HEIMANN, R. & MARKSTEIN, R. (1973). Choline acetyltransferase in corneal epithelium. Exp. Eye Res. 15, 395-399.

GNÄDINGER, M., WALZ, D., von HAHN, H.P. & GRÜN, F. (1967). Acetylcholine-splitting activity of abraded and cultivated corneal epithelial cells. Exp. Eye Res. 6, 239-242.

GOLDMAN, J.N., DOHLMAN, C.H. & KRAVITT, B.A. (1969). The basement membrane of the human cornea in recurrent epithelial erosion syndrome. Trans. Am. Acad. Ophthalmol. Otolaryngol. 73, 471-481.

GOSPODAROWICZ, D., MESCHER, A.L., BROWN, K.D. & BIRDWELL, C.R. (1977). The role of fibroblast growth factor and epidermal growth factor in the proliferative response of the corneal and lens epithelium. Exp. Eye Res. 25, 631-649.

GRANT, W.M. (1958). Further studies on facility of flow through the trabecular meshwork. Archs Ophthal., N.Y. 60, 523-533.

GRANT, W.M. (1963). Experimental aqueous perfusion in enucleated human eyes. Archs Ophthal., N.Y. 69, 783-801.

GREEN, K. (1965). Ion transport in isolated cornea of the rabbit. Am. J. Physiol. 209, 1311-1316.

GREEN, K. (1966). Ion transport across the isolated rabbit cornea. Exp. Eye Res. 5, 106-110.

GREEN, K. (1967a). Solute movement across the constituent membranes of the cornea. Exp. Eye Res. 6, 79-92.

GREEN, K. (1967b). Observations on corneal potential. Exp. Eye Res. 6, 93-96.

GREEN, K. (1968). Relation of epithelial ion transport to corneal thickness and hydration. Nature. 217, 1074-1075.

GREEN, K. (1969). Dependence of corneal thickness on epithelial ion transport and stromal sodium. Am. J. Physiol. 217, 1169-1177.

GREEN, K. & PADGETT, D. (1979). Effect of various drugs on pseudofacility and aqueous humor formation in the rabbit eye. Exp. Eye Res. 28, 239-246.

GREEN, K. & PEDERSON, J.E. (1972). Contribution of secretion and filtration to aqueous humour formation. Am. J. Physiol. 222, 1218-1226.

GREGORY, H. (1975). Isolation and structure of urogastrone and its relationship to epidermal growth factor. Nature. 257, 325-327.

GREINER, J.V., COVINGTON, H.I. & ALLANSMITH, M.R. (1979). The human limbus. A scanning electron microscopic study. Archs Ophthal., N.Y. 97, 1159-1165.

GRIERSON, I. (1976). The morphology of the outflow apparatus of the eye with particular reference to its structural appearance at various levels of intraocular pressure. Ph.D. Thesis. Glasgow University.

GRIERSON, I. & LEE, W.R. (1973). Erythrocyte phagocytosis in the human trabecular meshwork. Br. J. Ophthal. 57, 400-415.

GRIERSON, I. & LEE, W.R. (1975). Pressure-induced changes in the ultrastructure of the endothelium lining Schlemm's canal. Am. J. Ophthal. 80, 863-884.

GRIERSON, I., LEE, W.R. & ABRAHAM, S. (1978). Effects of pilocarpine on the morphology of the human outflow apparatus. Br. J. Ophthal. 62, 302-313.

GRIERSON, I., LEE, W.R. & ABRAHAM, S. (1979). The effects of topical pilocarpine on the morphology of the outflow apparatus of the baboon (Papio cynocephalus). Invest. Ophthal. Vis. Sci. 18, 346-355.

GUYENET, P., LEFRESNE, P., ROSSIER, J., BEAUJOUAN, J.C. & GLOWINSKI, J. (1973). Inhibition by hemicholinium-3 of [1-^{3}H] acetylcholine synthesis and [^{3}H] choline high-affinity uptake in rat striatal synaptosomes. Mol. Pharmacol. 9, 630-639.

GWIN, R.M., GELATT, K.N. & CHIOU, C.Y. (1979). Adrenergic and cholinergic innervation of the anterior segment of the normal and glaucomatous dog. Invest. Ophthal. Vis. Sci. 18, 674-682.

HAGA, T. & NODA, H. (1973). Choline uptake systems of rat brain synaptosomes. Biochim. biophys. Acta 291, 564-575.

HANNA, C. & O'BRIEN, J.E. (1960). Cell production and migration in the epithelial layer of the cornea. Archs Ophthal., N.Y. 64, 536-539.

HARNISCH, J.P., HOFFMANN, F. & DUMITRESCU, L. (1975). Side effects of local anaesthetics on the corneal epithelium of the rabbit eye. Albrecht v. Graefes Arch. Ophthal. 197, 71-81.

HARRIS, J.E. & GRUBER, L. (1956). The hydration of the cornea III. The influence of the epithelium. Am. J. Ophthal. 42(ii), 330-337.

HARVEY, A.M., LILIENTHAL, J.L. & TALBOT, S.A. (1941). On the effects of the intra-arterial injection of acetylcholine and prostigmine in normal man. Johns Hopkins Hosp. Bull. 69, 529-546.

HASSÓN-VOLOCH, A. & SIMAS, D. (1969). TLC identification of ACh after biosynthesis with the electric organ. Ann. Acad. brasil Ciênc. 41, 235-237.

HEBB, C.O. (1956). Choline acetylase in the developing nervous system of the rabbit and guinea pig. J. Physiol. 133, 566-570.

HEDBYS, B.O. (1961). The role of polysaccharides in corneal swelling. Exp. Eye Res. 1, 81-91.

HELLAUER, H.F. (1950). Sensibilität und Acetylcholingehalt der Hornhaut Verschiedener Tiere und des Menschen. Z. vergl. Physiol. 32, 303-310.

HENKES, H.E. & WAUBKE, T.N. (1978). Keratitis from abuse of corneal anaesthetics. Br. J. Ophthal. 62, 62-65.

HENROTTE, J. & WEEKERS, J.F. (1972). Étude clinique des lésions cornéennes dues à l'application locale et prolongée d'anesthesiques. Archs Ophthal., Paris. 32, 449-456.

HEYDENREICH, A. (1958). In: Die hornhautregeneration. Marhold, Halle.

HIRSCH, M. (1978). Fine structure and functions of the corneal endothelium. J. Fr. Ophthalmol. 1, 61-71.

HO, P.C., DAVIS, W.H., ELLIOTT, J.H. & COHEN, S. (1974). Kinetics of corneal epithelial regeneration and epidermal growth factor. Invest. Ophthalmol. 13, 804-809.

HODSON, S. (1974). The regulation of corneal hydration by a salt pump requiring the presence of sodium and bicarbonate ions. J. Physiol. 236, 271-302.

HODSON, S. (1977). The endothelial pump of the cornea (editorial). Invest. Ophthal. Vis. Sci. 16, 589-591.

HODSON, S. & MILLER, F. (1976). The bicarbonate ion pump in the endothelium which regulates the hydration of rabbit cornea. J. Physiol. 263, 563-577.

HOGAN, M.J., ALVARADO, J.A. & WEDDELL, J.E. (1971). Histology of the Human Eye. An atlas and textbook. Philadelphia; London; Toronto: W.B. Saunders Co.

HOLLAND, M.G., von SALLMANN, L. & COLLINS, E.M. (1956). A study of the innervation of the chamber angle. Am. J. Ophthal. 42(ii), 148-161.

HOLLAND, M.G., von SALLMANN, L. & COLLINS, E.M. (1957). A study of the innervation of the chamber angle II. The origin of trabecular axons revealed by degeneration experiments. Am. J. Ophthal. 44(ii), 206-221.

HOLLY, F.J. (1978). Biophysical aspects of epithelial adhesion to stroma. Invest. Ophthalmol. Vis. Sci. 17, 552-557.

HOLMBERG, Å. (1959). The fine structure of the inner wall of Schlemm's canal. Archs Ophthal., N.Y. 62, 956-958 and 1047-1056.

HOLMBERG, Å. & BÁRÁNY, E.H. (1966). The effect of pilocarpine on the endothelium forming the inner wall of Schlemm's canal: An electron-microscopic study in the monkey Cercopithecus aethiops. Invest. Ophthalmol. 5, 53-58.

HOLMSTEDT, B. (1967). Mobilization of acetylcholine by cholinergic agents. Ann. N.Y. Acad. Sci. 144, 433-458.

HOWARD, R.O. & WILSON, W.S. (1972). Development of acetylcholine, choline acetyltransferase and acetylcholinesterase in rabbit corneal epithelium. Br. J. Pharmac. 46, 567P-568P.

HOWARD, R.O., WILSON, W.S. & DUNN, B.J. (1973). Quantitative determination of choline acetylase, acetylcholine and acetylcholinesterase in the developing rabbit cornea. Invest. Ophthalmol. 12, 418-425.

HOWARD, R.O., ZADUNAISKY, J.A. & DUNN, B.J. (1975). Localisation of acetylcholinesterase in the rabbit cornea by light and electron microscopy. Invest. Ophthalmol. 14, 592-603.

HOYES, A.D. & BARBER, P. (1976). Ultrastructure of the corneal nerves in the rat. Cell Tissue Res. 172, 133-144.

HUECK, A. (1839) quoted by TRIPATHI, R.C. (1974). Comparative physiology and anatomy of the aqueous outflow pathway. In: Davson, H. The Eye. Vol. 5. New York, San Franscisco, London: Academic Press.

HULL, D.S., GREEN, K., BOYD, M. & WYNN, H.R. (1977). Corneal endothelium bicarbonate transport and the effect of carbonic anhydrase inhibitors on endothelial permeability and fluxes and corneal thickness. Invest. Ophthalmol. Vis. Sci. 16, 883-892.

INOMATA, H. & BILL, A. (1977). Exit sites of uveoscleral flow of aqueous humor in cynomolgus monkey eyes. Exp. Eye Res. 25, 113-118.

INOMATA, H., BILL, A. & SMELSER, G.K. (1972a). Aqueous humor pathways through the trabecular meshwork and into Schlemm's canal in the cynomolgus monkey (Macaca irus). An electron microscopic study. Am. J. Ophthal. 73, 760-789.

INOMATA, H., BILL, A. & SMELSER, G.K. (1972b). Unconventional routes of aqueous humor outflow in cynomolgus monkey (Macaca irus) Am. J. Ophthal. 73, 893-907.

JAKUS, M.A. (1964). Ocular fine structure. Selected electron micrographs. London : J. & A. Churchill Ltd.

JOHNSTONE, M.A. (1974). Pressure-dependent changes in configuration of the endothelial tubules of Schlemm's canal. Am. J. Ophthal. 78, 630-647.

JOHNSTONE, M.A. & GRANT, W.M. (1973). Pressure dependent changes in structures of the aqueous outflow system of human and monkey eyes. Am. J. Ophthal. 75, 365-383.

KANDORI, F. & OKAMOTO, T. (1960). Ein elektronenmikroskopisher Befund der Kammer winkels des Kaninchenauges, Der erste Bericht. Yonago Acta Med. 4, 152-156.

KAUFMAN, P.L. & BÁRÁNY, E.H. (1976a). Loss of acute pilocarpine effect on outflow facility following surgical disinsertion and retrodisplacement of the ciliary muscle from the scleral spur in the cynomolgus monkey. Invest.Ophthalmol. 15, 793-807.

KAUFMAN, P.L. & BÁRÁNY, E.H. (1976b). Residual pilocarpine effects on outflow facility after ciliary muscle disinsertion in the cynomolgus monkey. Invest. Ophthalmol. 15, 558-561.

KAUFMAN, B., GAY, H. & HOLLAENDER, A. (1944). Distribution of mitoses in the corneal epithelium of the rabbit and the rat. Anat. Rec. 90, 161-178.

KAUFMAN, P.L. & LÜTJEN-DRECOLL, E. (1975). Total iridectomy in the primate in vivo : surgical technique and post operative anatomy. Invest. Ophthalmol. 14, 766-771.

KAYE, G.I., HOEFLE, F.B. & DONN, A. (1973). Studies on the cornea VIII Reversibility of the effects of in vitro perfusion of the rabbit corneal endothelium with calcium-free medium. Invest. Ophthalmol. 12, 98-113.

KAYE, G.I., MISHIMA, S., COLE, J.D. & KAYE, N.W. (1968). Studies on the cornea VII Effects of perfusion with a Ca^{2+}-free medium on the corneal endothelium. Invest. Ophthalmol. 7, 53-66.

KAYES, J. & HOLMBERG, Å. (1960). The fine structure of Bowman's layer and the basement membrane of the corneal epithelium. Am. J.Ophthal. 50, 1013-1021.

KHODADOUST, A.A. (1967). Techniques for the preparation of sheets of pure corneal epithelium. Am. J. Ophthal. 63, 942-945.

KHODADOUST, A.A., SILVERSTEIN, A.M., KENYON, K.R. & DOWLING, J.E. (1968). Adhesion of regenerating corneal epithelium. The role of basement membrane. Am.J.Ophthal. 65, 339-348.

KINOSHITA, J.H. (1962). Some aspects of the carbohydrate metabolism of the cornea. Invest. Ophthalmol. 1, 178-186.

KINOSHITA, J.H., MASURAT, T. & HELFANT, M. (1955). Pathways of glucose metabolism in corneal epithelium. Science, N.Y. 122, 72-73.

KINSEY, V.E. (1951). The chemical composition and the osmotic pressure of the aqueous humor and plasma of the rabbit. J. gen. Physiol. 34, 389-402.

KINSEY, V.E. & REDDY, D.V.N. (1964). Chemistry and dynamics of aqueous humour. In: Prince, J.H. The rabbit in eye research Springfield, Illinois : Charles C. Thomas.

KLYCE, S.D. (1975). Transport of Na, Cl and water by the rabbit corneal epithelium at resting potential. Am. J. Physiol. 228, 1446-1452.

KLYCE, S.D. (1977). Enhancing fluid secretion by the corneal epithelium. Invest. Ophthalmol. Vis. Sci. 16, 968-973.

KRAKAU, C.E.T. (1976). Measurements of the episcleral venous pressure using a new method. In: J.S. Cant., Vision and Circulation. London : Henry Kimpton.

KRELL, R.D. & GOLDBERG, A.M. (1975). Effect of choline acetyltransferase inhibitors on mouse and guinea-pig brain choline and acetylcholine. Biochem. Pharmac. 24, 391-396.

KUHAR, M.J., SETHY, V.H., ROTH, R.H. & AGHAJANIAN, G.K. (1973). Choline: selective accumulation by central cholinergic neurons. J. Neurochem. 20, 581-593.

KUWABARA, T. (1970). Fine Structure of the Eye. Boston : Howe Laboratory of Ophthalmology Harvard Medical School.

KUWABARA, T., PERKINS, D.G. & COGAN, D.G. (1976). Sliding of the epithelium in experimental corneal wounds. Invest. Ophthalmol. 15, 4-14.

LANDGREN, S., LILJESTRAND, G. & ZOTTERMAN, Y. (1954). Chemical transmission in taste fibre endings. Acta physiol. scand. 30, 105-114.

LANGHAM, M. (1951). Respiration of the cornea. J. Physiol. 115, 65P.

LANGHAM, M. (1952). Utilization of oxygen by the component layers of the living cornea. J. Physiol. 117, 461-470.

LATIES, A.M. (1969). Localisation in cornea and lens of topically-applied irreversible cholinesterase inhibitors. Am. J. Ophthal. 68, 848-857.

LATIES, A. & JACOBOWITZ, D. (1964). A histochemical study of the adrenergic and cholinergic innervation of the anterior segment of the rabbit eye. Invest. Ophthalmol. 3, 592-600.

LEE, W.R. & GRIERSON, I. (1975). Pressure effects on the endothelium of the trabecular wall of Schlemm's canal : a study by scanning electron microscopy. Albrecht v. Graefes Arch. Ophthal. 196, 255-265.

LELE, P.P. & WEDDELL, G. (1959). Sensory nerves of the cornea and cutaneous sensibility. Expl Neurol. 1, 334-359.

LEMP, M.A. (1976). Cornea and Sclera. Archs Ophthal. N.Y. 94, 473-490.

LEUENBERGER, P.M. & GNÄDINGER, M.C. (1972). Sur le rôle de l'épithélium dans la cicatrisation cornéenne. Archs Ophthal. (Paris) 32, 33-42.

LIEB, W.A. (1960). Die Feinstruktur des Trabeculum der Kammerbucht Ber. Dtsch Ophthalmol. Ges. 62, 160-168.

LILJESTRAND, G. & ZOTTERMAN, Y. (1954). The effect of certain choline esters on the chemoreceptor activity in the carotid body. Acta physiol. scand. 31, 203-214.

LINNÉR, E. (1955). The outflow pressure in normal and glaucomatous eyes. Acta Ophthal. 33, 101-116.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. J. biol. Chem. 193, 265-275.

LÜTJEN-DRECOLL, E. & ROHEN, J.W. (1970). Über die endotheliale Auskleidung des Schlemmschen Kanals in Silberimprägnationsbild (The Silverlines of Schlemm's canal). Albrecht v. Graefes Arch. Ophthal. 180, 249-266.

LYONS, J.S. & KROHN, D.L. (1973). Pilocarpine uptake by pigmented uveal tissue. Am. J. Ophthal. 75, 885-888.

MACINTOSH, F.C. (1941). The distribution of acetylcholine in the peripheral and central nervous system. J. Physiol. 99, 436-442.

MACRI, F.J. (1967). The pressure dependence of aqueous humor formation. Archs Ophthal., N.Y. 78, 629-633.

MAJNO, G. & PALADE, G.E. (1961). Studies on inflammation I. The effect of histamine and serotonin on vascular permeability: An electron microscopic study. J. biophys. biochem. Cytol. 11, 571-605.

MARCHBANKS, R.M. (1968). The uptake of [^{14}C] choline into synaptosomes in vitro. Biochem. J. 110, 533-541.

MAREN, T.H. (1976). The rates of movement of Na^+, Cl^- and HCO_3^- from plasma to posterior chamber: Effect of acetazolamide and relation to the treatment of glaucoma. Invest. Ophthalmol. 15, 356-364.

MARGOLIS, R. & FEIGELSON, P. (1963). Purification and characterisation of a genetically determined rabbit serum esterase. J. biol. Chem. 238, 2620-2627.

MAUDGAL, P.C. & MISSOTTEN, L. (1978). Histology and histochemistry of the normal superficial corneal epithelium of rabbit. Albrecht von Graefes Arch. Ophthal. 205, 167-174.

MAUMENEE, A.E. & SCHOLZ, R.O. (1948). Studies on the physiology, biochemistry and cytopathology of the cornea in relation to injury by mustard gas and allied toxic agents. III. The histopathology of the ocular lesions produced by the sulfur and nitrogen mustards. Johns Hopkins Hosp. Bull. 82, 121-147.

MAURICE, D.M. (1957). The structure and transparency of the cornea. J. Physiol. 136, 263-286.

MAURICE, D.M. (1960). In: Duke-Elder, W.S. & Perkins, E.C. The transparency of the cornea. Oxford: Blackwell.

MAURICE, D.M. (1969). The cornea and sclera In: Davson, H. The Eye Vol. 1. London, New York : Academic Press.

MAURICE, D.M. (1972). The location of the fluid pump in the cornea. J. Physiol. 221, 43-54.

MAURICE, D.M. & RILEY, M.V. (1970). The Cornea. In: Biochemistry of the Eye Ed. Graymore, C.N. London, New York : Academic Press.

McCAMAN, R.E. & APRISON, M.H. (1964). The synthetic and catabolic enzyme systems for acetylcholine and serotonin in several discrete areas of developing rabbit brain. In: Progress in Brain Research Vol. 9., The Developing Brain ed. Himwich, H.E. New York; Amsterdam; London : Elsevier Publ. Co.

MINDEL, J.S. & MITTAG, T.W. (1976). Choline acetyltransferase in ocular tissues of rabbits, cats, cattle and man. Invest. Ophthalmol. 15, 808-814.

MINDEL, J.S. & MITTAG, T.W. (1977). Variability of choline acetyltransferase in ocular tissues of rabbits, cats, cattle and humans. Exp. Eye Res. 24, 25-33.

MINDEL, J.S. & MITTAG, T.W. (1978). Suppression of corneal epithelial choline acetyltransferase activity by lid closure. Exp. Eye Res. 27, 359-364.

MINDEL, J.S., MITTAG, T.W. & GREEN, J.P. (1973). Acetylcholine in rabbit, feline, bovine and human cornea. Abstract A.R.V.O.

MINDEL, J.S., SZILAGYI, P.I.A., ZADUNAISKY, J.A., MITTAG, T.W. & ORELLANA, J. (1979). The effects of blepharorrhaphy induced depression of corneal cholinergic activity. Exp. Eye Res. 29, 463-468.

MISHIMA, S. & KUDO, T. (1967). In vitro incubation of rabbit cornea. Invest. Ophthalmol. 6, 329-339.

MITTAG, T.W., HARRIS, L.S., COHN, K., GALIN, M.A. & EHRENPREIS, S. (1970). Analysis of cholinesterases of intact cat cornea, ciliary body, lens and retina. Invest. Ophthalmol. 9, 742-748.

MOSES, R.A., PARKISON, G. & SCHUCHARDT, R. (1979). A standard large wound of the corneal epithelium in the rabbit. Invest. Ophthalmol. Vis. Sci. 18, 103-106.

NEUFELD, A.H. (1978). Influences of cyclic nucleotides on outflow facility in the vervet monkey. Exp. Eye Res. 27, 387-397.

van OER, S. (1961). Über die Beziehung des Acetylcholins der Hornhautepithels zur Erregungsübertragung von diesem auf die sensiblen Nervenden. Pflüger. Arch. ges. Physiol. 273, 325-334.

OLSEN, J.S. & NEUFELD, A.H. (1979). The rabbit cornea lacks cholinergic receptors. Invest. Ophthalmol. Vis. Sci. 18, 1216-1225.

ORZALESI, N., BONOMI, L., DEL FIACCO, G., FOSSARELLO, M. & CARTA, S. (1980). Ultrastructural study of the trabecular meshwork of the rabbit in normal conditions and after corticosteroid-induced ocular hypertension. European Glaucoma Society, Abstracts.

OTORI, T. (1967). Electrolyte content of the rabbit corneal stroma. Exp. Eye Res. 6, 356-367.

PATERSON, S.J. & WILSON, W.S. (1977). Uptake of choline by rabbit corneal epithelium. Br. J. Pharmac. 59, 499P.

PATIL, P.N. (1974). Iris pigmentation and atropine mydriasis. Pharmacologist 16, 311-315.

PAVLIČ, M. (1967). The inhibitory effect of Tris on the activity of cholinesterases. Biochim. biophys. Acta, 139, 133-137.

PEDERSON, J.E. & GREEN, K. (1973). Aqueous humour dynamics : experimental studies. Exp. Eye Res. 15, 277-297.

PEDLER, C. (1962). The fine structure of the corneal epithelium. Exp. Eye Res. 1, 286-289.

PEI, Y.F. & RHODIN, J.A.G. (1971). Electron microscopic study of the development of the mouse corneal epithelium. Invest. Ophthalmol. 10, 811-825.

PETERSON, R.A., LEE, K.-J. & DONN, A. (1965). Acetylcholinesterase in the rabbit cornea. Archs Ophthal., N.Y. 73, 370-377.

PETERSON, W.S., JOCSON, V.L. & SEARS, M.L. (1971). Resistance to aqueous outflow in the rhesus monkey eye. Am. J. Ophthal. 72, 445-451.

PFISTER, R.R. & BURSTEIN, N. (1976). The effects of ophthalmic drugs, vehicles and preservatives on corneal epithelium : a scanning electron microscope study. Invest. Ophthalmol. 15, 246-259.

PODOS, S.M., MINAS, T.F. & MACRI, F.J. (1968). A new instrument to measure episcleral venous pressure. Comparison of normal eyes and eyes with primary open-angle glaucoma. Archs Ophthal., N.Y. 80, 209-213.

POTTER, L.T. (1968). Uptake of choline by nerve endings isolated from the rat cerebral cortex. In: The interaction of Drugs and Subcellular Components on Animal Cells. ed. Campbell, P.N. London: Churchill.

PRINCE, J.H. (1964). The rabbit in eye research. Springfield, Illinois: Charles C. Thomas.

RAVIOLA, G. (1974). Effects of paracentesis on the blood-aqueous barrier : an electron microscope study on Macaca mulatta using horse radish peroxidase as a tracer. Invest. Ophthalmol. 13, 828-858.

REDDY, D.V.N., ROSENBERG, C. & KINSEY, V.E. (1961). Steady state distribution of free amino acids in the aqueous humours, vitreous body and plasma of the rabbit. Exp. Eye Res. 1, 175-181.

REIM, M. & CATTEPOEL, H. (1970). Biochemischer Vergleich von frischer und lyophilisierter Bindehaut. Ber Zusammenkumft Dtsch Ophthalmol. Ges. 70, 381-387.

RILEY, M.V. (1971). The role of the epithelium in control of corneal hydration. Exp. Eye Res. 12, 128-137.

RILEY, M.V. (1977). Anion-sensitive ATPase in rabbit corneal endothelium and its relation to corneal hydration. Exp. Eye Res. 25, 483-494.

ROBERTSON, D.M. & WINKELMANN, R.K. (1970). A whole-mount cholinesterase technique for demonstrating corneal nerves : observations in the albino rabbit. Invest. Ophthalmol. 9, 710-715.

ROETTH, A. de (1950). Respiration of the cornea. Archs Ophthal., N.Y. 44, 666-676.

ROHEN, J.W. (1969). New studies on the functional morphology of trabecular meshwork and the outflow channels. Trans. Ophthal. Soc. U.K. 89, 431-447.

ROHEN, J.W., LÜTJEN, E. & BÁRÁNY, E. (1967). The relation between the ciliary muscle and the trabecular meshwork and its importance for the effect of miotics on aqueous outflow resistance. A study in two contrasting monkey species, Macaca irus and Cercopithecus aethiops. Albrecht v. Graefes Arch. Ophthal. 172, 23-47.

ROHEN, J.W. & van der Zypen, E. (1968). The phagocytic activity of the trabecular meshwork endothelium. Albrecht v. Graefes Arch. Ophthal. 175, 143-160.

ROSSIER, J. (1977). Choline acetyltransferase : a review with special reference to its cellular and subcellular localization. Int. Rev. Neurobiol. 20, 283-337.

ROWELL, P.P. & SASTRY, B.V.R. (1977). Human placental cholinergic system : effects of cholinergic blockade on amino acid uptake in isolated placental villi. Fedn Proc. 36, 981.

RUSKELL, G.L. (1961). Aqueous drainage paths in the rabbit. A neoprene latex cast study. Archs Ophthal., N.Y. 66, 861-870.

SAKIMOTO, G. (1979). Smooth muscle cells associated with the aqueous drainage channel in the rabbit eye. Jap. J. Ophthal. 23, 162-173.

SALAZAR, M. & PATIL, P.N. (1976) An explanation for the long duration of mydriatic effect of atropine in the eye. Invest. Ophthalmol. 15, 671-673.

SAVAGE, C.R. & COHEN, S. (1973). Proliferation of corneal epithelium induced by epidermal growth factor. Exp. Eye Res. 15, 361-366.

SAVAGE, C.R., INAGMI, T. & COHEN, S. (1972). The primary structure of epidermal growth factor. J. biol. Chem. 247, 7612-7621.

SCHLEMM, F. (1830). Ueber einen Kreisformigen dünnhäutigen Kanal in der verbindungsstelle von sclerotica und Cornea in menschlichen Auge. Z. Ophthal. 1, 543-546.

SCHRIER, B.K. & SHUSTER, L. (1967). A simplified radiochemical assay for choline acetyltransferase. J. Neurochem. 14, 977-985.

SCHRØDER, H.D. & SPERLING, S. (1977). Polysaccharide coating of human corneal endothelium. Acta Ophthal. 55, 819-826.

SCHWALBE, G. (1870). Untersuchungen über die Lymphbahnen des Auges und ihre Bergrenzungen. Arch. mikrosk. Anat. Entw. Mech. 6, 261-362.

SCHWEITZER, S. & WRIGHT, S. (1938). Action of prostigmine and acetylcholine on respiration. Q. J. exp. Physiol. 28, 33-47.

SEARS, M.L. (1960). Outflow resistance of the rabbit eye : Technique and effects of acetazolamide. Determination by constant rate anterior chamber infusion in vivo. Archs Ophthal. N.Y. 64, 823-838.

SEGAWA, K. (1968). Porosity of Schlemm's canal and Sondermann's canals. Med. J. Shinshu Univ. 13, 57-65.

SEMENOVA, N.F. (1976). Diurnal fluctuations in responses of mouse corneal epithelial cells to vinblastin. Bull. biol. Méd. Exp. U.R.S.S. 80, 1368-1370.

SHABO, A.L. & MAXWELL, D.S. (1972). Observations on the fate of blood in the anterior chamber. Am. J. Ophthal. 73, 25-36.

SHABO, A.L., REESE, T.S. & GAASTERLAND, D. (1973). Post mortem formation of giant endothelial vacuoles in Schlemm's canal of the monkey. Am. J. Ophthal. 76, 896-905.

SHAFFER, R.N. (1961). In : Newell, F.W. Glaucoma Transactions of the Fifth Conference pp 234-237. New York : Josiah Macy Jr. Foundation.

SHELDON, G.M. (1971). Misuse of corneal anesthetics. Can. med. Ass. J. 104, 528.

SIMON, J.R. & KUHAR, M.J. (1976). High affinity choline uptake: ionic and energy requirements. J. Neurochem. 27, 93-99.

SORIMACHI, M. & KATAOKA, K. (1975). High affinity choline uptake: an early index of cholinergic innervation in rat brain. Brain Res. 94, 325-336.

SORSBY, A., STONE, J., LEARY, G.A. & SHERIDAN, M. (1960). Changes in the depth of the anterior chamber and in the radius of curvature of the front surface of the lens during growth. Observations on the rabbit. Br. J. Ophthal. 44, 467-471.

SPEAKMAN, J.S. (1959a). Aqueous outflow channels in the trabecular meshwork in man. Br. J. Ophthal. 43, 129-138.

SPEAKMAN, J.S. (1959b). Endothelial cell vacuolisation in the cornea. Br. J. Ophthal. 43, 139-146.

SPEAKMAN, J.S. (1960). Drainage channels in the trabecular wall of Schlemm's canal. Br. J. Ophthal. 44, 513-523.

SPERLING, S. & JACOBSEN, S.R. (1980). The surface coat on human corneal endothelium. Acta Ophthal. 58, 96-102.

SRINIVASAN, B.D., WORGUL, B.V., IWAMOTO, T. & EAKINS, K.E. (1977). The reepithelialization of rabbit cornea following partial and complete epithelial denudation. Exp. Eye Res. 25, 343-351.

STEVENSON, R.W. (1974). The role of acetylcholine in the mammalian corneal epithelium. Ph.D. Thesis. University of Glasgow.

STEVENSON, R.W. & WILSON, W.S. (1974). Drug-induced depletion of acetylcholine in the rabbit corneal epithelium. Biochem. Pharmac. 23, 3449-3457.

STEVENSON, R.W. & WILSON, W.S. (1975). The effect of acetylcholine and eserine on the movement of Na^+ across the corneal epithelium. Exp. Eye Res. 21, 235-244.

STORMONT, C. & SUZUKI, Y. (1970). Atropinesterase and cocainesterase of rabbit serum : localisation of the enzyme activity in isozymes. Science, N.Y. 167, 200-202.

SVEDBERGH, B. (1974). Effects of artificial intraocular pressure elevation on the outflow facility of the ultrastructure of the chamber angle in the vervet monkey. (Cercopithecus ethiops). Acta Ophthal. 52, 829-846.

SVEDBERGH, B. (1976). Protrusions of the inner wall of Schlemm's canal. Am. J. Ophthal. 82, 875-882.

SVEDBERGH, B. & BILL, A. (1976). In: Aspects of the aqueous humour drainage. Svedbergh, B. (1976) Thesis. Acta University of Uppsala.

TAYLOR, J.M., COHEN, S. & MITCHELL, W.M. (1970). Epidermal growth factor : High and low molecular weight forms. Proc. natn. Acad. Sci. U.S.A. 67, 164-171.

TENG, C.C. (1961). The fine structure of the corneal epithelium and basement membrane of the rabbit. Am. J. Ophthal. 51, 278-297.

TERVO, T. & PALKAMA, A. (1978). Innervation of the rabbit cornea. A histochemical and electron-microscopic study. Acta anat. 102, 164-175.

THOFT, R.A. & FRIEND, J. (1975a). Permeability of regenerated corneal epithelium. Exp. Eye Res. 21, 409-416.

THOFT, R.A. & FRIEND, J. (1975b). Biochemical aspects of contact lens wear. Am. J. Ophthal. 80, 139-145.

THOFT, R.A. & FRIEND, J. (1977). Biochemical transformation of regenerating ocular surface epithelium. Invest. Ophthalmol. Vis. Sci. 16, 14-20.

TRIPATHI, R.C. (1971a). Mechanism of the aqueous outflow across the trabecular wall of Schlemm's canal. Exp. Eye Res. 11, 116-121.

TRIPATHI, R. (1971b). Ultrastructure of the exit pathway of the aqueous in lower mammals. (A preliminary report on the 'angular aqueous plexus'). Exp. Eye Res. 12, 311-314.

TRIPATHI, R.C. (1972). Aqueous outflow pathway in normal and glaucomatous eyes. Br. J. Ophthal. 56, 157-174.

TRIPATHI, R.C. (1973). Pressure dependency of the aqueous outflow (letter to the editor). Am. J. Ophthal. 76, 402-403.

TRIPATHI, R.C. (1974). Comparative physiology and anatomy of the outflow pathway. In: The Eye Vol. 5. ed. Davson, H. & Graham, L.T. New York, London : Academic Press.

TRIPATHI, R.C. (1977a). The functional morphology of the outflow systems of ocular and cerebrospinal fluids. Exp. Eye Res. 25,(Suppl.), 65-116.

TRIPATHI, R.C. (1977b). Uveoscleral drainage of aqueous humour. Exp. Eye Res. 25(Suppl.), 305-308.

TRIPATHI, R.C. & COLE, D.F. (1976). Uveoscleral drainage in the rabbit. Presented at the Annual Meeting of the Association for Research in Vision and Ophthalmology, Sarasota, April 1976 and quoted in Tripathi (1977b).

TURSS, R., FRIEND, J. & DOHLMAN, C.H. (1970). Effect of a corneal fluid barrier on the nutrition of the epithelium. Exp. Eye Res. 9, 254-259.

UUSITALO, R. (1972). The action of physostigmine, morphine, cyclopentolate and homatropine on the secretion and outflow of aqueous humour in the rabbit eye. Acta physiol. scand. 86, 239-249.

VAREILLES, P., CONQUET, P. & LE DOUAREC, J.-C. (1977). A method for the routine intraocular pressure (IOP) measurement in the rabbit : range of IOP variations in this species. Exp. Eye Res. 24, 369-375.

VRÁBEC, F. (1954). L'innervation du système trabéculaire de l'angle irien. Ophthalmologica, Basel, 128, 359-364.

WÅLINDER, P.-E. & BILL, A. (1969). Influence of intraocular pressure and some drugs on aqueous flow and entry of cycloleucine into the aqueous humor of Vervetmonkeys (Cercopithecus ethiops). Invest. Ophthalmol. 8, 446-458.

WEGEFARTH, P. (1914). Studies on cerebro-spinal fluid. V. The drainage of intraocular fluids. J. med. Res. 31, 119-147.

WEIMAR, V.L. & HARAGUCHI, K.H. (1966). The development of enzyme activities in corneal connective tissue cells during the lag phase of wound repair. II. Formalin-resistant oxidase-like reaction. Invest. Ophthalmol. 5, 14-21.

WILLIAMS, J.D. & COOPER, J.R. (1965). Acetylcholine in bovine corneal epithelium. Biochem. Pharmac. 14, 1286-1289.

WILSON, W.S. & COOPER, J.R. (1972). The preparation of cholinergic synaptosomes from bovine superior cervical ganglia. J. Neurochem. 19, 2779-2790.

WILSON, W.S., DUNCAN, A.J. & JAY, J.L. (1975). Effect of benzalkonium chloride on the stability of the precorneal tear film in rabbit and man. Br. J. Ophthal. 49, 667-669.

WOLF, J. (1968a). Inner surface of regions in the anterior chamber taking part in the regulation of the intraocular tension, including demonstration of the covering viscous substance. Documenta Ophth. 25, 113-149.

WOLF, J. (1968b). The secretory activity and the cuticle of corneal endothelium. Documenta Ophth. 25, 150-194.

WOLFF, E. (1952). Pacchionian-like bodies in the human canal of Schlemm. Br. J. Ophthal. 36, 100-103.

WULLE, K.G. & RICHTER, J. (1978). Electron microscopy of the early embryonic development of the human corneal epithelium. Albrecht v. Graefes Arch. Ophthal. 209, 39-49.

YAMAMURA, H.I. & SNYDER, S.H. (1973). High affinity transport of choline into synaptosomes of rat brain. J. Neurochem. 21, 1355-1374.

ZANDER, E. & WEDDELL, G. (1951). Observations on the innervation of the cornea. J. Anat. 85, 68-99.

ZIMMERMAN, T.J., GARG, L.C., VOGH, B.P. & MAREN, T.H. (1976). The effect of acetazolamide on the movements of anions into the posterior chamber of the dog eye. J. Pharmac. exp. Therap. 196, 510-516.

ZOTTERMAN, Y. (1953). Special Senses : thermal receptors. A. Rev. Physiol. 15, 357-372.

ZWEIFACH, B.W. (1961). Functional behavior of the microcirculation p.96. Springfield, Illinois: Charles C. Thomas.

APPENDIX

Appendix 1

Chemicals and drugs used.

Chemical	Manufacturer/Supplier
Acetic acid (glacial)	Hopkin & Williams Ltd.
Acetone	Hopkin & Williams Ltd.
Acetylcholine chloride	Sigma
Acetylcholine perchlorate	Sigma
Acetylcholinesterase (electric eel type)	Sigma
Acetythiocholine chloride	Sigma
L-adrenaline acid tartrate	Sigma
AG 1 X 8	Bio Rad Laboratories
Albumin (Bovine Serum)	Sigma
Amethocaine (Minims)	Smith & Nephew
L-ascorbic acid	Sigma
Atropine sulphate	British Drug Houses
Benoxinate (Minims)	Smith & Nephew
BW 284 C51	Burroughs Wellcome Ltd.
Calcium chloride	Hopkin & Williams Ltd.
Calcium ionophore A 23187	Lilly
Choline chloride	Sigma
Compound 48/80	Sigma
Cupric sulphate	Hopkin & Williams Ltd.
Diazepam (Valium injection)	Roche
Disodium hydrogen phosphate	Hopkin & Williams Ltd.
Dithiobisnitrobenzoate	Sigma

Ecothiopate iodide (Phospholine)	Ayerst
Epithelial growth factor (mouse)	Imperial Chemicals Industry Ltd. (estimated purity 50%)
Ethylene diaminetetra acetic acid	Sigma
Ethylmethylketone	British Drug Houses Ltd.
Folin Ciocalteu reagent	British Drug Houses Ltd.
Formic acid	Hopkin & Williams Ltd.
D-glucose	Hopkin & Williams Ltd.
Halothane	May & Baker Ltd.
Heparin Sodium (Mucous)(Pularin)	Duncan Flockhart Ltd.
Heptan-3-one	Kodak Eastman
Hexamethonium bromide	May & Baker Ltd.
Hydrochloric acid	Hopkin & Williams Ltd.
Hyoscine hydrobromide	British Drug Houses Ltd.
Lignocaine hydrochloride (Xylocaine)	Astra Chemicals Ltd.
Magnesium chloride	Hopkin & Williams Ltd.
Mecamylamine hydrochloride	Merck, Sharpe & Dohme Ltd.
Neostigmine methylsulphate	Sigma
Nicotine hydrogen tartrate	British Drug Houses Ltd.
Nitrogen, liquid	British Oxygen Company.
Nitrous oxide	British Oxygen Company
Oxotremorine	Aldrich Chemicals Ltd, Milwaukee.
Oxygen	British Oxygen Company.
Pancuronium bromide	Organon
Phenoxybenzamine hydrochloride	Sigma
Potassium chloride	Hopkin & Williams Ltd.

Potassium phosphate	Hopkin & Williams Ltd.
Potassium sodium tartrate	Hopkin & Williams Ltd.
Scintol 2 (see appendix p.163)	Koch Light Laboratories Ltd.
Sodium bicarbonate	Hopkin & Williams Ltd.
Sodium carbonate	Hopkin & Williams Ltd.
Sodium chloride	Hopkin & Williams Ltd.
Sodium chloride (Minims)	Smith & Nephew Ltd.
Sodium ethacrynate	Merck, Sharpe & Dohme Ltd.
Sodium hydroxide	Hopkin & Williams Ltd.
Sodium tetraphenylboron	Sigma
Sucrose	Hopkin & Williams Ltd.
Tetrodotoxin base	Sigma
Toluene	Koch Light Laboratories Ltd.
Tris (hydroxymethyl) aminomethane	(Tris) Sigma
Triton X 100	Koch Light Laboratories Ltd.
(+) - tubocurarine	Burroughs Wellcome Ltd.
Verapamil hydrochloride (Cordilox)	Abbott Laboratories Ltd.

All radioactively labelled compounds were obtained from the Radiochemical Centre, Amersham.

All chemicals were of Analar quality, except when stated otherwise.

Appendix 2

Reagent mixtures.

1. Toluene-Triton X scintillant

Triton X 100		1665 ml
Scintol 2		250 ml
Toluene	ad	5000 ml

2. Scintol 2 : a concentrated solution in toluene of PPO and Dimethyl POPOP where PPO is 2,5 - Diphenyloxazole and Dimethyl POPOP is 1,4 - Di - 2 - (4 - methyl - 5 - phenyloxazolyl) - benzene.

3. Iodoplatinate solution

Chloroplatinate		2 g
Distilled water	ad	20 ml

added to

Potassium iodide		120 g
Distilled water	ad	400 ml
diluted	ad	500 ml with distilled water

4. Draggendorf's reagent (modified)

Bismuth carbonate		500 mg	} the concentrate
Hydrochloric acid (conc)		1.5 ml	
Potassium iodide		3.0 g	
Distilled water	ad	10.0ml	

The concentrate	1 part	the spray
Distilled water	4 part	
Hydrochloric acid (conc)	0.5 ml	

5. Solution C for protein determination

Solution C — 98 parts of solution A
2 parts of solution B

These are mixed on the day of use.

Solution A	Sodium carbonate	2 g
	Sodium hydroxide (0.1N)	ad 100ml
Solution B	Cupric sulphate	0.5 g
	Sodium potassium tartrate	1.0 g
	Distilled water	ad 100ml

6. Dithiobisnitrobenzene (DTNB) solution

DTNB	79.2 mg	
Potassium phosphate (0.1M, pH 7.0)	20.0 ml	dissolved
Sodium bicarbonate	30.0 mg	

7. Acetylthiocholine solution

Acetylthiocholine chloride 0.5mM in potassium phosphate buffer (0.1M, pH 8.0).

GLA... UN...