A STUDY OF SOME INDICATORS OF CORNEAL METABOLIC ACTIVITY IN PERIODS OF CONTACT LENS WEAR

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SUMMARY

A STUDY OF SOME INDICATORS OF CORNEAL METABOLIC ACTIVITY IN PERIODS OF CONTACT LENS WEAR

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The study describes a series of experiments that improve existing techniques and develop new techniques for the determination and monitoring of contact lens wear effects on the cornea. Three main aspects are studied:

(1) Oxygen requirement

(2) Carbon dioxide production

(3) Lactic acid production and dynamics.

Experimental topics include: oxygen consumption and carbon dioxide efflux rates from the corneal epithelial surface, lactic acid production and dynamics across the limiting layers in normal and anoxic cornea, lactic acid dynamics in a soft lens polymer and the use of soft lenses as a sample collection medium. In all the experiments attention is paid to produce techniques suitable for use in the clinical environment.

The experimental data indicates that oxygen consumption rate is a reliable indicator of corneal metabolic activity and that there is no significant diurnal variation. However, measurement of carbon dioxide efflux rate is unsuitable for use as a diagnostic test due to the insensitivity and bulk of the electrode. Lactic acid concentrations in all tissues of the anterior segment of the eye, with the exception of the stroma plus endothelium, increase after eight hours of epithelial anoxia, thus measurement of corneal lactic acid concentration can be used to monitor corneal metabolic activity. However, the only sample suitable for in vivo use is the tears. In tears collected by capillary tube, measurement of lactic acid concentration using thin layer chromatography is insufficiently accurate and enzymatic assay provides only qualitative information. Hydrated Sauflon 85 is freely permeable to lactic acid and is suitable for use as a collecting medium for the lactic acid component of tears. Lactic acid collected from tears using Sauflon 85 soft contact lenses shows an increase in tear fluid lactic acid after eight hours epithelial anoxia.

Contact lenses, corneal metabolism, lactic acid, oxygen, carbon dioxide.

CONTENTS

SECTION 1

1.1 GENERAL INTRODUCTION

DUKE-ELDER (1970) has described the origins of contact lenses. Although optical theory can be traced back as far as Leonardo da Vinci, its relevance to the use of contact lenses as visual aids is remote. DUKE-ELDER (1970) further states that more direct relevance can be seen in the work of Descartes in the 17th century and Hershall in the 19th century, where early optical theory is linked to the needs of the emerging clinician.

The publication of a doctoral thesis by MÜLLER to the University of Kiel in 1889 contains the first known use of the expression "corneal lens". The work itself is concerned with the use of corneal devices in the correction of high myopia. DUKE-ELDER (1970) suggests that another advance in this period came with the recommendation of contact lenses for use in the treatment of keratoconus, aphakia and a prosthetic/ cosmetic lenses by Fick.

Despite the impact of the work of MÜLLER and FICK, contact lenses found much less widespread use than anticipated. One reason for this lack of enthusiasm can be ascribed to the problems found in the wearing of contact lenses (SABELL, 1978). FICK and MÜLLER both described disappointing tolerance to contact lenses. MÜLLER believed pressure of the haptic portion of the lens on the conjunctiva to be the cause. FICK when analysing his intolerance described the phenomena of a faint blue haze around bright lights; symptoms that have since become known as Sattlers Veil (DALLOS, 1946). FICK also noticed that regular use of contact lenses allowed the wearer to become more tolerant to the

lenses and secondly that air trapped behind the contact lens on insertion retarded the onset of visual clouding. FICK further ascribed the epithelium as the site of the observed clouding.

Thus at the end of the 19th century the foundations of contact lens use in the correction of visual errors and neutralisation of corneal irregularities had been laid. The successes and limitations of their use had also been realised. Further developments in the theory and use of contact lenses did not occur until the 1930's with the realisation of lack of understanding of corneal topography and anterior segment physiology. Attempts to improve patient comfort by the use of fitting sets with ranges of curvature and diameter, and the use of eye impressions to create accurate casts of the eye surface from which moulds could be produced rapidly followed (OBRIG, 1938).

The use of full clearance optic lenses were successful in allowing longer wearing times. Unfortunately corneal clouding and Sattlers Veil still limited the use of these lenses. At this point the majority of contact lenses were manufactured from glass and were consequently heavy. The development of polymethyl methacrylate (PMMA) in 1938 by Obrig and the C.W. Dixon Company began the next stage of advancement.

Although the optical properties of glass are superior to PMMA the relative gain in ruggedness and the reduction in weight to one third that of glass allowed a reduction in size of contact lenses to form the "corneal lens". These lenses when fitted have a maximum diameter less than the diameter of the corneal limbus and an inside curvature flatter than the longest curvature of the cornea. These lenses have

certain advantages over scleral lenses in that they neither encroach on to the sclera, nor is the sclera subject to pressure from the lens. The flat lens-cornea relationship allows for exchange of air and tears beneath the lens relieving pressure of the lens on the cornea.

Unfortunately even with small PMMA corneal contact lenses Sattlers Veil still develops although the time taken varies considerably. On removal of the contact lens the phenomena slowly disappears. These findings stimulated a number of studies to determine the effects of contact lenses on corneal function.

One possible factor which might affect corneal function is the contact between cornea and lens. Using a lens with the major part of the corneal segment removed showed that the presence of the lens between the cornea and atmosphere is responsible for the development of Sattlers Veil, since no opacities occur when the modified lenses are worn (SMELSER, 1952; DALLOS, 1946). Other experiments aimed at determining the cause and effect of Sattlers Veil concluded that contact lenses interfere with the optical properties of the cornea by increasing corneal hydration (SMELSER, 1952; KINSEY, 1952).

The increase in corneal hydration has a number of causes, interference with oxygen supply from the atmosphere to the corneal epithelium is an important factor (SMELSER, 1952; SMELSER and OZANICS, 1952; LANGHAM, 1952). Other less severe causes are alterations of the pH and osmotic balance at the epithelial surface caused by contact lens solutions and excessive tearing (DALLOS, 1946; KINSEY, 1952; POTTS, 1962).

Following identification of the origins of corneal distress (caused by contact lens wear) modifications to existing contact lenses took place. These changes allowed gaseous exchange between the air and the layer of tears contained between the back surface of the contact lens and the epithelial surface. These modifications have brought the wearing of hard contact lenses to the position they currently hold, where the clinician aims to equip the patient with contact lenses capable of being worn comfortably all day.

In parallel with the search for a comfortable hard lens has been a search for alternative materials suitable for use as contact lenses. One alternative material is hydrophilic glycol methacrylate gel (WICHTERLE and LIM, 1960). These gels are perfectly transparent, soft, elastic, chemically stable and biocompatible. In their early form these hydrophilic lenses contained 40% water. In the clinical situation, in comparison to hard lenses, these lenses though far from ideal perform well with regard to corneal physiology (PRAUS et al., 1969; HAMANO et al., 1972). Further advances in plastics technology have allowed the manufacture of hydrophilic lenses with increasing water contents that are less detrimental to the cornea.

The materials used in the manufacture of contact lenses have advanced in terms of patient acceptability, allowing longer wearing times and even continuous wear. Similarly the understanding of corneal function has advanced allowing a greater understanding of contact lens effects on the cornea. With the increase in understanding of the effects of contact lenses on normal corneal function has been an increase in the expectations of both patient and clinician. Both demand new and

increasingly sophisticated materials that will reduce the detrimental effects of contact lenses on the eye. Unfortunately at this time even the most advanced materials are seen to affect the normal functioning of the cornea. It must be accepted that contact lenses have some effect on normal corneal function, however by carefully monitoring of corneal function and the symptoms of changes induced by contact lenses, serious effects of contact lenses can be avoided.

Unfortunately the majority of methods used in the study of normal corneal functioning and the effects of contact lenses are in vitro techniques, or techniques that are in themselves detrimental to the eye. Those non-invasive techniques used in the study of normal corneal function lack the sensitivity of in vitro techniques.

The aims of this project are to develop non-invasive techniques and study normal corneal function using these techniques. Having gained a knowledge of normal corneal function to modify and develop the techniques further, until they are of sufficient accuracy to detect the subtle changes in corneal function caused by modern contact lens wear. To observe contact lens effects on corneal function using the monitoring techniques.

By satisfying the above aims it is hoped to produce a number of monitoring techniques suitable for use by the clinician in a clinical environment. Thus allowing early detection of any possibly detrimental effects.

1.2 LITERATURE SURVEY

Introduction

The cornea and sclera together form the tough outer tunic of the eye , they withstand the intraocular pressure from within and protect the contents from mechanical injury from without (SPOONER, 1957). The cornea is also the major component in the optical system of the eye. Of the total power of the eye approximately $\frac{3}{4}$ is contributed by the interface between the cornea and the air. The cornea is curved and transparent with surfaces of good optical standard, when covered by tear fluid.

The maintenance of normal corneal structure requires the constant action of the fluid transport system that is dependent on metabolic energy. A detailed knowledge of the anatomy, nutrition and energy producing mechanisms of the cornea is required before the location and function of the fluid pump can be determined. Having described the normal functioning of the cornea, the effects of external influences on normal corneal function can be determined and discussed. The effects of more specific external interferences such as contact lenses can then be related more accurately to tissue functions involving nutrition, metabolism and the control of hydration.

Once the effects of contact lenses on normal corneal function are known, efforts can be made to reduce the interference or to monitor the effects more closely to allow minimisation of the effect.

1.3 CORNEAL ANATOMY

(a) General Features

In the human eye the cornea is limited at the anterior surface by the Pre-corneal tear film and at the posterior surface by the Aqueous Humour. Peripherally the cornea blends with the conjunctiva, episclera and sclera in a transitional zone, the limbus. The cornea viewed at the anterior surface is an elliptical meniscus, the horizontal diameter (11.7 millimetres (mm.)) being slightly greater than the vertical diameter (10.6 mm.) (HOGAN et al., 1971). The central cornea has a thickness of 0.52 mm. (MAURICE and GIARDINI, 1951 (a)) thickening towards the periphery to 0.67 mm. at the limbus. In the rabbit the cornea has a horizontal diameter of 15.0 mm. and a vertical diameter of 13.8 mm. The central thickness is 0.37 mm. thickening to 0.45 mm. at the limbus (PRINCE et al., 1960).

The cornea has a unique isolated anatomical position and is devoid of blood vessels except at the periphery. Thus the reactions which take place in this isolated domain must therefore occur at temperatures appreciably below that of the blood (MAPSTONE, 1968).

The cornea seen under light microscopy has a number of substructures:-

Anterior	- Epithelium	50 Microns (μ)
	- Bowman's membrane	10 μ
	- Stroma	480 μ
	- Descemet's membrane	5 μ
Posterior	- Endothelium	5 μ

The dimensions are for normal adult human cornea (HOGAN et al., 1971).

(b) Epithelium

This is the outermost layer of the cornea and in terms of contact lens wear the most important since this is the only part of the cornea that comes into direct contact with the lens.

Light microscopy reveals that in man it is composed of 5 to 6 superimposed layers of cells. These cells can be further sub-divided on morphological grounds into:-

(i) a single row of basal columnar cells.

(ii) an intermediary layer of regular wing or polygonal cells, 2 - 3 cells thick.

(iii) and 2 layers of flattened plate-like surface cells. (HOGAN et al., 1971). There are however certain species differences. In the rabbit for example up to 6 cell layers in the surface layer are found (KAYE and PAPPAS, 1962).

The basal cells sit on a fine basement membrane attached to it by numerous desmosomes (PEDLER, 1962; JAKUS, 1961, 1964). These cells are tall, polygonal and contain a roughly spherical nucleus and a paucity of cell organelles. Adjacent cells are held together by desmosomes though of a different anatomical appearance to those at the basement membrane (PEDLER, 1962). The cell membranes are interdigitated and separated by a gap of 100 - 200 Ångstroms (Å), (WHITEAR, 1960).

The wing cells form 2 - 3 layers of polygonal shaped cells that are closely interlocked. Their lateral extensions are thin and wing-like, joining adjacent and basal cells. They arise from the division and forward migration of basal cells into the wing cell layer (HANNA and O'BRIEN, 1960). As the cells migrate to the surface the nuclei flatten, the cytoplasm becomes more dense, intra-cellular vesicles appear and the number of desmosomes increases.

The superficial cells are flat, overlapping, squamous plates with elongated nuclei that run parallel to the surface. They contain few organelles but many intracellular vesicles. These cells are formed from a further flattening and migration of the wing cells. Cells are constantly being sloughed off the surface, to be replaced by migrating wing cells. Surface cells possess many desmosomes holding adjacent cells together. These cells also possess a zona occludens that runs along the length of the two adjacent cells (OKINAMI et al., 1976). The anterior surface of these cells is covered in microvillae and microplicae (PFISTER, 1973; PFISTER and BURSTEIN, 1977; BLÜMCKE and MORGENROTH, 1967; KAYE and PAPPAS, 1962), that are thought to be involved in tear fluid retention although this opinion is questioned (MISHIMA, 1965; EHLERS, 1965).

Generations of new epithelial cells are produced mainly in the basal layer and to a lesser extent in the second cell layer (MACHEMER, 1966). Autoradiographic studies indicate that the life span of epithelial cells is between 3.5 and 7 days (FRIEDENWALD and BUSCHKE, 1944; HANNA and O'BRIEN, 1960). In the rat 14.5% of epithelial cells are renewed daily (BERTALANFFY and LAU, 1962) given a total turnover or regeneration

time of approximately 7 days. A similar regeneration time occurs in man.

(c) Stroma and Bowman's Membrane

Bowman's membrane is a cell free homogenous layer that lies immediately below the basement membrane of the epithelium. It consists of fine collagen fibres 260 Å in diameter in man. In other mammals the zone is thin and represents a condensation of the stromal surface (KAYES and HOLMBERG, 1960). The area surrounding the fibrils is filled with a mucoprotein ground substance. Though classified separately Bowman's membrane is really an extension of the stroma, its fibres being continuous with those of the stroma. The matrix of Bowman's membrane and the stroma possess similar biochemical components (WISLOCKI, 1952).

The stroma constitutes approximately 88% of the total corneal thickness in man. It is composed of collagenous lamellae within which lie fibroblast cells and ground substance. Fibroblasts occupy between 2 and 3% of the corneal stroma in man (MAURICE and RILEY, 1968) and rabbit (OTORI, 1967). The cells lie flattened in the plane of the tissue within or between the lamellae in the anterior irregular zone but only between the lamellae in the regular posterior layers (GOLDMAN and BENEDEK, 1968). There are approximately 200 lamellae in man, the mean thickness being 1.5 to 2.5 μ and width 9 to 260 μ . Lamellae lie one upon the other, running at various angles in alternate layers (JAKUS, 1964: PAYRAU et al., 1967). The lamellae run uninterrupted from limbus to lumbus and are continuous with the lamellae of the sclera. The lamellae are formed from long fibrils of circular cross

section and uniform diameter embedded in an abundant matrix of glycoprotein, mucoprotein and substances free of collagen. Fibrillar diameter varies from 210 - 650Å (KAYES and HOLMBERG, 1960; JAKUS, 1961).

(d) Descemet's Membrane and Endothelium

Under light microscopy with polar illumination Descemet's membrane appears to be formed from stratified layers (BAUD and BALAVOINE, 1953). Under electron microscopy it is seen as a collection of stratified layers lying parallel to the surface. Each layer is formed from a repeating hexagonal unit with dense nodes marking the angles (KAYE and PAPPAS, 1962; JAKUS, 1956, 1961; FEENEY and GARRON, 1961).

The endothelium is a single layer of cells covering the posterior surface of the cornea. Seen in reflection through a slit lamp it is composed of mostly hexagonal cells (MAURICE and RILEY, 1968; LAING et al., 1975). Each cell possesses an oval nucleus that lies centrally within the cell. The cytoplasm is filled with mitochondria and numerous Golgi bodies, with a granular endoplasmic reticulum found near the intercellular borders. The cell membrane is a typical unit membrane possessing numerous pinocytotic vesicles at the anterior and posterior sides. The surface of the posterior cell membrane shows 20 - 30 microvillae per cell (WOLF, 1969; BLÜMCKE and MORGENROTH, 1967). The intercellular borders follow a tortuous route towards the basal surface, lying on Descemet's membrane, and show complex interdigitations. The lateral cell membranes are separated by a gap of approximately 200Å along most of their length. At the posterior end of the intercellular border is an area of dense cytoplasm

and a narrowing of the intercellular gap to 100Å. This structure is referred to as a terminal bar or zona occludens. A flap-like projection of the cell usually covers the apical end of the intercellular space above the terminal bar (JAKUS, 1962; KAYES and HOLMBERG, 1960; KAYE and PAPPAS, 1962; KAYE et al., 1962; DONN et al., 1961; IWAMOTO and SMELSER, 1965; OKINAMI et al., 1976).

Human corneal endothelial cells are unable to divide by mitosis (MACHEMER, 1966) so that repair can only take place by a spreading of existing cells. Rabbit endothelium however can produce new cells in response to injury.

(e) Aqueous Humour and Precorneal Tear Film

The Aqueous Humour extends from the rear surface of the cornea to the ciliary muscle and lens. In man it has a volume of between 170 and 220 microlitres (µl.) (JONES and MAURICE, 1963; HEIM, 1941). It acts as a mechanical force directed against the cornea thus maintaining the shape of the cornea. A possible structural component is in the form of a very thin polysaccharide coating that lies on the surface of the corneal endothelium. This coating is between 600 and 1500Å thick and appears stable. Whether this layer is a component of the Aqueous Humour or a secretion of the endothelium is unknown. However, it is likely to play a part in the maintenance of normal corneal hydration (GREEN and OTORI, 1970 (a); SCHRØDER and SPERLING, 1977).

The precorneal tear film is a thin fluid layer that covers the exposed portion of the eye. Immediately following a blink it has a thickness

of approximately 8 μ (MAURICE, 1962; EHLERS, 1965). The precorneal tear film has a trilaminar structure and is stable but compressible (WOLFF, 1946, 1954; MISHIMA, 1965; DOHLMAN, 1971). It has three layers, an outer lipid layer, middle aqueous layer and an inner hydrophilic layer. The outer lipid layer has the function of limiting evaporation (MISHIMA, 1965; MISHIMA et al., 1966). It is formed from the secretions of the Meibomian glands situated in the lid margins. The middle layer forms the main component of the precorneal tear film, being 6.5 to 7.5 μ thick. Mucin derived from the conjunctival Goblet cells absorbed on to the hydrophobic corneal surface forms the innermost layer and creates a hydrophilic surface over which the overlying aqueous and lipid layers spread (WOLFF, 1954; HOLLY, 1973).

Blinking is necessary to maintain the precorneal tear film since no movement of the film occurs and there is no lachrymal fluid flow down the cornea between blinks (EHLERS, 1965). Tear fluid is formed in the lachrymal glands, and in the normal human the secretory rate is 10 to 20 μl per minute ($\mu l\ min^{-1}$). (NORN, 1965; FRANCOIS and NEETENS, 1973). The main function of the tear film is to wet the corneal surface and to protect the epithelium from osmotic changes due to evaporation.

1.4 CORNEAL SWELLING AND HYDRATION

In most mammalian species the proportion of water in the whole cornea lies within a range of 75 to 80% and is carefully maintained, as are thickness and hydration (DUANE, 1949 (a); DAVSON, 1949; HARRIS and NORDQUIST, 1955; FRENCH and DUMA, 1963; OTORI, 1967). The only

noticeable change in the normal animal being a slight decrease in thickness due to evaporation when the eye-lids are opened, the change being approximately 4% of the normal thickness in both rabbit and man (MISHIMA and MAURICE, 1961 (a), (b); MANDELL and FATT, 1965).

If the temperature of the isolated eyeball is allowed to fall to room temperature the cornea is seen to swell due to an imbibition of Aqueous Humour into the corneal stroma (HARRIS, 1960). The hydration of the cornea therefore varies depending on the state of the corneal environment. The limiting layers of the cornea, the epithelium and endothelium, are capable of only slight swelling, their cellular nature restricts the stresses that can be placed without permanently damaging the cells.

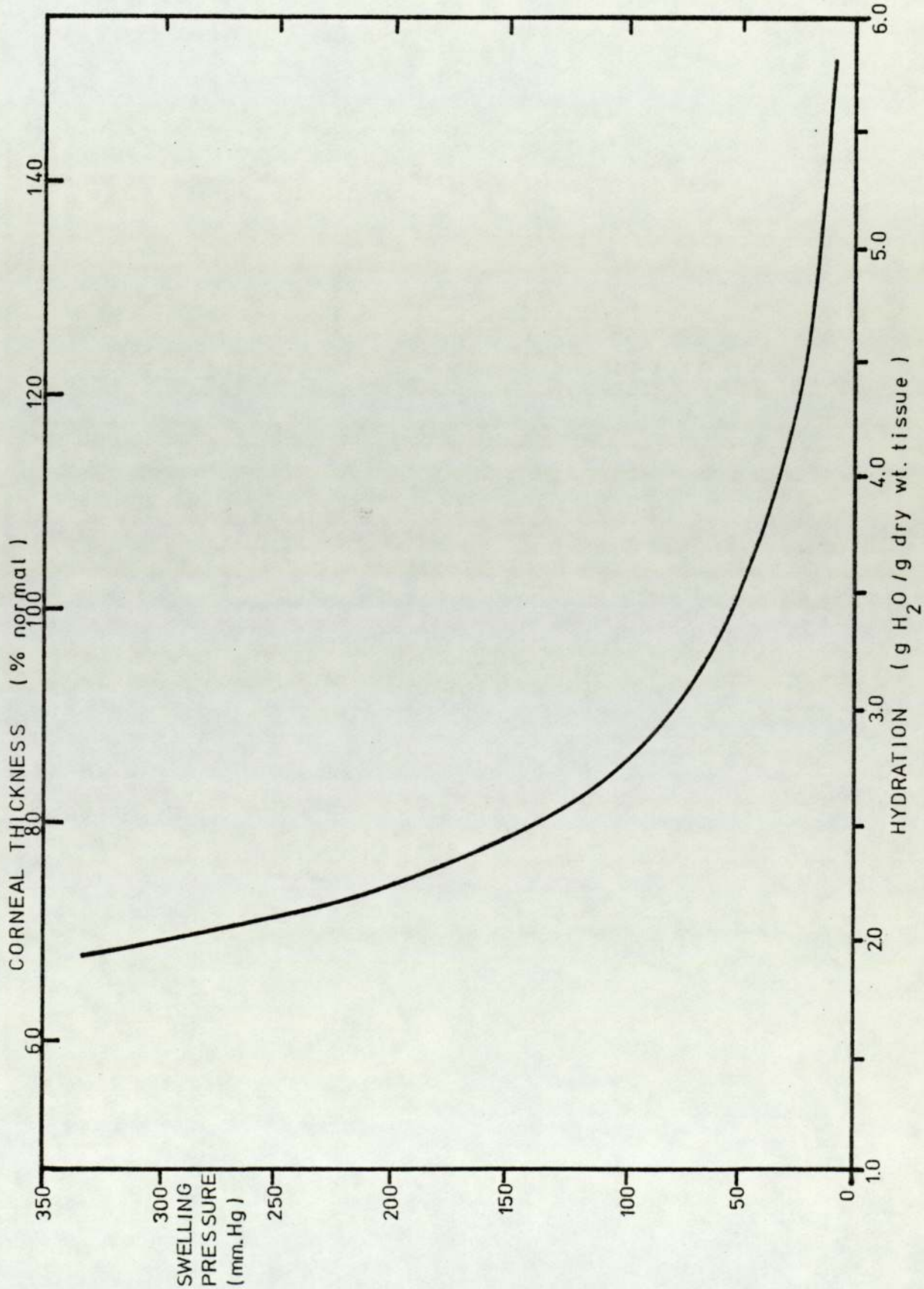
In contrast the stroma (due to its anatomy) behaves differently to the limiting layers when placed in water or hypotonic aqueous solutions. The isolated stroma will absorb fluid when placed in distilled water or solutions of non-electrolytes of small molecular weight (KINSEY and COGAN, 1942). Pieces of cat whole cornea will swell up to 5.5 times by weight when immersed in water. In saline solution the swelling is slower but continues for longer reaching 3 times its weight in 1% saline solution after one day and 10 times its weight after a week. Longer immersions result in even greater degrees of tissue swelling. In a similar situation human cornea will also swell, but to a lesser extent. The upper limit seems to be 2.5 times its initial weight (EHLERS, 1966). The greatest component in such swelling is in the stromal tissues of the cornea.

Swelling of the stroma will take place in the living animal if either of the limiting layers is destroyed. In the rabbit the stroma will swell to approximately twice its normal thickness with the epithelium removed, but more if the endothelium is destroyed (MAURICE and GIARDINI, 1951 (b)). The difference is due to the effect of intraocular pressure acting on the endothelial surface to oppose the swelling when the endothelium is intact, when removed fluid is forced into the stroma (ANSETH and DOHLMAN, 1957). When the stroma is swelling the lamellae cannot stretch thus preventing the stroma from expanding outwards. The stroma must swell inwards at the expense of the anterior chamber.

The pressure developed by the stroma has been described by various authors (KINSEY and COGAN, 1942; ANSETH and DOHLMAN, 1957; HEDBYS and DOHLMAN, 1963; HEDBYS et al., 1963). The measured swelling pressure, or imbibition pressure, for a cornea of normal thickness falls when the cornea swells and rises when it dehydrates, Figure 1. The rate of fluid imbibition across the swelling surfaces has been measured by FATT and GOLDSTICK (1965), who have shown that the movement of fluid can be predicted quantitatively from the value of swelling pressure and the resistances to the flow of fluid offered by the tissue.

Within the stroma swelling takes place uniformly over the whole thickness except for the anterior irregular layers. Over a limited range the stromal expansion takes place within the lamellae. The site of the expansion seems to be the ground substance surrounding the collagen fibrils since the water binding capacity is reduced after the stromal

Figure 1 Swelling pressure of the Ox cornea at various stages of hydration. After HEDBYS and DOHLMAN (1963).



mucoids are extracted (HERINGA et al., 1940; WOODIN, 1954). Precipitation by cetylpyridinium chloride or hyaluronidase digestion of stromal acidic glycosaminoglycans also reduces stromal swelling (HEDBYS, 1961; GREEN et al., 1976). Swelling must occur in the ground substance surrounding the fibrils and the swelling pressure must be developed in the same space, since there is no change in the state of hydration of the collagen fibrils.

The ionic strength of the stromal interstitial fluid is seen to be hypertonic to the Aqueous Humour and plasma (DAVSON, 1949; OTORI, 1967; MISHIMA and HEDBYS, 1967). This ionic excess is due to an excess of cations over anions in the extracellular fluid of the stroma (GREEN and FRIEDMAN, 1971; GREEN, 1969 (a), (b); GREEN et al., 1971). The stromal glycosaminoglycans possess fixed negative charges and it is suggested that cation binding to these fixed charges is responsible for the cation excess. It is also suggested that the swelling pressure - stromal thickness relationship is dependant on a mutual repulsion of the negative charges on the glycosaminoglycans. The cation excess must be maintained, any changes in the ion concentrations of the stroma will affect the stromal swelling and hydration of the stroma and ultimately the cornea (OTORI, 1969; HODSON, 1971 (a)). The thickness (representative of swelling) and hydration of the cornea show a linear proportionality within a set range (HEDBYS and MISHIMA, 1966). As the hydration and therefore the thickness of the stroma increases in proportion, eventually a point is reached where the passage of light through the stroma is no longer uniform and scattering occurs (MAURICE, 1957). Oedema of the epithelium can also lead to a scattering of light with a resulting fall in transparency (POTTS and

FRIEDMAN, 1959; POTTS, 1962; ZUCKER, 1966).

The corneal stroma therefore has a natural tendancy to imbibe water from its surroundings. To maintain the normal transparency of the cornea, this natural imbibition must be confounded (PAYRAU et al., 1967).

1.5 CORNEAL PERMEABILITY

Hydration, thickness and transparency of the cornea are all interrelated. The maintenance of these features and hence function within normal limits demands an intact epithelial and endothelial surface (HARRIS, 1957; STANLEY et al., 1966). If either of these membranes are damaged rapid swelling of the stroma occurs, (MAURICE and GIARDINI, 1951 (b); MISHIMA and HEDBYS, 1967), though there is some species variation in the effect of epithelium removal (DOANE and DOHLMAN, 1970).

It is possible for water to move across the corneal epithelium, this layer offers only a small resistance to the diffusional flux of water (COGAN and KINSEY, 1942 (a); DONN et al., 1963). Using various methods a number of authors have measured the value of water permeability of the epithelium (MISHIMA and HEDBYS, 1967; GREEN and GREEN, 1969; RILEY, 1971; STANLEY and WINSTON-SALEM, 1972; GREEN and DOWNS, 1973; WILSON and FATT, 1974). The endothelium is also permeable to water (DONN et al., 1963; STANLEY et al., 1966; MISHIMA and TRENBERTH, 1968; HEDBYS and MISHIMA, 1969; STANLEY and WINSTON-SALEM, 1972). The value for the permeability of the endothelium is much greater than that of the epithelium (GREEN and

GREEN, 1969). In comparison to the epithelium and endothelium the resistance of the stroma to water passage is negligible (MISHIMA and HEDBYS, 1967; GREEN and GREEN, 1969). The permeability to water of corneal tissues can be demonstrated by a net transfer of water under osmotic and hydrostatic pressure gradients across the whole thickness of the cornea (COGAN and KINSEY, 1942 (a), (b)).

Though water movement can be shown across the cornea under osmotic and hydrostatic pressure gradients, no movement of solutes is seen to occur. It is suggested that the corneal limiting layers show semi-permeability and that a net volume of flow of water can be created by an osmotic pressure gradient (VON BAHR, 1956). Measurements of the permeability of the limiting layers to ions and other fat insoluble substances show that the resistance to these substances is extremely high (MAURICE, 1953).

Small solutes move more slowly in the stroma than in free solution (MAURICE, 1961), however the rate of diffusion of small solutes is similar to that of water, as a result small solutes, within the confines of the stroma, are osmotically inert. Larger molecules, for example haemoglobin (MAURICE, 1969), diffuse more slowly and as a result if dissolved in the solution bathing the stroma they become osmotically effective and can prevent the cornea from swelling (SMELSER, 1952).

The epithelium shows a high resistance to the movement of all ionic substances (COGAN and HIRSCH, 1944; COGAN et al., 1944; MISHIMA and HEDBYS, 1967; MAURICE and RILEY, 1968; RILEY, 1971; STANLEY and

WINSTON-SALEM, 1972). It is probable that these substances enter the epithelium by the intercellular spaces, with the outer cell layer presenting the greater resistance to movement since the flattened form increases the path distance from one cell face to the next and also reduces the total length of cell borders at the surface.

The endothelium shows a high resistance to the movement of ionic substances. However the resistance is less than that shown by the epithelium (MAURICE, 1951, 1953, 1961; GREEN and GREEN, 1969; DONN et al., 1963). The obstruction offered by the endothelial layer depends mainly on the size of the solute particle and is little affected by charge or temperature (MAURICE and WATSON, 1965; SERY et al., 1962; MISHIMA and TRENBERTH, 1968; GREEN and DOWNS, 1976). An exception is the movement of sugars across the endothelium, which is more rapid than predicted, energy-dependant and for which a special mechanism is postulated (HALE and MAURICE, 1969; CSAKY, 1965). The barrier presented by the posterior surface of the cornea lies almost entirely in the cell layer. Descemet's membrane has a resistance which is negligible when compared to the endothelium (MAURICE, 1961), and large molecules can pass freely (DONN et al., 1963). The fact that the obstruction offered by the endothelium is dependant on particle size and not charge and temperature suggests that the greater part of the penetration is through inert pores. These pores are thought to be the intercellular spaces (MAURICE, 1953, 1961). However the presence of zonula occludens systems in the endothelium (HODSON, 1968; IWAMOTO and SMELSER, 1965), place doubt on intercellular spaces as being the only pathway of solute movement, and suggest that some fluid flow may take place through the cells (KAYE et al., 1973 (a), (b)).

It can be concluded that the endothelium and epithelium show semi-permeability to small ions and that a net volume flow of water across the cornea can be created by an osmotic or hydrostatic pressure gradient; that the limiting layers are permeable to water but they are sufficiently impermeable to act as barriers to free diffusion, the endothelium is more permeable than the epithelium; and that the stroma will swell when exposed to aqueous solutions *in vitro* or when the limiting layers are damaged *in vivo*.

1.6 CONTROL OF CORNEAL HYDRATION AND THICKNESS

If the limiting layers of the cornea are completely impermeable to Aqueous Humour and tears the stroma cannot swell since it is denied access to fluid. COGAN and KINSEY (1942 (a), (b)) appreciated that the limiting membranes need only be perfectly semi-permeable, in that they should be totally impermeable to salts, to balance swelling pressure and maintain corneal thickness. These authors further suggested that the difference in tonicity between the interstitial fluid of the stroma and the tears or Aqueous humour (both were then considered as being hypertonic to the plasma) could maintain corneal thickness.

Such a hypothesis has since been shown to be untenable. A hypertonic Aqueous Humour would be ineffective since salts and water can penetrate the endothelium into the less concentrated stromal fluid allowing the stroma to swell. The subsequent retention of these salts which are not free to leave the tissue fluid results in further swelling (DAVSON, 1949; MAURICE, 1951). A hypertonic tear fluid could maintain

normal thickness by constantly draining fluid from the stroma (GREEN and DOWNS, 1973), the small quantity of salts that could cross the epithelium could pass out across the relatively more permeable endothelium without the tissue becoming appreciably concentrated. Such a hypothesis implies firstly that tear fluid is substantially hypertonic and secondly that if the epithelium were bathed in isotonic or hypotonic fluids swelling could result. When tested experimentally it is clear that corneal thickness is maintained constant when bathed in solutions isotonic to blood, and only limited swelling occurs in hypotonic solutions. Therefore such an osmotic mechanism is clearly not employed to regulate corneal hydration in mammals (VON BAHR, 1956; MISHIMA and MAURICE, 1961 (b)).

A continuous inbibition of fluid by the stroma as a result of its swelling pressure is to be expected even when the cellular layers are intact. At low temperatures the in vitro cornea swells, whereas at body temperature it is able to maintain its thickness even though the external conditions are identical. Some mechanism must therefore operate to oppose the tendency to swell. The dependance of this mechanism on temperature suggests an active rather than passive removal of the fluid constantly leaking into the stroma. Since the entry of fluid into the stroma follows a pressure gradient its subsequent removal must occur against this gradient and therefore a source of metabolic energy is required to provide the necessary power. It is presumed therefore that an active process is involved without the necessity of identifying the movement of any substance against an electrochemical gradient (DAVSON, 1949).

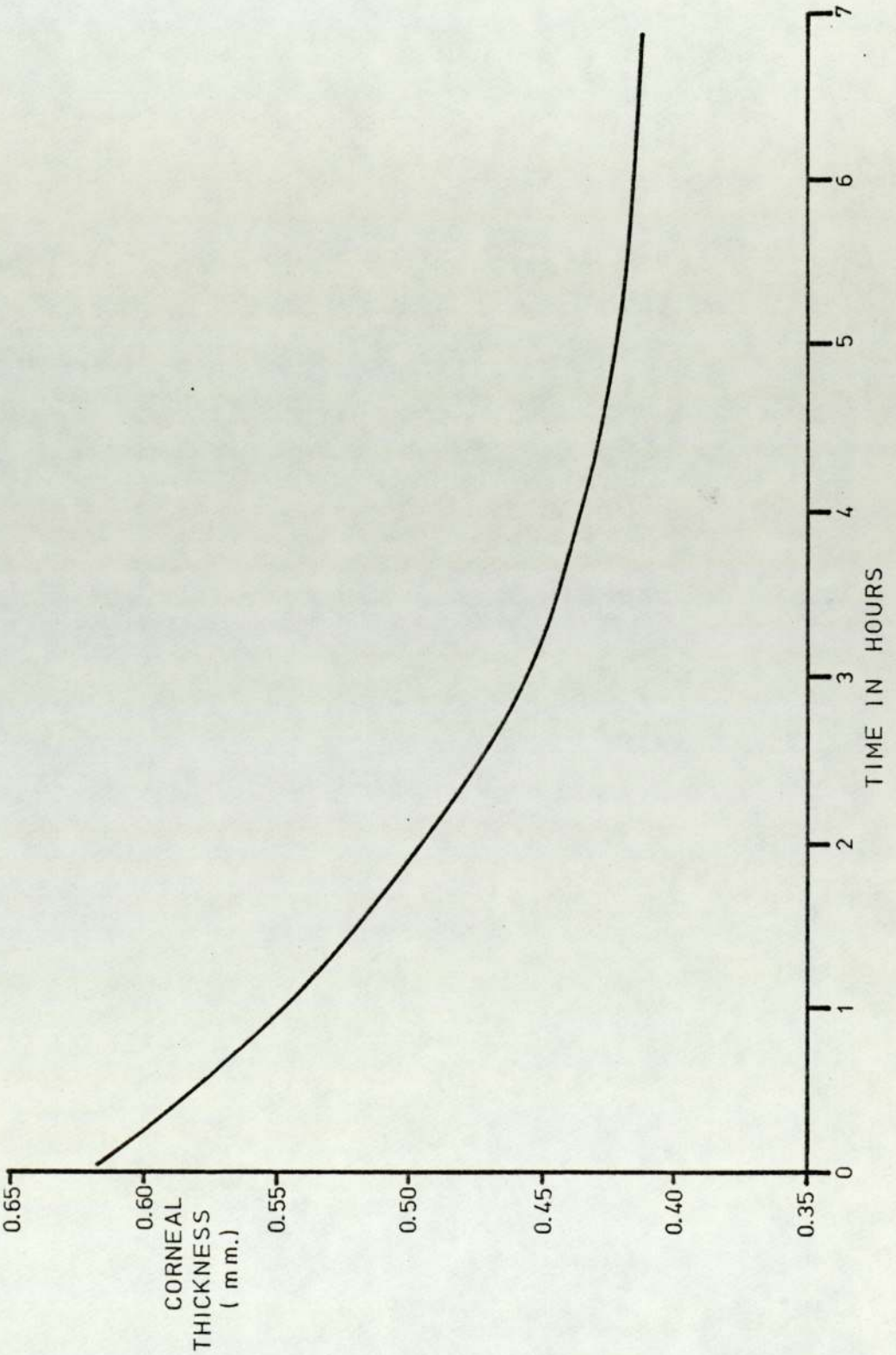
Demonstration of an active process does however require evidence that fluid is transported out of the stroma and that this is linked to the metabolic activities of the corneal tissues. It is not sufficient to show that the tissue swells or shows an increase in rate of swelling when its metabolism is interfered with, since this could result from a decrease in the passive resistance of the limiting layers to water movement.

The cornea of a rabbit eye swells if its metabolism is supressed by refrigeration below 10° centigrade (°C) for a number of hours in a damp chamber. The cornea will return to its normal thickness if subsequently incubated at physiological temperature (DAVSON, 1955; SCHWARTZ et al., 1954; HARRIS and HORDQUIST, 1955; LANGHAM and TAYLOR, 1956; DONN et al., 1959 (a); MISHIMA and KUDO, 1967; DIKSTEIN and MAURICE, 1972; KIKKAWA, 1969), Figure 2. Such a temperature reversal is seen in isolated preparations of cornea, the limbus can therefore be ignored as a possible site of the active process.

The precise location of the active corneal hydration control has been and remains the subject of much literature and discussion.

The first tissue to be implicated in the control of corneal hydration is the epithelium. A potential exists across the epithelium, lens side being positive with respect to the outer surface (DONN et al., 1959 (a); MODRELL and POTTS, 1959; MAURICE, 1967; FEE and EDELHAUSER, 1970). It is suggested that this potential is generated in the superficial layers of the corneal epithelium (MAURICE, 1967).

Figure 2 Temperature reversal of thickness in the in vitro rabbit cornea. After MISHIMA and KUDO (1967).



Experiments using radioactively labelled sodium ions show that the flux of this ion is responsible for most of the potential difference. Altering the sodium ion concentration of the perfusing solutions in an epithelial preparation indicates the presence of an active sodium pump present in the epithelium (LINDEMANN, 1963; GREEN, 1966 (a), (b); EHLERS and EHLERS, 1968; GREEN and OTORI, 1970 (b); AKAIKE and HORI, 1970). If the transepithelium potential difference is abolished, an increase in corneal hydration results (GREEN, 1966 (b); GREEN, 1968; GREEN and OTORI, 1970 (b)). Thus it has been postulated that an active epithelial sodium pump is essential for maintenance of corneal hydration. Active extrusion of sodium ions into the stroma would render it hypertonic with regard to the Aqueous Humour. This stromal excess of sodium ions might then neutralise the mutually repulsive charges on the glycosaminoglycans allowing the molecules to take up a more compact formation (GREEN, 1966 (b); FRIEDMAN and GREEN, 1971). The swelling pressure of the stroma would thereby be reduced in accord with the hypothesis of DAVSON (1955); DONN et al., (1959 (a), (b)); SMELSER (1962) and STEVENSON and WILSON (1975).

However MISHIMA and KUDO (1967) showed that the presence of the epithelial layer is not essential for the control of corneal hydration. That the presence of an intact epithelium is not obligatory to maintenance of corneal hydration has been shown by other authors who suggest that the epithelium plays a passive barrier-leak role, and that active sodium transport across the epithelium is not essential for a deturgesced cornea (RILEY, 1971; DIKSTEIN and MAURICE, 1972).

Chloride ions have been shown to be actively transported out of the cornea towards the tears in the rabbit (VAN DER HEYDEN et al., 1975; KLYCE et al., 1973; KLYCE, 1975), presumably as part of the sodium pump mechanism. KLYCE (1977) has shown that in the swollen cornea where the endothelial surface is sealed, chloride ion flux can thin the swollen cornea by transporting water out of the cornea. The amount of water moved is however very small compared to the quantity of water moved by the endothelium, that is necessary to maintain a normally hydrated cornea. The precise role of the epithelial active transport mechanisms is therefore still unknown.

Far more conclusive evidence exists on the role of the endothelium as the major factor in the control of corneal hydration (RILEY, 1971; TRENBERTH and MISHIMA, 1968; DONN, 1966). A complete temperature-reversal effect can be demonstrated in an in vitro preparation of cornea with the epithelium removed, but not with the endothelium absent (MISHIMA and KUDO, 1967; MISHIMA et al., 1969; DIKSTEIN and MAURICE, 1972; DOANE and DOHLMAN, 1970). An isolated preparation of endothelium and stroma is capable of moving water from the stroma to the Aqueous Humour against a pressure gradient (MAURICE, 1972).

The translocation of fluid from the stroma to the Aqueous Humour and this manifestation of the endothelial pump is termed the "fluid-pump" of the cornea. The nature of the fluid-pump has been the subject of many investigations. Many reviews of the mechanism of action of the fluid-pump and its role in maintaining the deturgescence of the cornea have been written (HARRIS, 1967; HODSON, 1971 (a), 1974; COGAN and KINSEY, 1942 (b); DOHLMAN, 1971; SHAPIRO and CANDIA , 1973;

FISCHBARG and LIM, 1974; FRIEDMAN, 1972, 1973 (a), (b), (c); GREEN, 1969 (b)).

The endothelial fluid-pump shows a number of features that have led to a greater understanding of its function:-

(a) A spontaneous electrical potential (lens side negative) exists across the endothelium (BARFORT and MAURICE, 1972; FISCHBARG, 1972, 1973; HODSON, 1974, 1977).

(b) The fluid pump is totally inhibited by 10^{-5} Molar (M) Ouabain, a sodium, potassium, adenosine triphosphatase (ATP ase) inhibitor (LAMBERT and DONN, 1964; BROWN and HEDBYS, 1965; LANGHAM and KOSTELNICK, 1965; TRENBERTH and MISHIMA, 1968; HODSON, 1974). The absence of sodium ions likewise blocks pump action (HODSON, 1971 (b), 1974; DIKSTEIN and MAURICE, 1972; FISCHBARG and LIM, 1974).

(c) The fluid-pump is partially yet reversibly inhibited in the presence of carbonic anhydrase inhibitors (FISCHBARG and LIM, 1974) and in the absence of bicarbonate ions (HODSON, 1971 (b), 1974; HODSON and MILLER, 1976; HULL et al., 1977).

(d) There is a reversible decrease in endothelial potential difference and arrest of fluid transport in the absence of potassium ions (FISCHBARG, 1972, 1973; FISCHBARG and LIM, 1974). An increase in hydrogen ion concentration (FISCHBARG and LIM, 1974) or a decrease in temperature likewise inhibit the fluid-pump (BARFORT and MAURICE, 1972, 1974; MAURICE, 1972). A change in chloride ion

concentration is without effect (HARRIS and NORDQUIST, 1955).

(e) Anions derived from glucose metabolism are incapable of maintaining the fluid pump (RILEY et al., 1977).

A number of models have been proposed linking together all the features exhibited by the endothelial fluid-pump (FISCHBARG, 1973; FISCHBARG and LIM, 1974; HODSON, 1971 (b), 1974, 1977). The precise nature of the pump is still not fully understood, however there is agreement among current authors as to the likely nature.

Bicarbonate ions are pumped into the Aqueous Humour by the endothelium. Under physiological conditions $\frac{2}{3}$ of the substrate is provided by exogenous bicarbonate ions (HCO_3^-) derived from the corneal stroma. The remainder is provided by exogenous carbon dioxide, which is converted to HCO_3^- by carbonic anhydrase located directly underneath the posterior membrane of the endothelium (SILVERMAN and GERSTER, 1973; LONNERHULM, 1974; YOKOTA and WALLER, 1975; HODSON and MILLER, 1976), across which the active flux of bicarbonate ions pass. It is thought that the endothelial potential difference is generated by an electrogenic HCO_3^- transport. The mechanism by which HCO_3^- are actively pumped across the posterior membrane is unknown. Endothelial ATP-ase activity is stimulated in the presence of HCO_3^- (RILEY, 1977 (a)); this could link the pump mechanism to the energy producing systems of the endothelium.

The method by which HCO_3^- flux and water translocation are coupled is also unknown. It is likely that endothelial structure is important

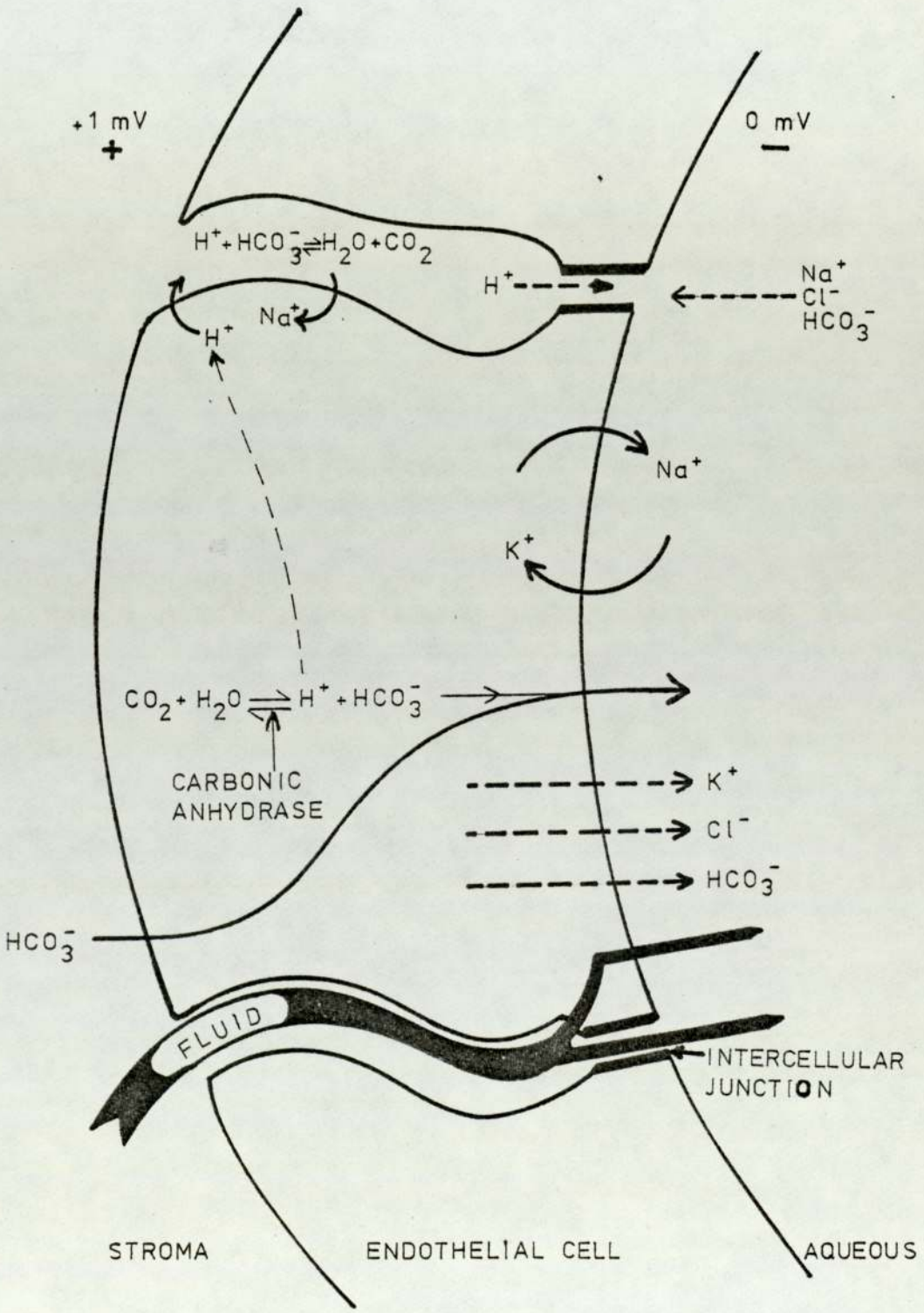
since ATP-ase activity is identified at the lateral and apical cell membranes in the endothelium (KAYE and TICE, 1966; LEUENBERGER and NOVIKOFF, 1974; ROGERS, 1968; TERVO and PALKAMA, 1974).

Though HCO_3^- are responsible for the net flux across the endothelium it seems likely that this is not the only "ion-pump" active in the corneal endothelium. There is evidence for a sodium pump in the endothelium (HODSON, 1971 (b); DIKSTEIN and MAURICE, 1972; FISCHBARG and LIM, 1974), however the presence of a membrane potential (lens side negative) suggests that only an anion is extruded. It is possible that the cell has an active sodium pump, but that the activity is symmetrical across the membrane and not polar. Sodium transport is seen to occur across the lateral margins of endothelial cells (KAYE et al., 1968; KAYE and TICE, 1966). It is possible that this produces the required symmetry, and the absence of any trans-endothelial electrical activity associated with the active sodium transport, Figure 3.

1.7 SUBSTRATE REQUIREMENT AND SUPPLY

Many authors have shown that the mechanisms that maintain normal corneal hydration require a supply of energy (LANGHAM, 1955; LANGHAM and TAYLOR, 1956; HARRIS and NORDQUIST, 1955; DAVSON, 1955; MISHIMA and KUDO, 1967; DIKSTEIN and MAURICE, 1972). The cornea contains many cells, some concerned with the active transport process, others with the production of new cells at the epithelium. All these cells are living and therefore have a requirement for nutrients.

Figure 3 A model for the "Fluid Pump" at the corneal endothelium. After FISCHBARG and LIM (1974); HODSON and MILLER (1976).



Energy is produced in the cornea by a number of pathways. The metabolic patterns of the corneal layers vary, reflecting their different functions and energy requirements. The normal functioning of the respective layers is however dependent to a certain extent on the structural integrity of the cornea, and in some aspects the layers are interdependent. As a result of the requirement for structural integrity and the interdependance of the corneal layers, evaluation of information obtained from a study of isolated layers in terms of whole cornea function is restricted. In the main however information taken from isolated layers is valuable and its use is valid in the assessment of normal corneal function.

The requirement of corneal cells are similar to the needs of any living mammalian cell; oxygen, an energy source and an amino-acid supply. Amino-acids are supplied by the Aqueous Humour, absorbtion taking place across the endothelium (RILEY, 1977 (b)). In the normal cornea most energy is obtained from the breakdown of glucose to lactic acid and carbon dioxide (MISHIMA and HEDBYS, 1968).

Glucose Supply and Consumption

There are three possible pathways of glucose supply to the cornea:-

(a) Tears

(b) Aqueous Humour

(c) Limbus

The concentration of glucose in the tears is low, 2.6 milligrams per 100 millilitres, (mg $100ml^{-1}$) (GIARDINI and ROBERTS, 1950; EHLERS,

1965; REIM et al., 1967 (a)). This is a factor of approximately ten times less than the glucose concentration of the Aqueous Humour (WALKER, 1933; REIM and LICHTE, 1965; REIM et al., 1967 (a); HALE and MAURICE, 1969; BRUUN-LAURSEN and LORENTZEN, 1973; STEVENS-ANDREWS, 1976 (a)). The low concentration of glucose and the impermeability of the epithelium to small solutes reduce the supply of glucose to the epithelium from the tears to such a small flux that it is wholly inadequate to satisfy the needs of the cornea for the maintenance of normal cellular function.

The limbus is also unable to supply glucose to the majority of the cornea due to the high resistance to lateral movement of solutes (MAURICE and WATSON, 1965; MISHIMA and HEDBYS, 1967; MAURICE and RILEY, 1968; THOFT and FRIEND, 1972). Lateral movement of glucose from the limbus may however supply the peripheral cornea with glucose (MAURICE, 1969; MISHIMA and HEDBYS, 1968).

The only remaining source of glucose supply is the Aqueous Humour. As stated earlier the glucose concentration of the Aqueous Humour is high, approximately ten times that of the tears. The precise concentration does however vary in disease (deBERARDINIS et al, 1965; BRUUN-LAURSEN and LORENTZEN, 1974) and with age (BRUUN-LAURSEN and LORENTZEN, 1975).

In vitro evidence for the Aqueous Humour as a source of glucose is shown in experiments where, increased hydration and loss of temperature-reversal effect ensue, on the removal of glucose from the bathing solutions (MISHIMA and KUDO, 1967). When non-permeable membranes are placed in the stroma, or air bubbles placed in the Aqueous Humour,

there is a decrease in the epithelial glucose concentration, which results in epithelial atrophy. This suggests that Aqueous Humour glucose normally provides the major metabolic substrate to this distant tissue in vivo (KNOWLES, 1961; POLLACK, 1962; BROWN and MISHIMA, 1966; TURSS et al., 1970, 1971; THOFT et al., 1971).

Measurements of glucose concentration in the Aqueous Humour and corneal tissue show a fall in glucose concentration from the Aqueous Humour to the stroma (REIM et al., 1967 (a); MISHIMA and HEDBYS, 1968; TURSS et al., 1971). Calculations of the potential rate of movement of glucose across the endothelium via simple diffusion indicate that it would be insufficient to satisfy the corneal glucose requirements (RILEY, 1969 (a), 1972; THOFT and FRIEND, 1972). The observed rate of glucose movement across the endothelium is much more rapid than would be expected on the basis of its molecular dimensions and the size of the diffusion gradient (MAURICE, 1969). An active movement of glucose from the Aqueous Humour into the stroma across the endothelium by a process of facilitated diffusion utilising a carrier and a metabolic process has therefore been suggested (HAIE and MAURICE, 1969).

Glucose is not the only substrate for the energy-producing metabolism of the cornea. Glycogen deposits present only in the epithelium of the cornea can be used as substrate (CALMETTES et al., 1956; HERRMANN and HICKMAN, 1948 (a), (b); KAMEI, 1959; LANGHAM, 1954; TURSS et al., 1970; THOFT and FRIEND, 1972; REIM and LICHTE, 1965; STEVENS-ANDREWS, 1976 (a)). If the balance of glucose metabolism and lactic acid production in the cornea is examined, there is a small discrepancy in that more lactic acid is produced than glucose metabolised.

This small component is due to a breakdown of glycogen (RILEY, 1969 (a); TSUTSUI et al., 1965). Glycogen is therefore continually being produced from the epithelial glucose pool and broken down to produce energy, though the turnover rate is slow (MISHIMA and HEDBYS, 1968; MAURICE and RILEY, 1970; MAURICE, 1969). This is the situation in the normal cornea, in the absence of a glucose supply the epithelial glycogen reservoir is depleted (TURSS, 1970, 1971; THOFT and FRIEND, 1972). The epithelial glycogen reservoir is capable of maintaining corneal hydration for approximately 34 hours in the absence of a glucose supply (HERRMANN and HICKMAN, 1948 (a); MISHIMA and HEDBYS, 1968; MAURICE and RILEY, 1970). Reduction of epithelial oxygen supply also reduces the epithelial glycogen pool, no glycogen can be identified in the epithelium after two hours contact lens wear (SMELSER and OZANICS, 1952, 1953; KINSEY, 1952).

In the cornea the product of glycolysis, pyruvate, is converted to lactic acid by the action of lactate dehydrogenase (LDH), and to carbon dioxide and water by the Krebs Citric Acid Cycle. The production of carbon dioxide from the breakdown of pyruvate is slower than the rate of pyruvate production, therefore there is a lactic acid excess in the tissues of the cornea. Since lactic acid can be used as a substrate in the Krebs Citric Acid Cycle by means of the reconversion to pyruvate, it can act as a substrate to the energy-producing mechanisms of the cornea and can be classed as a carbohydrate energy reserve (KUHLMAN and RESNIK, 1959). Lactic acid is produced in all the component tissues of the cornea at approximately equal rates on an equivalent tissue weight basis (MAURICE and RILEY, 1968). In the normal cornea lactic acid conversion to pyruvate does not usually

occur. Lactic acid produced from glycolysis is usually lost to the Aqueous Humour, with a small amount leaving the cornea via the epithelium to the tears (REIM et al., 1972; RUBEN and CARRUTHERS, 1972; RILEY, 1972). When glucose and glycogen are depleted, the lactic acid reservoir is capable of maintaining corneal respiration for a period of up to three hours (KUHLMAN and RESNIK, 1959).

Oxygen Supply and Consumption

One of the requirements for a stable corneal hydration is an adequate oxygen supply (DUANE, 1949 (b); DE ROETTH, 1950; LANGHAM, 1955; LANGHAM and TAYLOR, 1956; MISHIMA and TAKAHASHI, 1969; DIKSTEIN and MAURICE, 1972; FREEMAN, 1972). The quantity of oxygen consumed by corneal tissue has been the subject of a number of studies. Early work on the measurement of corneal oxygen consumption rate used Warburg respirometer techniques and determined the rates of oxygen consumption in whole cornea or in corneae with one or other of the limiting layers removed. Unfortunately respirometer techniques require the use of isolated corneae, and the determination of the oxygen consumption of the individual layers demands a splitting up of the cornea. Isolation of the tissue and inadequacies in the incubation medium can induce trauma in the tissue and affect corneal oxygen consumption. Separation of the tissue layers also eliminates any inter-layer metabolic inter-reactions which can affect measured values of oxygen consumption for the component layers.

Recently polarography has offered an alternative method for the measurement of oxygen consumption rate in corneal tissues.

Polarographic oxygen sensors allow the measurement of oxygen consumption rate both *in vitro* and *in vivo*. *In vitro* methods are similar to the Warburg gas analysis techniques. A second use of polarographic oxygen sensors is in the *in vivo* determination of oxygen consumption rate, by placing the sensor on the cornea and measuring the rate of depletion of oxygen from the membrane of the electrode. These *in vivo* values are not however strictly comparable to values found in the *in vitro* respiratory studies, since polarographic methods do not take into account the gas that passes through the cornea into the Aqueous Humour, and that supplied to the cornea by the Aqueous Humour.

In isolated corneae with the epithelium removed oxygen consumption is minimal compared to the whole cornea. This suggests that the epithelium is the major oxygen consuming tissue in the cornea (FREEMAN, 1972; FARRIS et al., 1965). Values of oxygen consumption rate of the cornea using different methods are shown on Table 1.

Measurements of oxygen consumption by the various layers of the cornea show that on gas consumed per surface area basis the epithelium consumes 40% of the total oxygen consumption (FREEMAN, 1972; FARRIS et al., 1965). On a gas consumed per unit weight basis the stroma is the least active, the epithelial consumption is ten times that of the stroma and the endothelium is approximately five times that of the epithelium (FREEMAN, 1972). The stroma consists of only 2% by weight of cells, therefore if the respiratory activity is calculated on a per cell basis, the stromal cells possess an oxygen consumption rate approximately equal to the epithelium.

Table 1

Corneal oxygen consumption rates for different species and methods of determination

AUTHOR	METHOD	SPECIES	CONSUMPTION RATE $\mu l O_2 cm^{-2} hr^{-1}$
BESSEY and WOLBACH 1939	WARBURG	RAT	40.0
LEE and HART 1944	"	"	24.9
ROBBIE et al 1947	"	RABBIT	4.6
DE ROETTH 1950	"	OX	7.6
LANGHAM 1952	"	RABBIT	8.6
NONAKA 1958	"	"	10.09
FUNATSU 1958	"	"	7.77
MATSUMOTO and KUDO 1960	POLAROGRAPHIC SENSOR	"	10.39
HILL and FATT 1963 (a)	"	HUMAN	4.8
HILL and FATT 1963 (b)	"	"	1.9
FARRIS et al 1965	"	RABBIT	1.05
HABERICH and DENNHARDT 1966	GAS ANALYSIS	OX	9.5
RILEY 1969 (a)	POLAROGRAPHIC SENSOR	RABBIT	11.6
JAUREGUI and FATT 1971	"	"	0.65
JAUREGUI and FATT 1972	"	HUMAN	2.8
FREEMAN 1972	"	RABBIT	9.54
EDELHAUSER 1974	"	"	7.56

The cornea is unusual in that it is totally avascular. Since an oxygen supply is essential to the normal functioning of the cornea, and since the cornea is transparent and the hydration balanced, the cornea must be receiving an adequate oxygen supply. The possible origins of oxygen supply are similar to those of glucose supply; tears, Aqueous Humour and limbal blood vessels.

An excised eye placed in a moist chamber will remain transparent for 24 hours, however if the oxygen tension in the chamber is decreased the cornea will rapidly become opaque. GUNDERSON (1939) has shown that complete peritomy has no effect on the cornea and concludes that the limbal blood vessels are of little importance as a nutritional supply to the cornea. The large distance separating the limbal blood vessels and the central cornea, make it unlikely on permeability considerations that sufficient oxygen can be obtained by the epithelium, from the limbus, to maintain its metabolism.

Low oxygen tension in the Aqueous Humour relative to the atmosphere, (BARR and ROETMAN, 1974), measurement of endothelial permeability to oxygen, and oxygen requirement of the epithelium, show that the Aqueous Humour is incapable of supplying adequate oxygen to the epithelium.

FISCHER (1930 (a), (b)) showed that oxygen will pass into the cornea from the atmosphere and that removal of the epithelium increases the rate of oxygen passage from the atmosphere to the Aqueous Humour. Other evidence implicating the atmosphere as the major source of oxygen to the cornea is given by LANGHAM (1952) who showed that

increasing the atmospheric oxygen tension leads to a fall in corneal lactic acid. This suggests that oxygen is diffusing into the cornea from the atmosphere. The converse is also true, reducing the atmospheric oxygen tension increases corneal lactic acid concentration (LANGHAM, 1951, 1952), therefore the cornea is taking oxygen from the atmosphere. This work also confirms the inability of the Aqueous Humour and limbus to supply adequate oxygen. The cornea therefore obtains most of its oxygen from the atmosphere by direct diffusion across the epithelium (HEALD and LANGHAM, 1953, 1956; LANGHAM, 1952). The fact that increasing the atmospheric oxygen concentration leads to a drop in lactic acid concentration within the cornea further suggests that the cornea is not utilising oxygen at a maximum rate under normal atmospheric conditions.

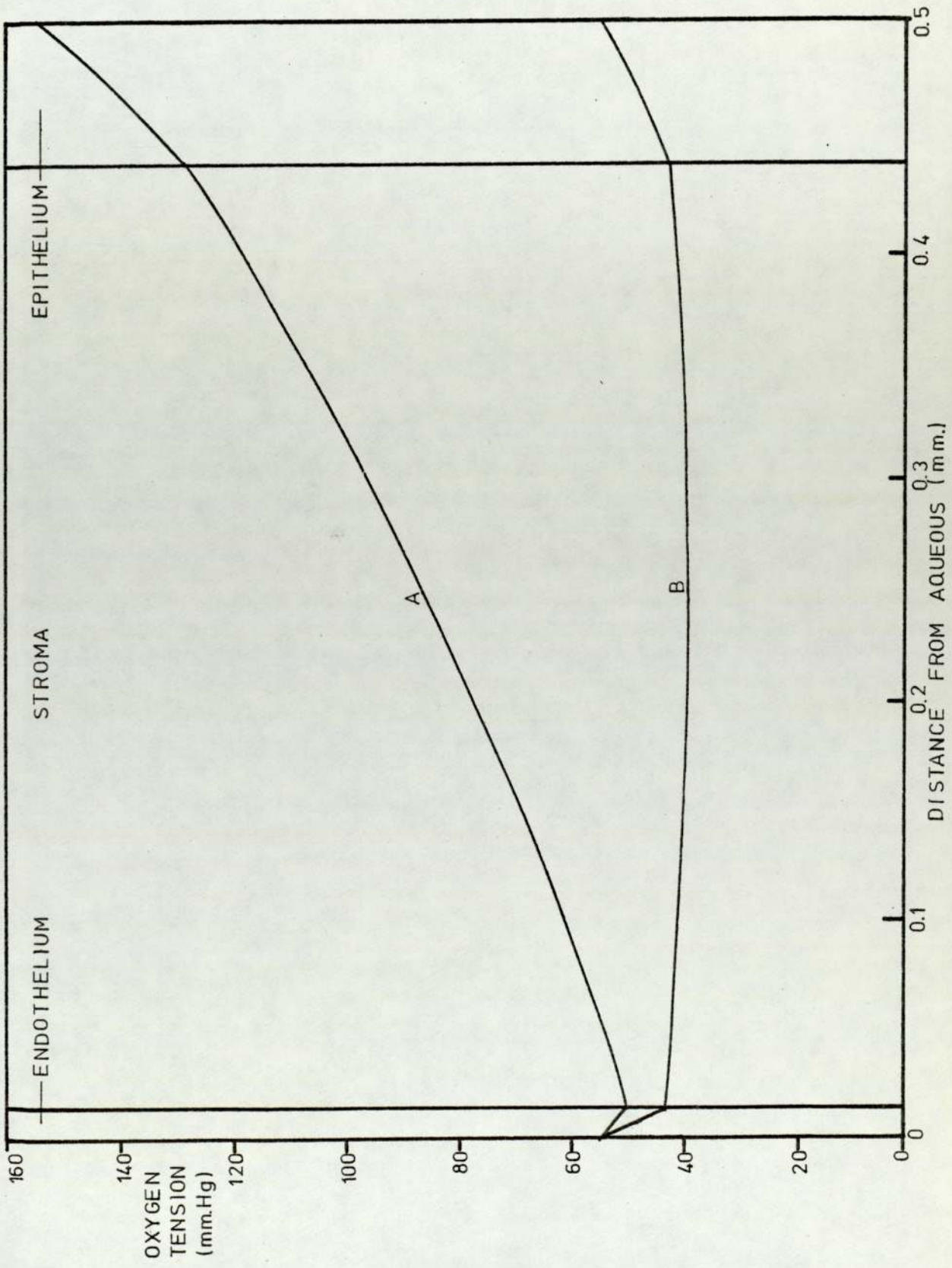
Having seen that the cornea obtains most of its oxygen by direct exchange with the atmosphere, FATT and BEIBER (1968) attempted to show the steady-state distribution of oxygen in the cornea. This work used previously published values for oxygen consumption by the component layers of the cornea, and the diffusion coefficient and solubility values for oxygen in the stroma given by TAKAHASHI and FATT (1965), and TAKAHASHI et al., (1967). Such a mathematical treatment shows a gradual decrease in oxygen tension with movement from the epithelium to the endothelium. The oxygen tension at the epithelial surfaces being 155 millimetres of mercury (mmHg), and at the endothelium surface slightly higher than 55 mmHg (the measured Aqueous Humour oxygen tension), indicating a flux of oxygen from the cornea into the Aqueous Humour at steady state.

RILEY (1969 (a)) pointed out that measuring the in vivo oxygen flux using polarographic oxygen sensors (FARRIS et al., 1965; KUDO and TAKAHASHI, 1967), could not take into account the oxygen contributed by the Aqueous Humour to corneal oxygen supply, seen in his in vitro work on rabbit cornea. RILEY (1969 (a)) considers the Aqueous Humour to be supplying 20% of the total oxygen supply.

FATT et al., (1974) state the limitations of the work by FATT and BEIBER (1968). These are that, the diffusion coefficient, solubility of oxygen and oxygen consumption values are those for stroma only. Due to the lack of evidence to the contrary FATT and BEIBER (1968) considered that on a unit cell basis, similar values for these parameters were held by the limiting layers of the cornea. Using measured values for oxygen permeability (product of solubility and oxygen diffusion coefficient), and oxygen consumption for the individual layers (FREEMAN and FATT, 1972; FREEMAN, 1972), FATT et al., (1974) repeated the work of FATT and BEIBER (1968). FREEMAN (1972) showed that the oxygen permeability of the epithelium is four times that of the endothelium. Using this difference FATT et al., (1974) obtained a normal open eye profile for the cornea, Figure 4. The only difference between the profiles of FATT and BEIBER (1968) and FATT et al., (1974) is that an oxygen flux exists in the cornea from the Aqueous Humour into the endothelium rather than from cornea to Aqueous Humour as found by the earlier authors.

Confirmation of the profile of FATT et al., (1974) is given by the oxygen profile measured in vivo by KWAN et al., (1972).

Figure 4 Calculated oxygen tension profile for rabbit cornea in the open eye (A) and closed eye (B). After FATT et al., (1974).



In the closed eye situation the main oxygen supply to the cornea is removed (HILL and FATT, 1964). The origin of the corneal oxygen must be reconsidered. The oxygen tension of the Aqueous Humour is 55 mmHg, although the range is extremely large and inter-author variations are great (FRIEDENWALD and PIERCE, 1937; HEALD and LANGHAM, 1956; DRECKHAHN and LORENZEN, 1958; KLEIFELD and NEUMANN, 1959; KLEIFELD and HOCKWIN, 1961; JACOBI, 1966; WEGNER and MØLLER, 1971; KWAN et al., 1972; BARR and SILVER, 1973). In the normal open eye situation the Aqueous Humour oxygen flux is seen to be small (FATT et al., 1974). The Aqueous Humour oxygen component will remain constant in the closed eye situation, however it is insufficient to maintain the epithelium and an alternative source must be found. The blood vessels that are present in abundance in the palpebral conjunctiva carry oxygenated blood. The oxygen tension at the palpebral conjunctival surface has been measured at between 55 and 70 mmHg, corresponding to that of the blood supply within the blood vessels (KWAN and FATT, 1971; FATT and BEIBER, 1968; WEGENER and MØLLER, 1971). The blood supply of the palpebral conjunctiva is capable of supplying oxygen to the cornea in the closed eye situation, and since no signs of corneal distress are visible after closing the eye for several hours, the palpebral conjunctiva must be capable of supplying adequate oxygen for maintenance of normal oxygen consumption (LANGHAM, 1954). The corneal oxygen tension profile of a closed eye is given on Figure 4.

1.8 PATHWAYS OF CARBOHYDRATE METABOLISM

The energy required for general cellular maintenance, and of corneal hydration in particular, depends primarily on the metabolism of glycogen, glucose and lactic acid (section 1.6). The pathways via which these substances are utilised in the different layers, has been established using histochemical enzyme localisation, quantitative enzyme assays and radioactive tracer techniques.

JAEGER (1953) showed dehydrogenase activity in bovine corneal homogenates, the activity in the epithelium, stroma and endothelium being 96, 1 and 3% respectively. This author suggested that the presence of dehydrogenase activity indicated an active Krebs Citric Acid Cycle, (Tricarboxylic acid cycle, T.C.A. cycle). That it is located in the epithelium, and that it is the main oxidative pathway. Specific dehydrogenase enzymes have been demonstrated in the cornea. One of the major enzymes in the T.C.A . cycle, succinic dehydrogenase (SDH) is found predominantly in the epithelium and endothelium, with only insignificant amounts in the stroma (DE BERARDINIS, 1951; KUSONOKI, 1960; BAUM, 1963). Likewise the enzyme responsible for the conversion of pyruvate to lactic acid, lactate dehydrogenase (LDH) is present in the cornea (KUHLMAN and RESNIK, 1958; KUHLMAN, 1959; MOORE and WORTMAN, 1959). Coenzymes I and II are present in the cornea, although Coenzyme II cannot be shown in the stroma (COGAN and KUWABARA, 1960; KUSONOKI, 1960). The dehydrogenases connected with anaerobic glycolysis (the Embden-Myerhoff Pathway (EMP)) and aerobic glycolysis (the Pentose Phosphate Pathway or Hexose Monophosphate Shunt (HMS)) are also present in the cornea although the aerobic pathway is absent in the stroma.

Quantitative enzyme analysis (to describe the distribution of enzymes associated with carbohydrate metabolism) has confirmed the conclusions of the histological evidence, that all three pathways of carbohydrate metabolism are present in all layers of the cornea (REIM et al., 1971; REIM and SCHRAMM, 1965). The activity of the HMS and TCA cycle in the epithelium varies between species, and the activity of the HMS in the stroma is low.

Early biochemical studies confirmed the enzymatic conclusions that the cornea possesses an EMP and TCA cycle (LEINFELDER, 1948; DUANE, 1949 (b); DE ROETTH, 1950, 1951; LANGHAM, 1954). The work of HERRMANN and HICKMANN (1948 (a), (b)) disagreed with the general findings, however a reassessment of their work by RILEY (1969 (b)) has shown their work to agree with the general findings.

The appearance of radioactive carbon dioxide derived from glucose labelled in the one or six position has confirmed the existance of a third pathway, the aerobic HMS (KINOSHITA, 1957, 1962; KINOSHITA and MASURAT, 1954, 1959; KINOSHITA et al., 1955; KUHLMAN and RESNIK, 1959; RILEY, 1969 (b); OLSEN et al., 1970).

The presence of a lactic acid pool in the corneal epithelium indicates that glycolysis is very active in this tissue, and that even in aerobic conditions the TCA cycle cannot keep up with the lactic acid production. It appears also that the triose phosphate product of the aerobic HMS is converted to lactic acid and contributes to the lactic acid pool of the cornea (KINOSHITA et al., 1955). Oxidation of glucose to carbon dioxide and water seems only to take place to any

appreciable extent in the epithelium. In the stroma and endothelium lactate utilisation plays a more significant role (KINOSHITA, 1962).

The HMS is extremely active in the corneal epithelium. It is estimated that under normal conditions more glucose is metabolised by the HMS than by the EMP (KINOSHITA, 1957, 1962; KINOSHITA and MASURAT, 1959; KUHLMAN and RESNIK, 1959). The precise ratio of aerobic to anaerobic glycolysis seems to be species variable (KINOSHITA et al., 1955; KUHLMAN and RESNIK, 1959; RILEY, 1969 (a)), the ratio of glucose oxidised by aerobic and anaerobic glycolysis is 70:30 (KUHLMAN and RESNIK, 1959).

The mechanism by which energy is released from carbohydrate metabolism and passed to the energy requiring tissues of the cornea is complex and not fully understood. High energy compounds; Adenosine triphosphate (ATP), Adenosine diphosphate (ADP), Adenosine monophosphate (AMP), Nicotinamide adenine dinucleotide (NAD), Nicotinamide adenine dinucleotide phosphate (NADP), Glutathione (GSH), are present in all the corneal layers (HERRMANN and MOSES, 1945; MORLEY and TOTH, 1961; REIM and LICHTE, 1965; REIM et al., 1966, 1967 (a), 1968).

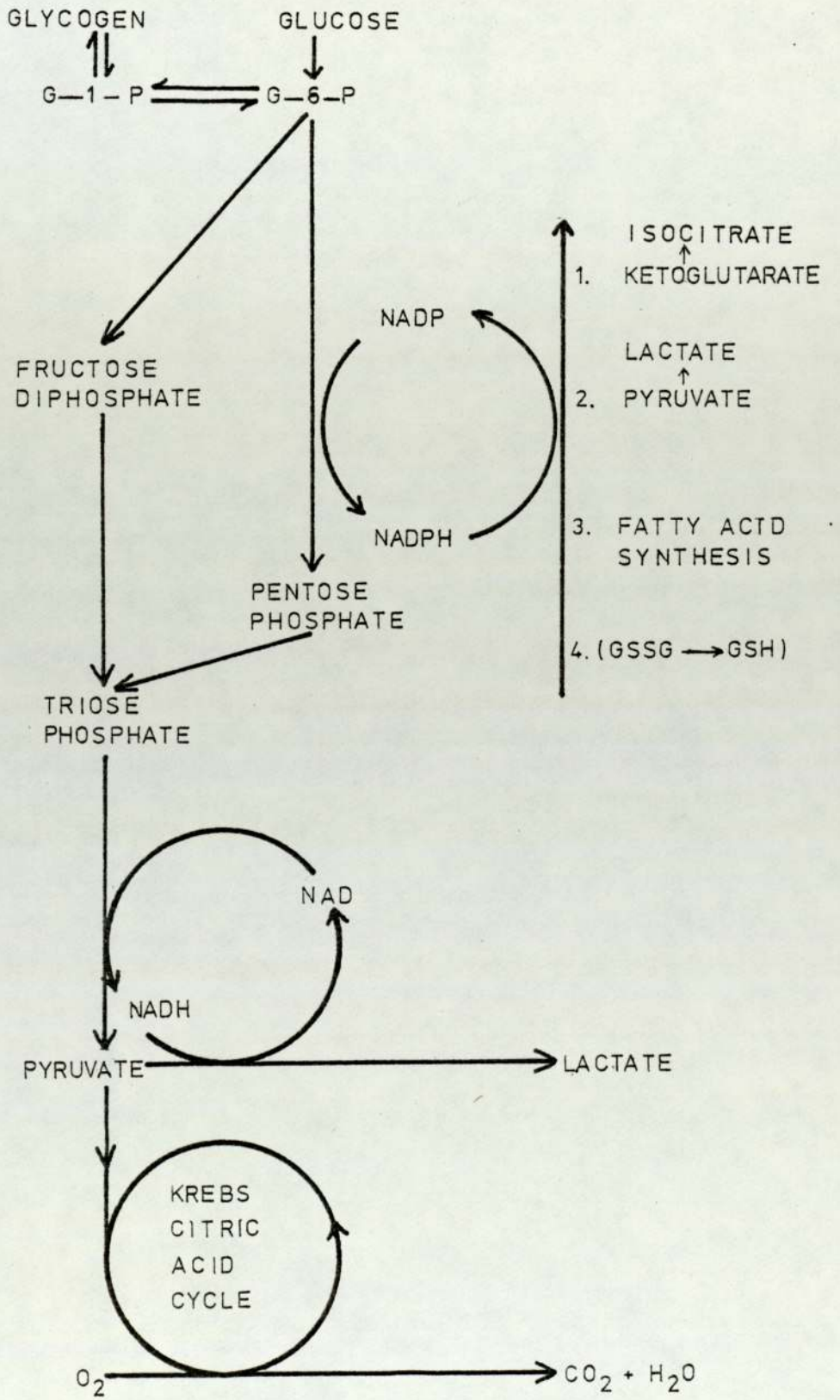
In the epithelium a functioning oxidative phosphorylation is evident from the presence of a high ATP/ADP ratio, which declines rapidly under anaerobic conditions (REIM and LICHTE, 1965), and from the stimulation of respiration by low concentrations of dinitrophenol (LANGHAM and TAYLOR, 1965).

A high proportion of glucose is metabolised in the cornea by the HMS. In this breakdown of glucose by the HMS reduced NADP (NADPH) is produced, that must be oxidised for appreciable amounts of carbon dioxide to be produced by later phases of metabolism, since the NADP pool is limited. The method of reoxidation of NADPH is not fully understood, however KINOSHITA (1962) has shown that LDH, present in large amounts in the corneal epithelium (KUHLMAN, 1959; MOORE and WORTMAN, 1959; GRAYMORE and McCORMICK, 1966; GRAYMORE and POWER, 1967, 1968), can function with this Coenzyme by forming lactate from pyruvate. Pyruvate can stimulate the HMS by oxidising NADPH with the formation of lactate (KINOSHITA, 1957, 1962). To obtain energy from reduced NADPH the reoxidation must proceed through the electron transmitting systems and the associated phosphorylation mechanisms. Whether the passage of energy from NADP or NAD to the electron transfer system is direct or through other Coenzymes or compounds is unknown.

The electron transport system at the mitochondrion uses oxygen to produce three moles of ATP per mole of oxygen. ATP is the high energy compound used in the functioning of the corneal cells. Though reduction of the Coenzyme by LDH seems to be the most efficient method in the rabbit, other mechanisms take priority in some other species (MAURICE and RILEY, 1970). A summary of the metabolic reactions taking place in the rabbit corneal epithelium is shown on Figure 5.

In the stroma the potential cellular activity of the enzymes of glucose metabolism is similar to the epithelium, on a cell to cell basis, suggesting that glucose metabolism proceeds at approximately the same rate per cell in each layer of the cornea (LANGHAM, 1954). The stromal

Figure 5 Pathways of glucose metabolism and hydrogen transfer in the corneal epithelium. After MAURICE and RILEY (1970).



cells appear unable to oxidise glucose via the HMS, although the overall yield of energy is similar to the epithelium. A possible explanation is that the oxygen potential found in the stroma is too low to drive the HMS. Oxidation via the TCA cycle is possible in the stroma, it may be that with the lack of oxygen, oxidised Coenzymes formed in the TCA cycle are passed to the epithelium for combination with oxygen, thus allowing the TCA cycle to function in the stroma (HERRMANN, 1961). The feasibility of such a transfer mechanism is questioned by BAUM (1963) and LEE and HART (1944).

The endothelium possesses all the enzymes necessary for the three metabolic pathways. The rate of respiration in this layer is approximately equal to that in the epithelium (DE ROETTH, 1950; LANGHAM, 1954), though again species variations are noticeable (MATSUMOTO and KUDO, 1960). The energy produced by glucose metabolism in the endothelium is much greater than, that required to drive the endothelial hydration control mechanisms, and since synthetic activity of these cells is small, that required for cell maintenance (HARRIS, 1957; MAURICE, 1951, 1969; MAURICE and RILEY, 1970). The function of the excess energy production is unknown. Oxygen supply to the endothelium to drive the TCA cycle and HMS pathways is obtained from the Aqueous Humour, as shown by FATT et al., (1974) and RILEY (1969 (a)).

In an attempt to identify the relationship between metabolism and the pump mechanisms in the cornea a number of studies have been performed, where the normal corneal balance is altered either by the use of inhibitors or by selective removal of metabolites or substrates. The effects of such treatments vary depending on whether the cornea is treated <u>in vitro</u> or <u>in vivo</u>.

Dinitrophenol administration has no effect on corneal hydration suggesting that oxidative phosphorylation is not essential for maintenance of the pump mechanism. Iodoacetate added to the epithelium is similarly ineffective (LANGHAM and TAYLOR, 1956).

Sodium removal and ouabain inhibition of sodium-potassium dependent ATPase leads to corneal swelling and a failure to show a temperature reversal effect (DIKSTEIN, 1971, 1973; LAMBERT and DONN, 1964; BROWN and HEDBYS, 1965; LANGHAM and KOSTELNIK, 1965; KAYE and DONN, 1965; ROGERS, 1968).

Incubation of the cornea in vitro with a bathing solution similar to the Aqueous Humour (WACHTYL and KINSEY, 1958), shows that when Glutathione is present in the incubation medium on the endothelial surface, glucose is the only substrate required for normal maintenance of corneal hydration (DIKSTEIN, 1971; DIKSTEIN and MAURICE, 1972; ANDERSON et al., 1974).

DAVSON (1955) has shown that, in the intact cornea, temperature reversal did not occur under conditions of anoxia. In the isolated cornea, anoxia produced an increase in corneal hydration (SCHWARTZ et al., 1954). Endothelial anoxia produces an increase in corneal hydration even when the epithelium is bathed in gas of a high oxygen tension. Similarly epithelial anoxia increases hydration in these isolated preparations (TAKAHASHI, 1967). The mechanism by which epithelial anoxia affects the hydration is unsure. It does not affect the oxygen supply to the endothelium, but it may induce epithelial disturbances leading to increased water permeability,

coupled with the reduced energy production by inhibition of the aerobic pathways.

The process maintaining normal corneal hydration shows a number of features:-

(a) It is dependent on energy derived from aerobic metabolism, glycolysis alone is insufficient to satisfy the metabolic demands of the tissue.

(b) There must be a constant oxygen supply to the endothelium.

(c) There must be an oxygen supply to the epithelium, however epithelial oxygen is not used to supply the endothelium.

(d) Glutathione must be present in the epithelium (WHIKEHART, 1975; RILEY and YATES, 1977). The role of oxygen in the epithelium may be to produce glutathione for use as an oxidising agent in the endothelium.

(e) It requires the presence of sodium ions.

(f) Mitochondrial ATP is not essential to the metabolic pump, but glycolysis must take place.

1.9 INTERFERENCE WITH SUBSTRATE SUPPLY

(a) Glucose

Although glycogen and lactate can be utilised, glucose is the main carbohydrate energy source employed in maintenance of corneal hydration

(HERRMANN and HICKMAN, 1948 (b); RILEY, 1969 (a)). Since glycogen and lactate are continually produced, this implies that a constant supply of glucose to the cornea is maintained. Glycogen is produced by conversion of glucose in a gluconeogenic pathway (STEVENS-ANDREWS, 1976 (b)). Lactic acid is produced as an end product of both aerobic and anaerobic glycolysis and is pooled in all the corneal tissues until it is required. It can then be converted to pyruvate and fed into the TCA cycle.

If the normal glucose supply is stopped either by removing the glucose component in the incubation medium in vitro, or by destroying the ciliary blood vessels in vivo, the concentration of glucose in the cornea falls (TURSS and SCHEBITZ, 1972). As a consequence of the fall in glucose concentration, glycogen and lactic acid carbohydrate reserves fall and the energy supply to the hydration control mechanisms will eventually fall (HERRMANN and HICKMAN, 1948 (b); LANGHAM, 1954; DE ROETTH, 1951; MISHIMA and KUDO, 1967). Removal of the glucose supply produces a fall in the ATP concentration of the cornea and an increase in the lactic acid production rate, although the reason for the latter phenomena is not completely understood (TURSS et al., 1970; SCHÜTTE et al., 1972 (a), (b)).

(b) Oxygen

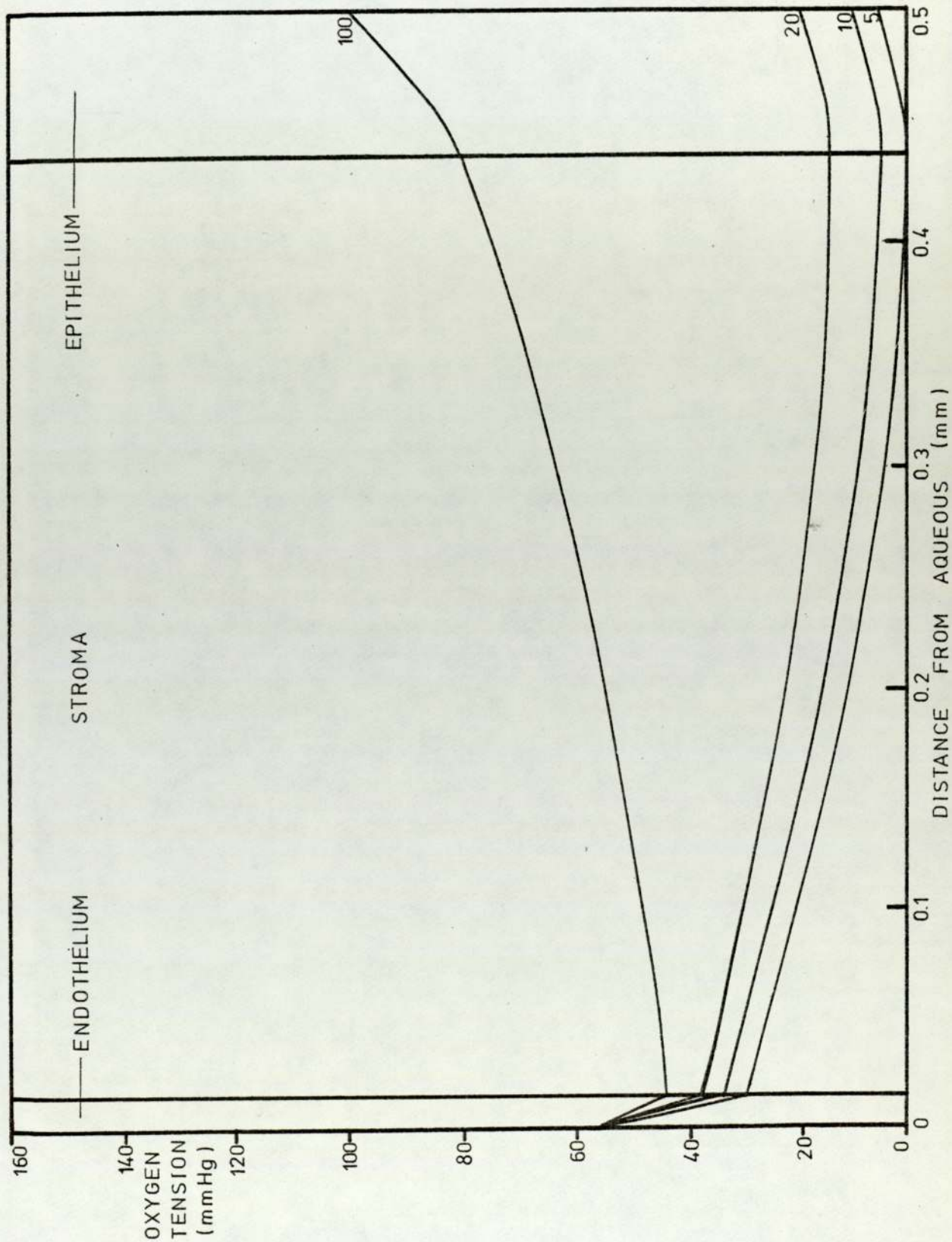
Maintenance of cellular integrity and deturgescence in the cornea requires a continual supply of carbohydrate and of oxygen (DE ROETTH, 1950; DUANE, 1949 (b); SMELSER, 1952; SCHWARTZ et al., 1954; DAVSON, 1955; HARRIS and NORDQUIST, 1955; LANGHAM and TAYLOR, 1956;

HARRIS, 1957; RILEY, 1969 (a)). Oxygen is used in the cornea as a substrate for the energy producing mechanisms present in the limiting layers of the cornea.

Removal of the oxygen supply to the cornea has a multiplicity of effects, the precise effect depending on the site of anoxia. A profile of oxygen distribution in the cornea where epithelial anoxia is induced by the passage of nitrogen gas over the epithelium is shown by FATT (1968 (a)) and re-calculated by FATT et al., (1974), Figure 6. All the effects of anoxia are a direct consequence of the interference with aerobic energy producing mechanisms.

When anoxia is induced in the cornea by reducing the oxygen supply to the epithelium and endothelium, the oxygen supply necessary to maintain the aerobic phase of corneal respiration, the HMS and TCA pathways and oxidative phosphorylation, is removed. As a consequence the only energy producing mechanism that can function is anaerobic glycolysis, the EMP pathway (MAURICE and RILEY, 1970; RILEY, 1969 (a); THOFT and FRIEND, 1972). There is some compensation by the cornea in anoxia demonstrable by a significant increase in the rate of glycolysis. This is known as the Pasteur effect (DE ROETTH, 1950, 1951; HERRMANN and HICKMAN, 1948 (a), (b); FISCHER, 1940). This increased rate of glycolysis must supply all the energy requirements of the cornea under anoxic conditions. As a result of the increased rate of glycolysis the rate of glucose consumption increases (THOFT and FRIEND, 1972; MAURICE, 1969). The rate of glucose supply to the epithelium is however limited and may possibly be reduced in anoxia, since

Figure 6 Calculated oxygen tension profile for rabbit cornea with varying oxygen tension at the epithelial surface. After FATT et al., (1974).



glucose supply itself is an energy requiring process (HALE and MAURICE, 1969). In anoxia the glucose concentration of the cornea, in particular the epithelium, falls rapidly. Eventually a point is reached where insufficient glucose is available to supply the metabolic requirements, the energy reserves of the cornea must then be used. Glycogen present only in the epithelium is mobilised to Glucose - 6 - Phosphate and enters the EMP to be converted to lactic acid (HERRMANN and HICKMAN, 1948 (b); SMELSER, 1952; SMELSER and OZANICS, 1952, 1953; SMELSER and CHEN, 1955; LANGHAM, 1952, 1954, 1960; UNIACKE and HILL, 1972). Lactic acid cannot be used as an energy reserve in anoxia since it can only be metabolised by the aerobic TCA cycle.

In anoxia therefore there is an increase in the rate of glycolysis and a decrease in glucose concentration and glycogen store. The lactic acid pool of the cornea is unable to be metabolised, therefore the size of the lactic acid pool increases due to the addition of lactic acid produced by the increased rate of glycolysis. Associated with the increase in lactic acid production is an overall increase in the lactate/pyruvate ratio due to the switch of the equilibrium of lactate to pyruvate in favour of lactic acid production (HERRMANN and HICKMAN, 1948 (b); DE ROETTH, 1950, 1951; LANGHAM, 1952, 1954; REIM et al., 1966; REIM and LICHTE, 1965; RILEY, 1969 (a)). Oxygen is also required to maintain oxidative phosphorylation, the consequence of oxygen lack can be seen at this level by an observable decrease in the ATP/ ADP ratio (REIM et al., 1966; REIM and LICHTE, 1965).

Oxygen is necessary for the normal functioning of the corneal hydration maintenance mechanisms, by removing this oxygen supply the ability of the cornea to maintain its normal hydration is endangered. Glycolysis is capable of producing approximately 35% of the normal energy production of the cornea (LANGHAM, 1960), however under conditions of anoxia the cornea swells therefore the energy released from glycolysis is insufficient to maintain the dehydrating mechanisms (PHILPOT, 1955; RILEY, 1969 (a); MANDELL and POLSE, 1969, 1971; POLSE and MANDELL, 1970 (a), (b); UNIACKE and HILL, 1972; UNIACKE et al., 1971, 1972). As discussed earlier the precise site of action of anoxic effects is uncertain, however the epithelium and endothelium are to some extent interdependent so any effect on one layer is likely to affect the other.

The enzymes involved with glucose metabolism in the cornea are also affected by anoxia. Parallel to the decrease in glycogen content and increase in hydration is a short term increase in LDH activity in all areas of the corneal epithelium. Following the increase is a slight decrease in activity in the basal zone of the epithelium (KING et al., 1971; UNIACKE et al., 1972). These authors suggest that the increase in activity reflects an increased LDH production to keep up with the increased rate of anaerobic glycolysis allowing rapid conversion of pyruvate to lactic acid and the resultant dehydrogenation of the Coenzyme. The reason for the fall in activity is not known, however it could possibly be a feedback inhibition by the high lactic acid concentrations on the LDH synthetic mechanisms. Moderate anoxia is seen to decrease SDH activity whereas severe anoxia increases its activity. SDH is one of the major enzymes of the TCA cycle

(RENGSTORFF and HILL, 1974 (a), (b)). The decreased activity is the result of interference with cellular metabolism, as has been shown by other authors (KAUFMAN and HILL, 1960). The increased level is seen as an accumulation of SDH as shown by other tissues in similar circumstances (WEIMAR and HARAGUCHI, 1966).

When the anoxic stress is removed the cornea rapidly returns to normal, the glycogen level increases as does the glucose concentration. Corneal hydration returns to normal and the lactic acid concentration in the cornea falls quickly to the pre-stress levels. The activity of epithelial LDH also returns quickly to normal (LOWTHER and HILL, 1973, 1974; DIKSTEIN and MAURICE, 1972). These factors show that within certain limits the changes induced in the cornea by anoxia are fully reversible.

One aspect not yet considered is that of carbon dioxide production. The end product of aerobic metabolism in all layers of the cornea is carbon dioxide. Production of carbon dioxide can be at two points in the aerobic metabolic pathway:-

(a) From the aerobic glycolysis of glucose by the HMS pathway.

(b) As the end product of the TCA cycle. (KINOSHITA, 1962).

Some carbon dioxide produced by metabolism of glucose dissolves in the tissue fluid, however most of the carbon dioxide is lost to the corneal bathing solutions, tears and Aqueous Humour. The carbon dioxide tension in air at standard temperature and pressure (STP) is 0.3 mmHg, however the carbon dioxide tension in the Aqueous Humour is approximately the same as that of the blood, between 55 and 60 mmHg (DAVSON, 1962; CANTAROW and TRUMPER, 1945). A diffusion gradient

exists across the cornea, the direction of movement being from Aqueous Humour to tears. The resistance of corneal tissue to carbon dioxide is much less than that shown to oxygen, therefore carbon dioxide can move freely in the cornea. Carbon dioxide produced in the cornea by metabolism will not move against the concentration gradient and therefore passes into the tears.

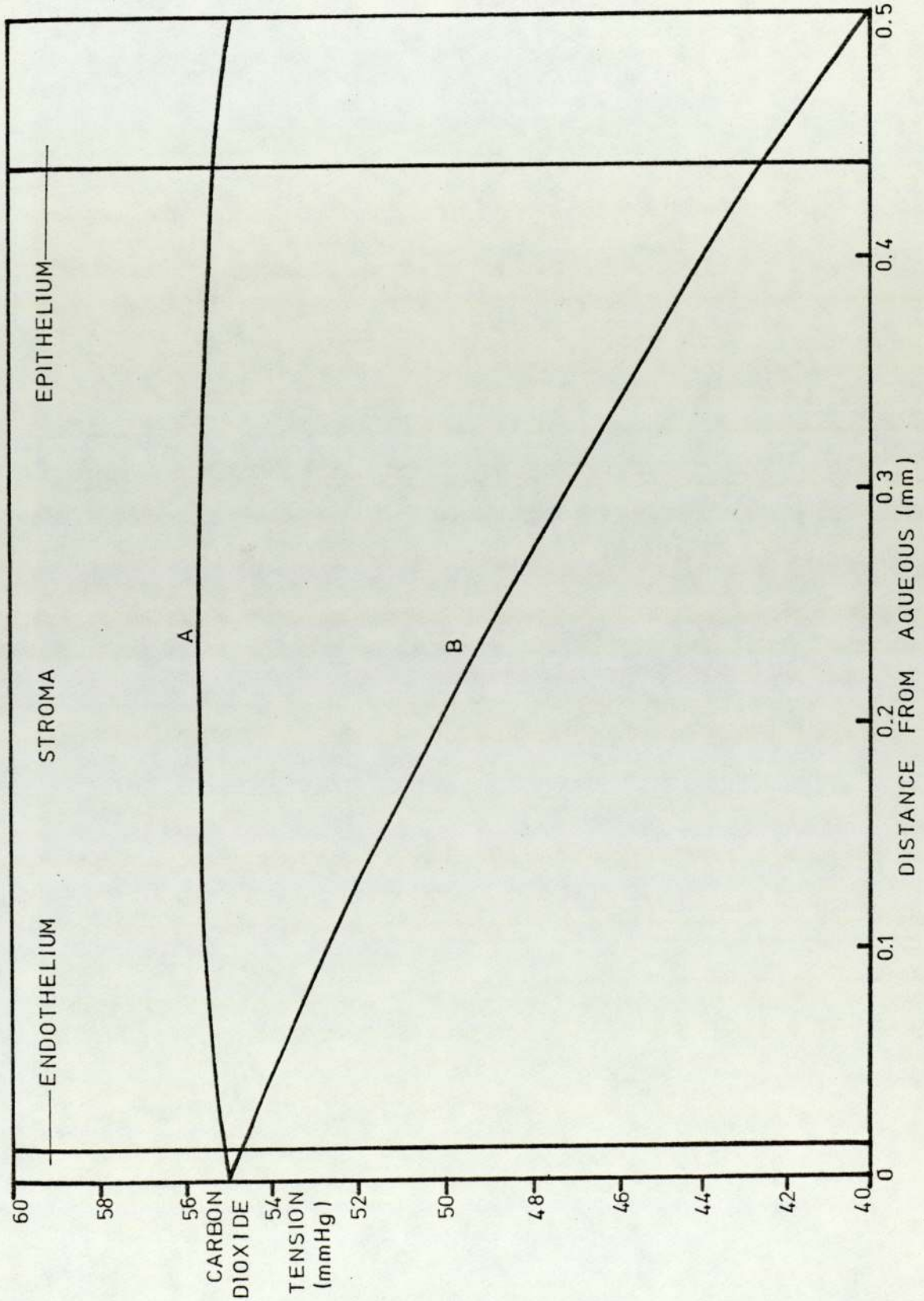
The size of the carbon dioxide flux at the epithelium has been measured, using gasometric techniques and carbon dioxide electrodes, and found to be between 20 and 30 microlitres of carbon dioxide per hour ($\mu l\ CO_2\ hr^{-1}$) (REDSLOB and TREMBLAY, 1933; FATT et al., 1964). HABERICH and DENNHARDT (1966) showed the production of carbon dioxide and consumption of oxygen to be approximately equal, confirming the respiratory quotient of one measured by DE ROETTH (1950). FATT and BEIBER (1968) calculated a profile of carbon dioxide tension in the cornea, Figure 7. These authors considered the carbon dioxide efflux rate to be equivalent to the rate of oxygen consumption, and diffusion and solubility coefficients of carbon dioxide in corneal tissue to be the same as in water. The calculated profile for the open eye confirmed the expected carbon dioxide flux from Aqueous Humour to the tears.

The measured carbon dioxide flux at the epithelial surface has two components:-

(a) The carbon dioxide moving from Aqueous Humour to tears down the diffusion gradient.

(b) The carbon dioxide produced from glucose metabolism.

Figure 7 Calculated Carbon Dioxide profile for the rabbit cornea in the open eye situation (B) and closed eye (A). After FATT and BEIBER (1968).



The Aqueous Humour component is however much greater than the metabolic component (FATT and BEIBER, 1968).

In the closed eye the situation is changed. The carbon dioxide tension at the epithelial surface is equal to that of the blood since the blood vessels of the palpebral conjunctiva are in close proximity to the epithelial surface. The carbon dioxide tension within the cornea is equal to that at the boundaries, reflecting the low resistance offered to carbon dioxide diffusion by the corneal tissues (FATT and BEIBER, 1968), Figure 7. When the local carbon dioxide tension in the cornea rises above that of the boundary layers, then carbon dioxide moves to the Aqueous Humour and tears equally.

With nitrogen gas at the epithelial surface the profile is almost identical to the open eye. The only difference is a lower carbon dioxide tension at the epithelium which increases the gradient from Aqueous Humour to tears, since the carbon dioxide tension of the Aqueous Humour remains almost constant (FATT, 1968 (a)). In anoxia the aerobic pathways are unable to function, carbon dioxide is no longer produced within the cornea, and therefore the corneal component of the epithelial flux diminishes.

1.10 CONTACT LENS EFFECTS

In the *in vivo* eye interference with normal corneal function, without interfering with corneal integrity, can only be by factors acting at the epithelial surface. In general the only major interference with corneal function, that acts at the epithelial surface, is the wearing

of contact lenses. Contact lenses can act on the cornea in many ways; by mechanical effects or by interference with substrate supply and removal of wastes from the cornea (BURNS and ROBERTS, 1970).

Contact Lenses and Oxygen Supply

The most important effect of contact lens wear on normal corneal function is through their interference with oxygen supply to the cornea.

In the normal cornea the oxygen tension of the Aqueous Humour is 55 mmHg and the atmosphere at the epithelial surface 155 mmHg. When the eye is closed the oxygen tension at the epithelial surface is reduced to that of the blood contained in the vessels of the palpebral conjunctiva. Oxygen profiles for both these conditions have been calculated by FATT et al., (1974). Under open or closed eye conditions no significant change in the thickness or hydration of the cornea is seen. Therefore it may be presumed that the cornea is receiving an adequate supply of oxygen. If the cornea is made anoxic by passing nitrogen gas over the epithelium, the cornea swells and hydration increases, therefore insufficient oxygen is available. These experiments however only provide information on the absolute oxygen requirements of the cornea.

The contact lens by a purely physical effect presents a barrier to normal oxygen supply to the cornea. Modifications of lens design and materials have attempted to minimise this effect. However, even the most acceptable lenses have a degree of interference. A knowledge of the precise oxygen requirements of the cornea for normal corneal functioning is required, to provide a minimum value of oxygen required

at the corneal surface, below which the cornea enters respiratory distress. Knowing the minimum oxygen requirement the usefulness of a particular contact lens can be predicted.

Reduction of the oxygen tension at the epithelial surface by gas mixtures of known oxygen tension shows that the ability of the cornea to consume oxygen at its normal rate falls when a certain ambient oxygen tension is reached. If this is the case, then reduction of oxygen tension not only reduces the quantity of oxygen available to the epithelium, but also reduces the rate at which the epithelium can take up the oxygen. Such an occurrence is important in terms of contact lens wear since the lens reduces the oxygen tension at the epithelial surface, and may even reduce it below the point of diminished consumption whereby the anoxic effects immediately become more severe.

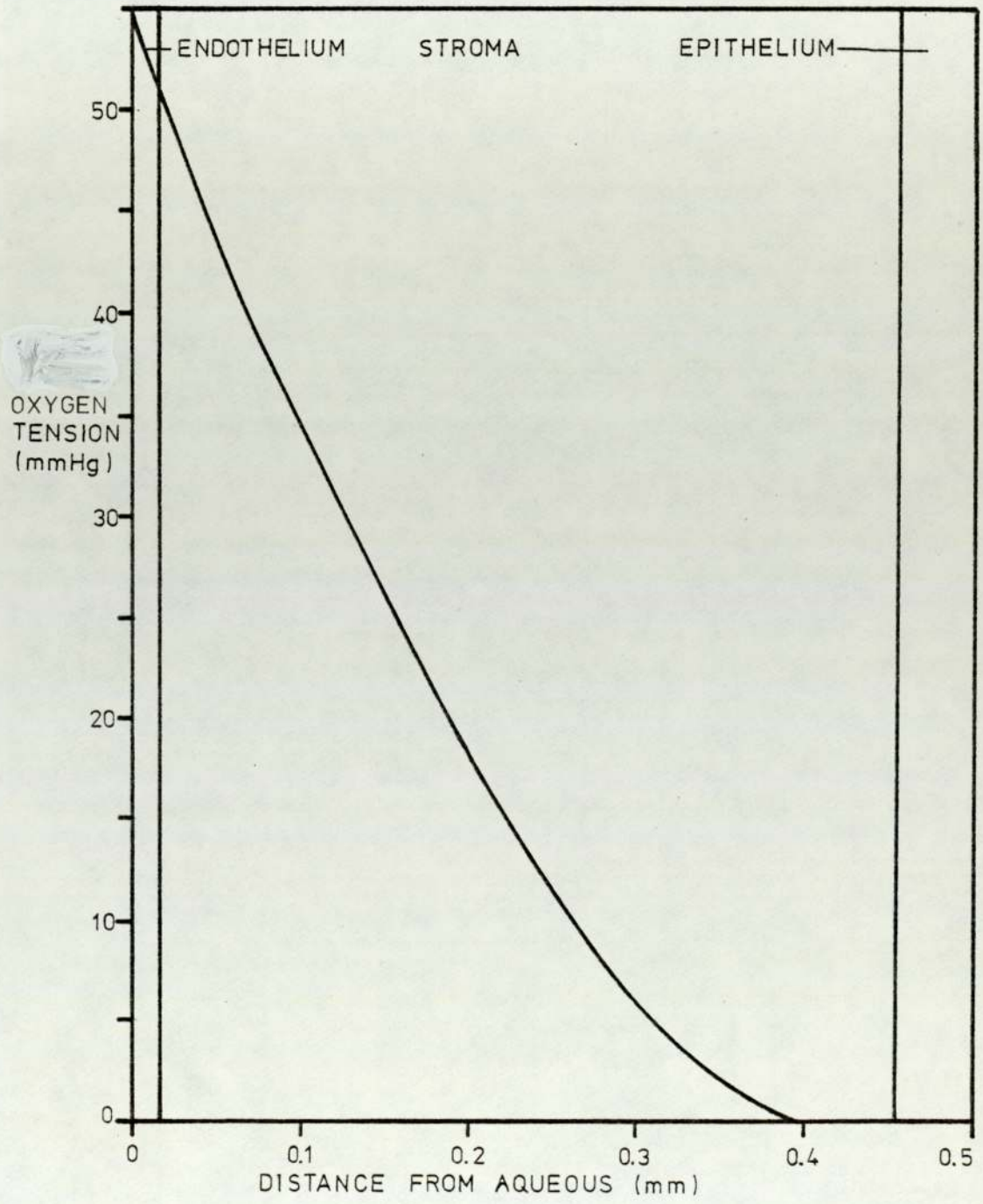
The point at which the consumption rate of the epithelium falls and anoxic effects are seen in the cornea is known as the critical oxygen tension. This has been measured by a number of authors using different methods. Thickness and hydration of the cornea show a linear proportionality (HEDBYS and MISHIMA, 1966). By monitoring corneal thickness with varying oxygen tensions at the epithelial surface, the minimum oxygen requirement for maintenance of normal corneal hydration is found to be 11 to 19 mmHg (POLSE and MANDELL, 1970 (b); UNIACKE et al., 1971; CARNEY, 1975). Below this critical oxygen tension the cornea hydrates and swells. By observing the level of SDH in the corneal epithelium while exposing the epithelial surface to reduced oxygen tensions for specific periods of time, a similar minimum oxygen tension requirement of 19 mmHg is found (RENGSTORFF et al., 1974 (a), (b); HILL et al., 1974).

The effect of placing an oxygen impermeable barrier over the cornea has been measured (HILL and FATT, 1964). In this situation the only source of oxygen supply is the stroma and Aqueous Humour. Under these conditions only a small supply of oxygen is available, the supply being sufficient to satisfy the corneal requirement for only 20 seconds. HILL and FATT (1964) also showed that the cornea isolated from the atmosphere cannot continue to respire at its normal rate, confirming the results of FATT et al., (1974) and TAKAHASHI et al., (1967).

Observation of the rate of oxygen consumption following the removal of an oxygen impermeable barrier from the corneal surface shows that the cornea is capable of building up an oxygen debt. The oxygen consumption increases on removal of the obstruction. A similar oxygen debt is demonstrated in the work of HABERICH and DENNHARDT (1966).

Using a treatment similar to that for the open eye, closed eye and anoxic eye, the oxygen tension profile across the cornea, when the atmospheric oxygen tension is removed by the wearing of an oxygen impermeable contact lens, has been calculated (FATT, 1968 (a); FATT et al., 1974). The profile obtained, Figure 8, shows that the oxygen tension at the epithelial surface falls to near zero, this profile is almost identical to that of eye exposed to nitrogen gas. In vivo measurements of the oxygen tensions in the stroma and Aqueous Humour, when the corneal epithelium is exposed to various gas mixtures and oxygen impermeable contact lenses, confirm the profiles of FATT (1968 (a)) and FATT et al., (1974), (HAMANO et al., 1972; BARR and SILVER, 1973).

Figure 8 Calculated oxygen tension profile for the rabbit cornea with an oxygen impermeable contact lens in place. After FATT (1968 (a)).



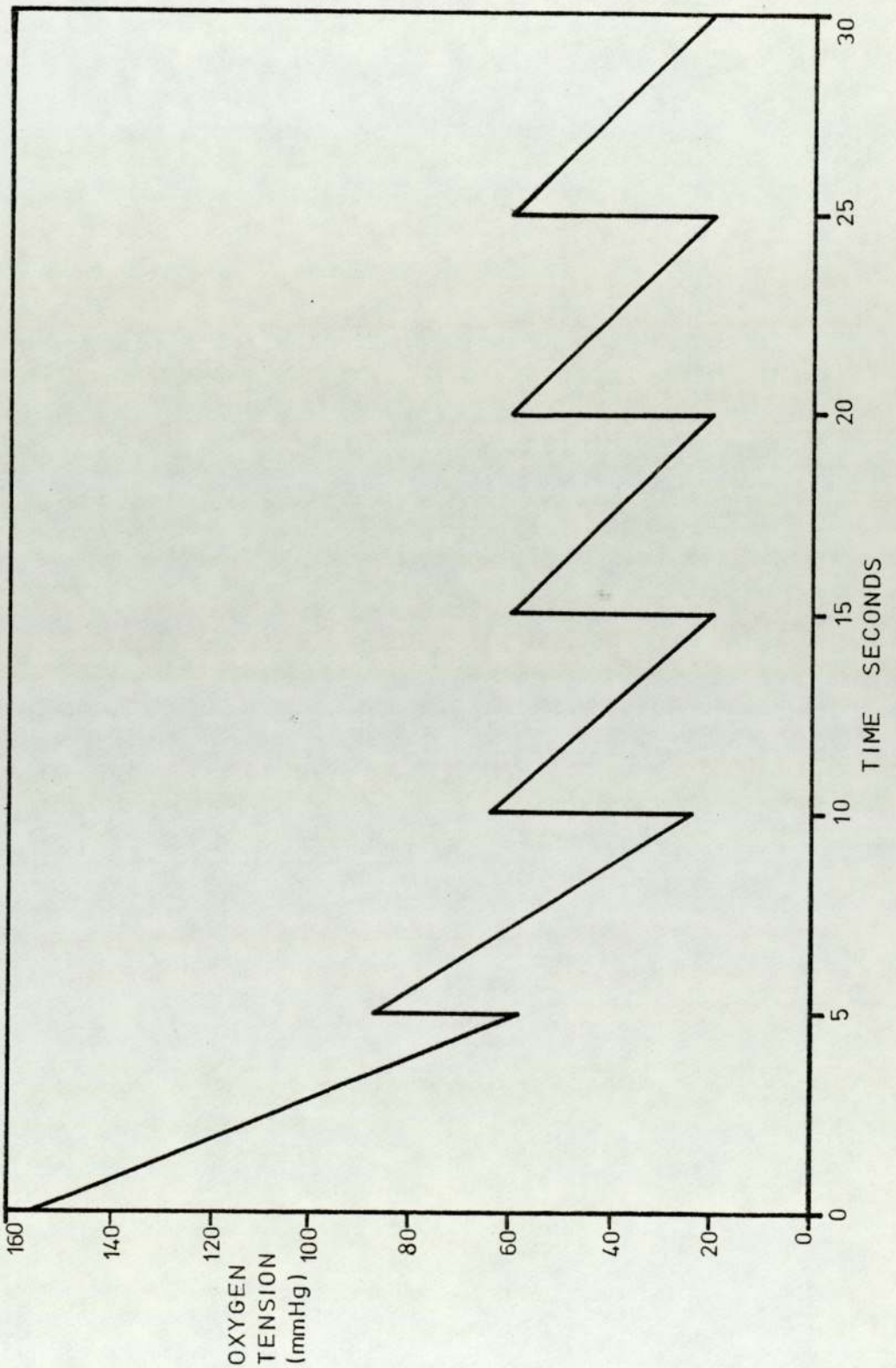
When a contact lens is fitted tightly to the cornea, the only path for oxygen supply to the epithelium must be through the lens itself. The oxygen permeability of the lens and "fit" will determine the success of a particular lens (MILLER, 1973; HILL and CUCKLANZ, 1967). The hard PMMA lens oxygen permeability is very small, the flux of oxygen through the lens is small and insufficient to supply the minimum oxygen requirement of the cornea (FATT et al., 1974; FATT and LIN, 1977). The oxygen tension under a hard contact lens will eventually approach zero as the oxygen in the tears, trapped between lens and epithelium, is consumed. The Aqueous Humour and stroma are incapable of supplying the oxygen needs of the epithelium and another source of oxygen supply must be found (HILL and CUCKLANZ, 1967; RUBEN, 1967; JAUREGUI and FATT, 1971; HILL et al., 1973).

Hard contact lenses trap a thin layer of tears between the back surface of the lens and the epithelial surface. The oxygen dissolved in this pool of tears is all that is available to the epithelium. The volume of the trapped tear reservoir is very small and as a consequence the oxygen contained is sufficient to maintain the epithelial supply for only a matter of seconds (FATT and HILL, 1970; HILL and AUGSBURGER, 1971). Modifications to hard contact lens design so that they rock on the cornea allows an exchange of tears at each blink. The rocking motion of the lens at each blink acts as a tear pump (CUCKLANZ and HILL, 1969; FATT, 1969; FATT and HILL, 1970; CARTER, 1972; BERGER, 1974). Tears pumped under the lens by the rocking motion contain oxygen, fresh oxygen is thus supplied at each blink. Such oxygen replacement mechanisms allow hard lenses to be worn for considerable

periods (JAUREGUI and FATT, 1971). Mathematical predictions of the movement of tears at blinks confirm the exchange of tears and the replenishment of tear oxygen, and that the oxygen supplied in this manner is sufficient to satisfy the minimum oxygen requirement of the epithelium (CUCKLANZ and HILL, 1969; FATT and HILL, 1970; BERGER, 1974), Figure 9. Measurement of oxygen tensions in the trapped tear film can be measured directly by using, small polarographic oxygen sensors (FATT and HILL, 1970) or the oxygen debt phenomena (JAUREGUI and FATT, 1971; HILL et al., 1972). These in vivo measurements confirm that in a well fitted hard contact lens, where efficient tear exchange is taking place, sufficient oxygen is supplied to the corneal epithelium for maintenance of normal corneal function.

The supply of oxygen to the corneal epithelium with the more recently developed hydrophilic soft contact lenses is totally different to that for hard lenses. Soft lenses when placed on the eye conform closely to the contours of the epithelial surface (HILL and CUCKLANZ, 1967; FATT, 1972 (a), (b)). Such conformity eliminates almost all mechanical exchange of tears at the lens edge induced by blinking. The oxygen requirement of the corneal epithelium must be met by the flux of oxygen moving through the lens itself, and must satisfy the minimum oxygen requirement of 19 mmHg. The oxygen tension at the corneal surface beneath an oxygen-permeable contact lens is seen to be a function of the oxygen transmissibility and lens thickness (FATT et al., 1969; FATT and ST. HELEN, 1971). To be successful the soft contact lens must have a high oxygen transmissibility and a minimum thickness.

Figure 9 Exhaustion course of oxygen from the reservoir of a hypothetical contact lens system on successive blink cycles. After BERGER (1974).



The oxygen transmissibility is known for a number of hydrophilic soft lens materials (FATT and ST. HELEN, 1971; FATT et al., 1969; TAKAHASHI et al., 1967; FATT et al., 1974). Using this information FATT et al., (1969) have calculated the oxygen profiles for a cornea with an oxygen permeable soft contact lens in place. When fitted tightly the profiles show that insufficient oxygen is passing through the lens to maintain the oxygen requirement. Soft lenses must as a consequence be fitted loose to allow some mechanical oxygen supply for movement of tears beneath the lens. Insufficient oxygen supply through soft contact lenses has been shown by other authors using different techniques (HILL and CUCKLANZ, 1967; HILL and AUGSBURGER, 1971; PETERSON and FATT, 1973).

When the eye is closed the oxygen flux through the soft contact lens is reduced still further since the oxygen gradient across the lens is reduced. The oxygen tension on the outer surface falls from 155 mmHg to 55 mmHg. The oxygen tension at the epithelial surface beneath the soft contact lens is reduced still further and normal corneal function can no longer be maintained.

More recently silicon rubber has found an application in the manufacture of contact lenses. Silicon rubber has a very high oxygen permeability compared to hydrophilic soft lens polymers. The movement of oxygen across a silicon rubber lens when placed on a cornea is greatly increased. The likelihood of successful wear is increased accordingly. Measurement of the epithelial surface oxygen tension under silicon rubber lenses shows that an adequate supply of oxygen can pass through the lens (HILL and AUGSBURGER, 1971; HILL and CUCKLANZ, 1967; HILL, 1974; FATT et al., 1969). Such is the oxygen permeability of this

material, that it is theoretically possible to supply adequate oxygen to the epithelium in the closed eye situation. Difficulties with the mechanical properties of silicon rubber restrict their use at present.

The success of contact lens wear depends on an adequate oxygen supply to the corneal epithelium. The various types of contact lens material have varying oxygen permeabilities, from the almost impermeable PMMA lenses to highly permeable silicon lenses. The variation in oxygen permeability is reflected in the varying levels of interference with normal corneal function. Removing the oxygen supply completely, induces many changes in corneal metabolism and function as described in section 1.8. Contact lenses by way of their interference similarly affect normal corneal metabolism and function, the precise effect varying with the particular type of lens, fit, size, oxygen transmissibility and wearing time (MILLER, 1973).

Tightly fitted hard contact lenses have the most severe effect. The changes parallel to those of induced total anoxia:-

(a) Increased lactic acid concentration in the epithelium, stroma, Aqueous Humour and tears (SMELSER and CHEN, 1955; MORLEY and McCULLOCH, 1961; PRAUS et al., 1969; DREIFUS et al., 1969; BURNS et al., 1971; RUBEN and CARRUTHERS, 1972; KILP, 1974).

(b) Decrease in the ATP/ADP ratio and an increase in the lactate/pyruvate ratio (THOFT and FRIEND, 1975).

(c) Decreased epithelial glucose and glycogen concentration, increased LDH activity and a biphasic SDH response (KISHIDA and OTORI, 1971; OTORI and KISHIDA, 1973).

Soft contact lenses show similar effects on corneal tissues as those shown by hard contact lenses. The degree of interference with corneal function is however diminished. The degree of interference is reduced still further by silicon contact lenses (DREIFUS et al., 1969; DREIFUS and PRAUS, 1970; PRAUS et al., 1969; HAMANO et al., 1972; AUGSBURGER et al., 1972; KILP, 1974; JEPPE and HILL, 1975).

Carbon dioxide efflux from the cornea in contact lens wear is restricted due to the barrier effect of the lens. However since less is being produced under conditions of induced anoxia the likelihood of acidosis due to dissolved carbon dioxide in the tears is unlikely. As the carbon dioxide in the cornea reaches that in the cornea of the Aqueous Humour the flux of carbon dioxide from the Aqueous Humour to the tears is reduced, reaching zero as equilibrium is reached.

As a result of the changes in corneal metabolites and enzyme activities caused by the contact lens-induced anoxia, increases in hydration and thickness of the cornea take place. Again the degree of change is dependent on the level of anoxia induced by the contact lens at the epithelial surface (SMELSER, 1952; SMELSER and CHEN, 1955; KINSEY, 1952; MANDELL et al., 1970; FARRIS et al., 1971; POLSE and MANDELL, 1971; FARRIS and DONN, 1972; UNIACKE, 1971; POLSE et al., 1975; MANDELL, 1976). Changes in the hydration of the cornea and corneal metabolites are rapidly reversible on cessation of contact lens wear (POLSE, 1972).

A number of studies monitoring changes induced by the wearing of contact lenses have concentrated on changes in the non-metabolic environment of the cornea. Such methods are of little use in the study of contact lens effects on the cornea where the aim is to gain a better understanding of corneal metabolic activity.

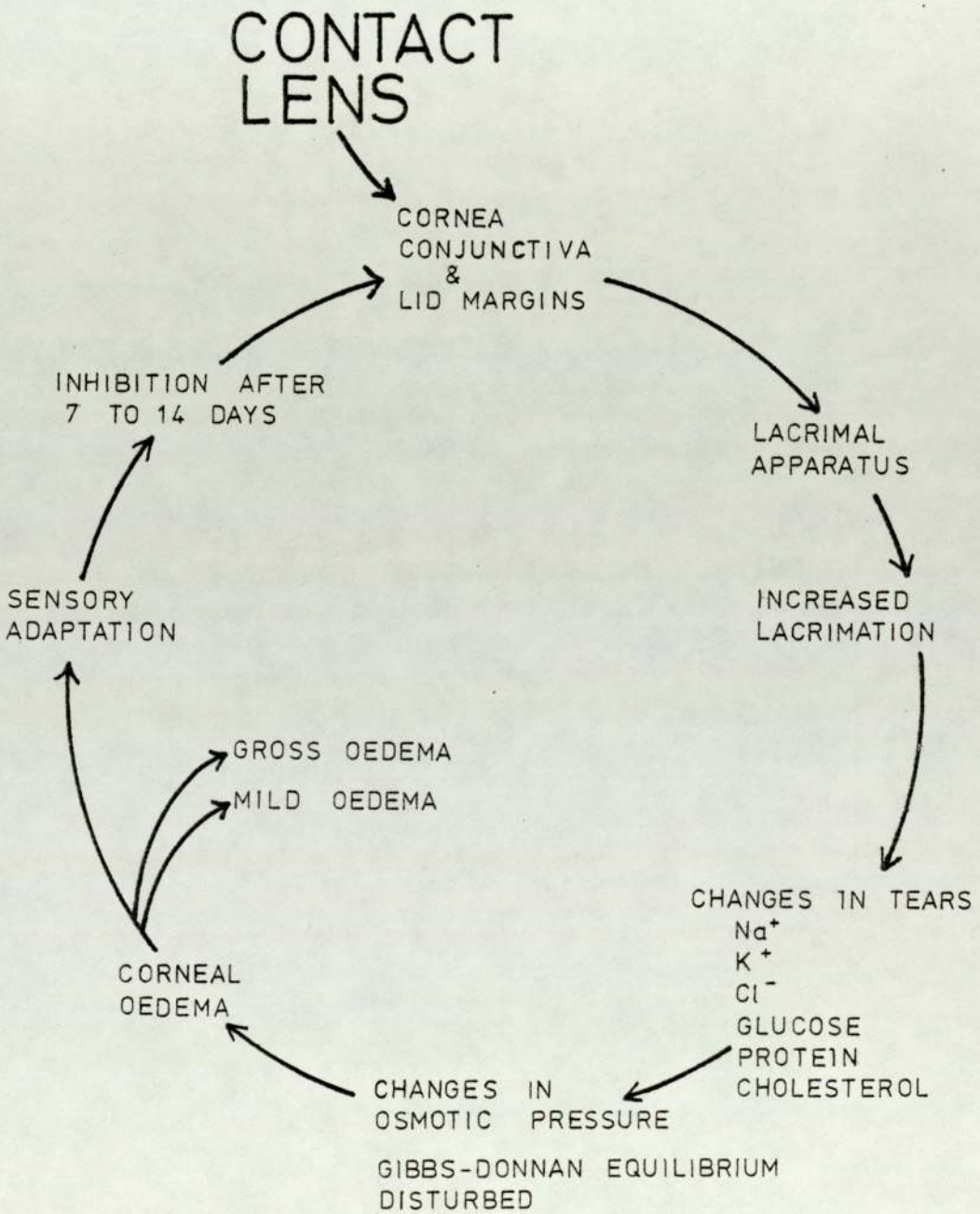
A fall in tear protein concentration is seen in contact lens wear (BALIK and KUBAT, 1968; HILL and UNIACKE, 1969; CALLENDER and MORRISON, 1974). A similar fall in tear cholesterol (HILL and YOUNG, 1973; YOUNG and HILL, 1973; HILL and WALLACE, 1973), and tear sodium ion concentration (LOWTHER et al., 1970) is seen in contact lens wear. These changes are attributable to an increased lacrimation. Increased lacrimination can also produce hypotonic tears (UNIACKE and HILL, 1970; TERRY and HILL, 1977), such hypotonic tears can increase corneal hydration to a limited extent (SMELSER, 1952; MANDELL et al., 1970; MILLER, 1973). Tear pH has been studied by CARNEY and HILL (1975, 1976), however only slight transient changes are observable. Figure 10.

1.11 COMMENT ON LITERATURE AND RESEARCH STRATEGY

The survey of previous literature has shown that there is a high level of understanding regarding corneal function, and of the metabolic requirements to maintain that function.

The effects of contact lens wear on normal corneal function are less well understood. The majority of information regarding metabolism and function, has been derived from experiments where either the tissue is maintained *in vitro* or removed from the animal following the experimental

Figure 10 Non-metabolic changes in the cornea associated with contact lens wear. After CALLENDER and MORRISON (1974).



period. Comparatively little information has been derived from *in vivo* experimentation.

The unique location of the cornea restricts *in vivo* observation of corneal metabolism and function to those events taking place at the anterior epithelial surface. *In vivo* observation has therefore concentrated on three main aspects; epithelial oxygen consumption rate, carbon dioxide efflux rate and tear lactic acid concentration.

Corneal oxygen consumption rate has been extensively used as a technique for determining the respiratory well being of the cornea, in the normal and contact lens wear situation, with some success. However there have been only a few studies of corneal carbon dioxide efflux rate and tear lactic acid concentration, and their use as indicators of the state of respiration and metabolism.

There is a similar lack of information regarding the use of these techniques in the clinical environment, as tools for the determination and monitoring of contact lens wear effects on the cornea.

To decrease the risk of damage to the cornea, by contact lenses; it is necessary to determine contact lens effects using *in vivo* techniques, and where possible to monitor and minimise these effects.

The present study attempts to improve the understanding of normal corneal metabolism and function by observing oxygen consumption rate, carbon dioxide efflux rate and tear lactic acid concentration. To use *in vivo* techniques, and where possible to modify the techniques such that their use in the clinical environment is practical.

SECTION 2

CORNEAL OXYGEN FLUX

Mean and diurnal Epithelial oxygen consumption rate in the *in vivo* rabbit and human cornea.

2.1 Introduction

The cornea similar to other tissues requires an adequate supply of oxygen (DUANE, 1949(b); DE ROETTH, 1950; LANGHAM and TAYLOR, 1956; FREEMAN, 1972; DIKSTEIN and MAURICE, 1972). Certain techniques can be employed to monitor corneal physiology and the effects of contact lenses on the normal functioning of the cornea. One such method is to study oxygen fluxes of the cornea.

A number of studies have successfully determined the rate of oxygen consumption by the cornea (FISCHER, 1940; DE ROETTH, 1950, 1951; LANGHAM, 1954, 1955), however all these experiments used excised corneal tissue. The work of HABERICH and DENNHART (1966) provides one possible exception in that the gasometric method of analysis required only the enucleation of the eye in order to create an effective seal between a collecting chamber and the corneal surface.

The problem of studying oxygen consumption *in vivo* could not be solved until the development of electrochemical methods of gas analysis. Electrometric methods for measuring oxygen consumption permit the direct determination of oxygen tension in both the liquid and gas phase, this offers certain advantages in terms of sensitivity, speed, accuracy and favourability of electrode environment (McKEOWN et al. 1967).

A membrane covering the oxygen sensor isolates the electrodes and electrolyte from the system under analysis. Continuous analysis of oxygen tension in the system being measured is possible and can be subjected to continuous electrical recording (CLARK, 1956, 1959).

Following their inception numerous applications have been found for polarographic oxygen sensors, in biology (BREZINA and ZUMAN, 1958; WEGENER and MØLLER, 1971; SOUTTER et al., 1975; FATT, 1976).

Many studies of ocular tissues have used polarographic sensors of various forms (KRAUSE and GOREN, 1956; DRECKAHN and LORENZEN, 1958; FATT, 1968(a); ERNEST, 1973). The work of HILL and FATT (1963(a)) is the first record of polarographic oxygen sensors used in the determination of corneal oxygen requirements. This work prompted others to use polarographic sensors to study oxygen consumption by the cornea, however these experiments differed in that the contact between sensor and cornea was direct (HILL and FATT, 1963(b); HILL and FATT, 1964; FARRIS et al., 1965; JAUREGUI and FATT, 1971, 1972).

The aim of this experiment is to use the membrane covered polarographic oxygen sensor system of CLARK (1956) as part of an apparatus for the determination of corneal oxygen consumption rate, suitable for in vivo use.

2.2 Theory of electrode operation

It is possible to reduce oxygen gas in a solution, by supplying electrons to a metal surface. In the polarographic system the metal

surface donating electrons to the oxygen in solution is referred to as the cathode. The reaction for the reduction of oxygen at the cathode surface can be given as:-

$O_2 + 2H_2O + 4\ e^- \longrightarrow 4\ OH^-$

(KOLTHOFF and JORDAN, 1952; LINGANE, 1961).

Thus as electrons are supplied to one of the electrodes constituting the polarographic sensor, reduction of oxygen to hydroxyl ions takes place. If a reference electrode is placed in the same solution then an electric current is produced. The anode in the Clark type sensor acts as the reference electrode. The anode accepts electrons from the solution and maintains a constant potential with respect to the solution.

The anode is silver and the reaction taking place at the anode can be given as:-

$Ag \longrightarrow Ag^+ + e^-$.

Usually the electrolyte of the sensor contains chloride ions and silver ions are deposited as the salt:-

$Ag^+ + Cl^- \longrightarrow AgCl$ (SEVERINGHAUS and BRADLEY, 1971).

However in this system silver is removed from solution by the methocel component of the electrolyte.

As the sensor is based on a polarographic principle, the meter used is an ammeter with a voltage bias source.

If the applied voltage is varied and current output noted, a plot of current output against applied potential produces a sigmoidal curve,

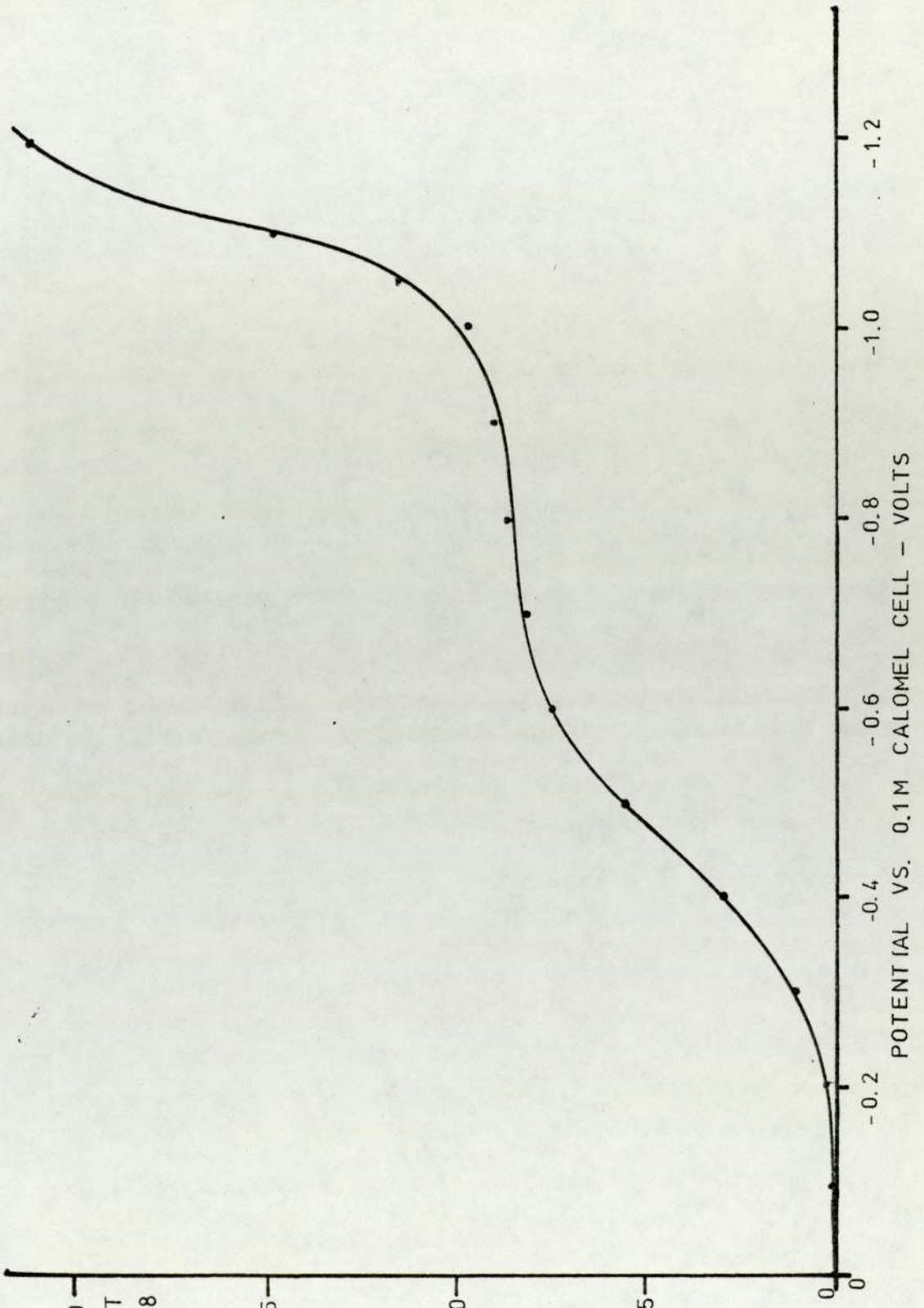
Figure 1. Above -0.2 volts (V.) the current increase is approximately proportional to applied potential difference (P.D.). At this point there is an adequate supply of oxygen available to the cathode and current is limited only by the potential applied. As the P.D. increases to -0.6V. a plateau occurs at which increasing the P.D. does not produce a significant increase in current. Above -1.0V. other electrode reactions occur and there is a steep increase in current output.

Oxygen diffusion to the sensor is maximal at a P.D. of -0.6V.. Whereas current output is almost independent of applied potential and wholly dependent on the rate of diffusion of oxygen to the sensor. At the plateau point the electrode is considered "polarised" and when used as a device for measuring oxygen tension in solution an electrode operating in this region is known as a polarographic sensor.

The cathode material is usually a noble metal, since side reactions in the solution near the cathode are undesirable. The sensor has two electrodes: a cathode acting as an electron donor, and an anode that accepts electrons from solution and compensates for variations in current obtained at a given oxygen tension. The anode acts as a half-cell reference electrode, independantly of the bathing solution, thus maintaining a constant voltage on the cathode.

Thus the sensor consists of cathode, anode, external polarising voltage and electrolyte. A problem arises in isolating anode, cathode and electrolyte from the solution under investigation. The system of CLARK (1956, 1959) solved this problem by placing the electrodes side by side and trapping a layer of electrolyte beneath an oxygen permeable

Figure 1 The current output, applied potential relationship for an oxygen microsensor. After Davies (1962).



membrane. This system with the modifications of FATT (1964 (a), (b); HILL and FATT, 1963 (b)) is that used in this study of corneal oxygen requirements.

The use of a membrane imposes a medium of low oxygen diffusion rate between the cathode and tissue solution. As a result covered sensors give a lower current output than a bare sensor for a given oxygen tension. The use of membranes also necessitates the effects of stirring and flow on current output to be taken into consideration.

Work by DE BOODT and KIRKHAM (1953) demonstrated that for an uncovered sensor, 95 per cent (%) of the decline in the diffusion gradient of oxygen between the medium and cathode occurred within six electrode diameters. This consideration also applies to covered electrodes if the oxygen diffusion coefficient, oxygen solubility coefficient of the membrane product, is equal to the sample medium. As membrane thickness increases and diffusion coefficient/oxygen solubility product falls electrode response time increases (FATT, 1964 (a), (b); STUCK et al., 1971). At a membrane thickness of approximately six electrode diameters where the diffusion coefficient/membrane oxygen solubility product is equal to that of the test solution the sensor will "see" only into the membrane and will be free of flow and stirring effects. Conversely if the membrane is less than six electrode diameters the sensor "sees" through the membrane into the test solution and is no longer free from flow and stirring effects. A membrane six electrode diameters thick with a diffusion coefficient/oxygen solubility coefficient product similar to the bathing solution is not feasible.

Designing the oxygen sensor for maximum stability with maximum response time requires a compromise between cathode size, membrane thickness and membrane material. Cathode size is important not only in determining membrane thickness but also because oxygen is actually consumed in the reduction reaction at the cathode surface, therefore the smaller the cathode the lower the oxygen consumption.

2.3 APPARATUS

Design Requirements

The *in vivo* measurement of oxygen consumption by the cornea using an oxygen sensor necessitates that certain features be included in the design:-

(a) Size - The electrode must fit into the palpebral aperture without inducing stress to the eye or patient.

(b) Electrical and chemical isolation - Safety demands that a scientific instrument be electrically isolated from the patient. The caustic nature of electrolytes requires that they be prevented from making contact with the eye.

(c) Response time - The time that the electrode is in contact with the eye must be kept to a minimum.

(d) Stability - Changes in current output other than those caused by changes in the oxygen tension of the bathing solution must be minimal.

Construction of small oxygen sensors demands specialist equipment and a high degree of technical skill. Thus the sensors had to be obtained from external sources. None of the instrument manufacturers

approached were able to provide a sensor with the stated requirements. A research team within the Department of Medical Physics at University College Hospital, London, under the direction of Dr D PARKER were however extremely generous in their offer to design and manufacture an oxygen sensor to the required specifications.

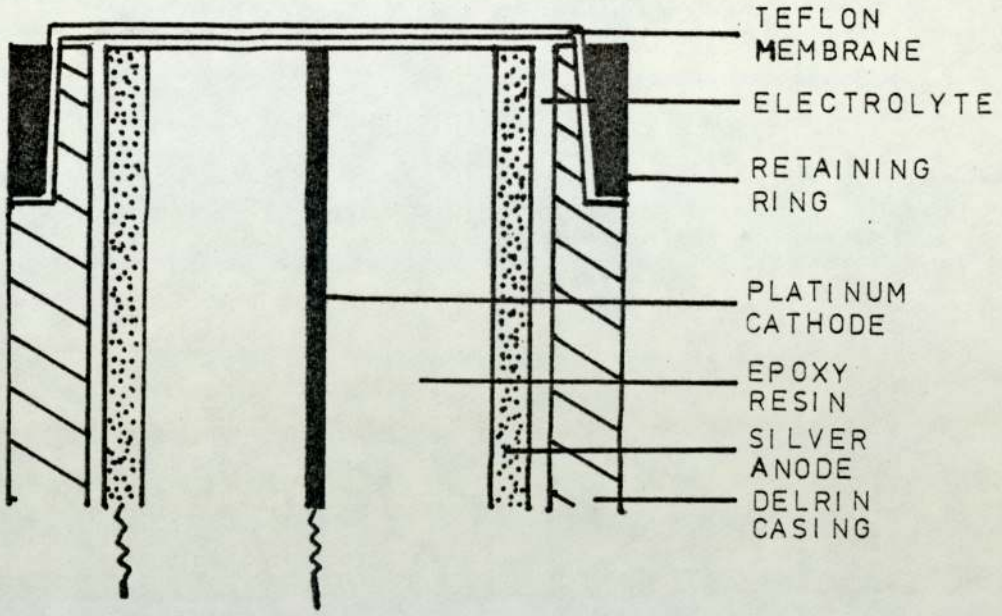
The sensor is of the Clark type with a platinum wire cathode, silver anode, Teflon (P.T.F.E.) membrane and an overall diameter of 5.0mm.

A 25 μ platinum wire is embedded in a specially formulated water resistant epoxy resin. Surrounding the resin is a ring of silver 2.8mm external diameter and 0.35mm thick. The electrolyte is a formulation of Instrumentation Laboratories (U.K.) Ltd., Catalogue Number 33013, containing 0.575 Molar (M) potassium hydroxide, in a water methocel mixture. A 12 μ Teflon membrane, D.606 (Radiometer, V.A. Howe Ltd.) is held in place over the electrodes with a stainless steel ring that fits tightly over a lathe cut nylon casing. The Teflon membrane acts as an oxygen reservoir and ensures that the change in current is controlled entirely by the change in the oxygen tension of the medium or tissue immediately below the cathode. Figure 2 shows a schematic representation of the sensor tip. Figure 3 shows a scale drawing of the electrode.

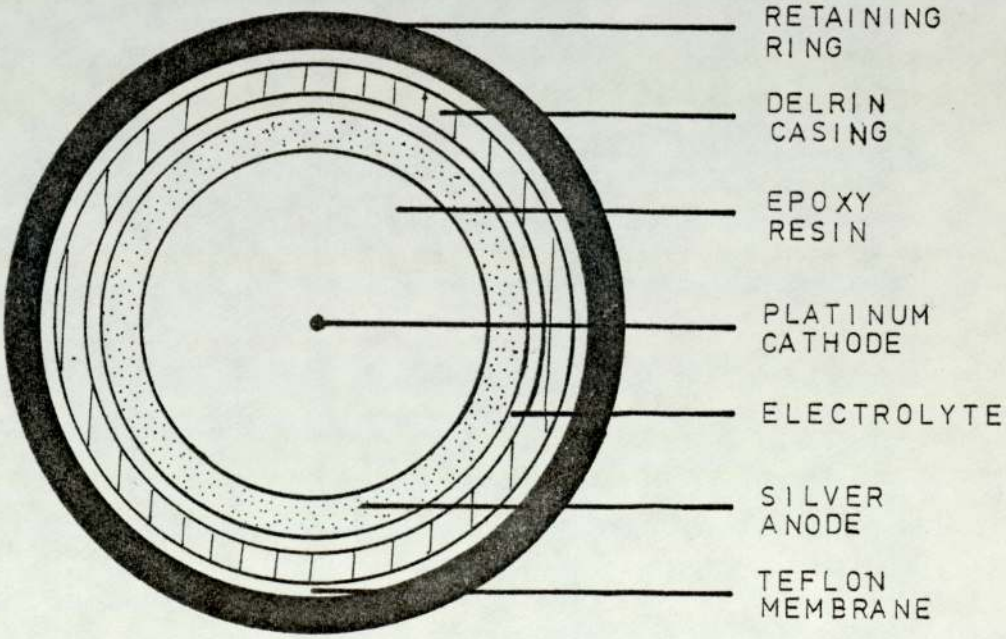
Peripheral Equipment

The sensor is connected to a high impedance amplifier ($Z_{IN} = 10^{14}\Omega$) generously loaned by Dr PARKER'S group. The amplifier carries controls for calibration, zero adjust and range selection. Attached to the amplifier is a constant voltage supply set at -0.8 V., this provides

Figure 2 A schematic of the Clark type oxygen sensor tip.

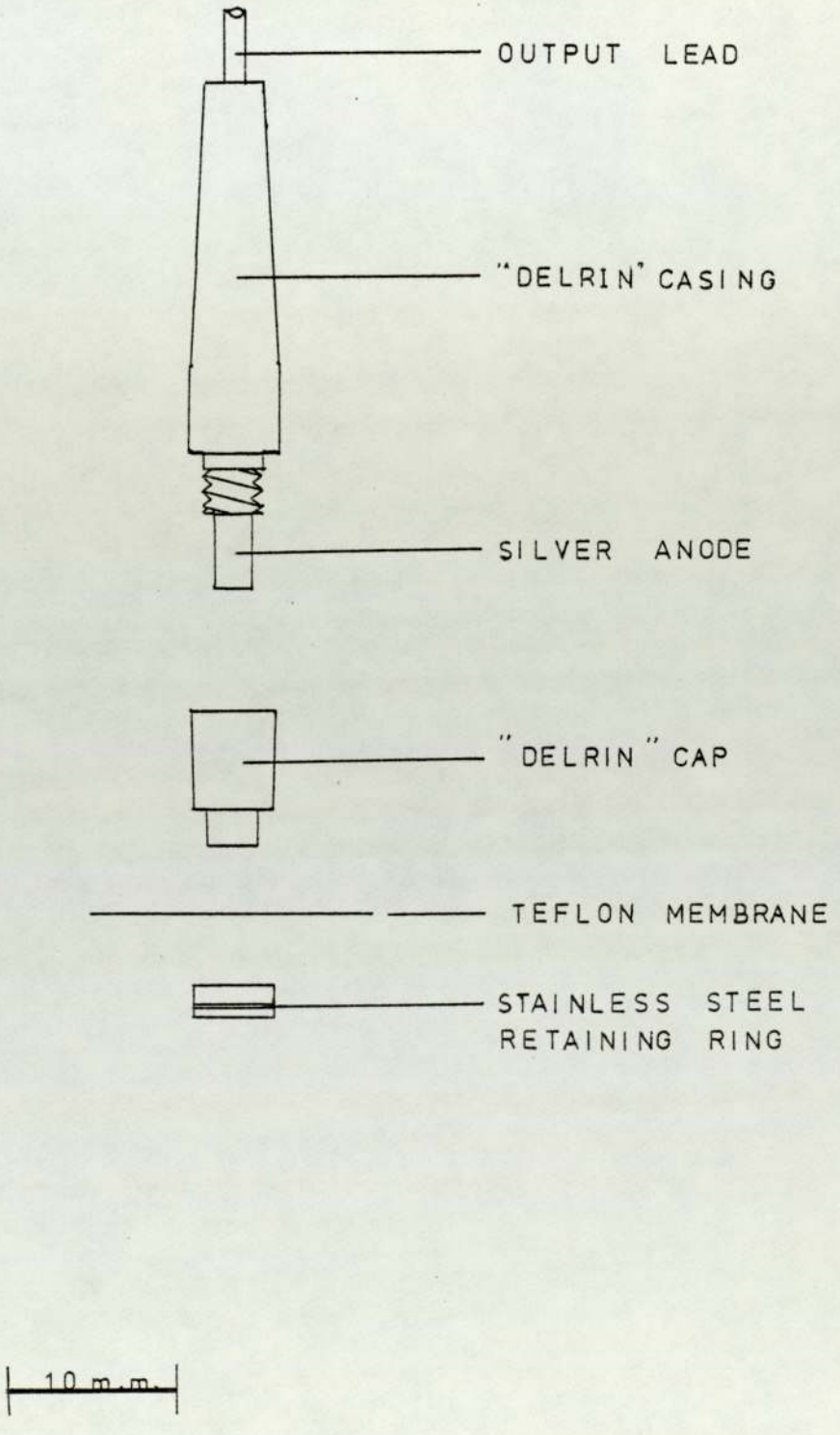


SIDE ELEVATION



PLAN

Figure 3 A scale drawing of the MkII oxygen sensor.



the polarising voltage for the sensor. A potential divider, i.e. variable resistance, adjusts amplifier output to match the chart recorder input voltage requirements.

The chart recorder is a Vitatron Model UR40 (Vitatron U.K. Ltd.) Figure 4 shows peripheral equipment for the determination of oxygen consumption rate.

Preparation for use

A 12 μ Teflon membrane is fitted over the nylon casing cap and held in place with a stainless steel retaining ring which passes over the membrane and is pushed tight against the nylon cap. The nylon cap has a slight shamfer allowing a "flush-fit" of the membrane retaining ring.

The cap is filled with electrolyte, care being taken at this point to exclude all air bubbles. The sensor body is screwed tightly into the cap after which the completed unit is rinsed thoroughly in distilled water to remove any traces of electrolyte.

Calibration

It is necessary to determine the relationship between oxygen tension of the test solution and current output for each sensor.

The relationship between current output and oxygen tension for oxygen sensors with large cathodes is linear, with zero current output at zero oxygen tension. Large cathode sensors can be calibrated using air and

Figure 4 Peripheral equipment to the oxygen sensor.

A = High impedance amplifier

B = Potentiometer box

C = Chart recorder.

nitrogen saturated solutions since current output in nitrogen saturated solution is equivalent to zero oxygen tension = 0.

Unfortunately sensors with small cathodes have a significant current output in oxygen free solutions. Furthermore the relationship between oxygen tension and current output above 150mm Hg is curvilinear. Calibration requires the use of either chemical methods or calibration curves (WINGO and EMERSON, 1975). The highest oxygen tension encountered in the determination of corneal oxygen consumption rate is that of air = 155mm Hg. The assumption has been made in this system that the response of the sensor is linear within the working range. Calibration of the sensor for use in this experiment is performed as for a large cathode sensor using air and nitrogen saturated solutions.

With the sensor in a nitrogen saturated 0.9% sodium chloride solution at 34 degrees centigrade (^{o}C), the amplifier is set to read zero using the set zero gain control. The sensor is transferred to an air saturated 0.9% sodium chloride solution at 34^{o}C and the amplifier set to read 155mm Hg. using the calibration control. The zero control on the chart recorder is adjusted to give a zero reading for zero amplifier output, and the potential divider adjusted such that 100% scale reading is obtained for an amplifier output corresponding to 155mm Hg.

Output from the sensor at 34^{o}C in a nitrogen saturated solution = 0.050 Nanoamps (10^{-9} amps), and in an oxygen saturated solution = 32.0 Nanoamps.

Response time for the sensor at 34°C is estimated from the time taken for a 95% response in moving from a nitrogen saturated solution to an air saturated solution and vice versa and is found to be less than five seconds.

Electrode Properties

(a) Temperature:-

Sensor current output has been shown to vary with temperature, (BERKENBOSCH and RIEDESTRA, 1963; CAREY and TEAL, 1965). It is possible to convert oxygen readings at one temperature to that which would be obtained at another, however the most simple method is to calibrate the sensor at the temperature of the system under study. The temperature at the corneal surface is 34°C as a result the sensor is calibrated in solutions maintained at 34°C in a Student water bath (Gallenkamp Ltd.).

(b) Mechanical pressure:-

Small cathode sensors are sensitive to mechanical pressure directed at the membrane (FATT, 1964 (a)). Possible reasons for pressure effects are distortion and compression of the membrane and electrolyte layer. The effect of mechanical pressure on the sensor used in this study is determined by pressing the sensor against a soft lens template covered with felt soaked in air saturated 0.9% sodium chloride solution with a pressure near to that with which the sensor will be held against the eye. Mechanical pressure is shown to have no significant effect on sensor current output.

(c) Cathode Oxygen Consumption:-

Oxygen sensors have an inherent oxygen consumption, the precise rate

varying between sensor systems (SEVERINGHAUS and BRADLEY, 1971; TAKAHASHI et al., 1967). Determination of the rate of oxygen consumption by the sensor used in this study is determined by pressing the sensor against an air saturated layer of felt soaked in 0.9% sodium chloride solution and observing any change in oxygen tension over a period of five minutes. No detectable change could be found. This agrees with the conclusions obtained from comparable systems, that the oxygen consumption of micro-cathode type sensors is negligible (HILL and FATT, 1963 (a), (b)).

Electrode Stability

(a) Short-term stability:-

It is necessary to determine the short-term stability of the sensor since calibration checks taken at random intervals after placing the sensor in a solution with a constant oxygen tension show changes in sensor current output, requiring adjustment of the calibration control. Placing the sensor in air saturated 0.9% sodium chloride solution at 34°C and taking hourly readings of oxygen tension, shows that for the sensor used in this study a constant output is obtained for up to six hours. Over six hours adjustments are necessary. A possible explanation of the observed drift after six hours is that Teflon has a tendency to stretch which could alter the membrane and electrolyte thickness.

A similar test to determine zero output drift by maintaining the sensor in air saturated 0.9% sodium chloride solution and placing the sensor in nitrogen saturated 0.9% sodium chloride solution every hour showed

no change in sensor zero output in a twelve hour test period.

(b) Long-term stability:-

Calibration adjustments were required at increasingly frequent intervals over a twelve month period. After twelve months the sensor current output could no longer be matched to the amplifier. Leakage of water through the nylon casing effectively short circuited the sensor tip. Drying by warming to 60°C in a desiccator increased the useful life of the electrode by a number of weeks, however a replacement had to be obtained after fourteen months of use.

Oxygen Sensor Mark II

This electrode possesses the same specifications as the Mark I electrode, however to improve the water resistant properties the outer casing is made from a highly water impermeable plastic, "Delrin", the generous gift of I.C.I. Ltd. Figure 5 shows the sensor in its component parts, Figure 6 shows the sensor assembled.

The smooth surface of Delrin poses a problem in that the stainless steel ring is incapable of holding the membrane rigidly in place. This is overcome by using two layers of Nescofilm (Fisons Ltd.) as a spacer. The method of preparation is to place three layers, one Teflon and two Nescofilm, across the sensor cap and to place the stainless steel retaining ring over all three layers until secure. The Nescofilm is then cut away, using a Number 12 scalpel, from the sensor tip to reveal the membrane beneath. Figure 7 illustrates the technique for mounting the membrane on the cap of the Mark II oxygen sensor.

Figure 5 An exploded view of the MkII oxygen sensor.

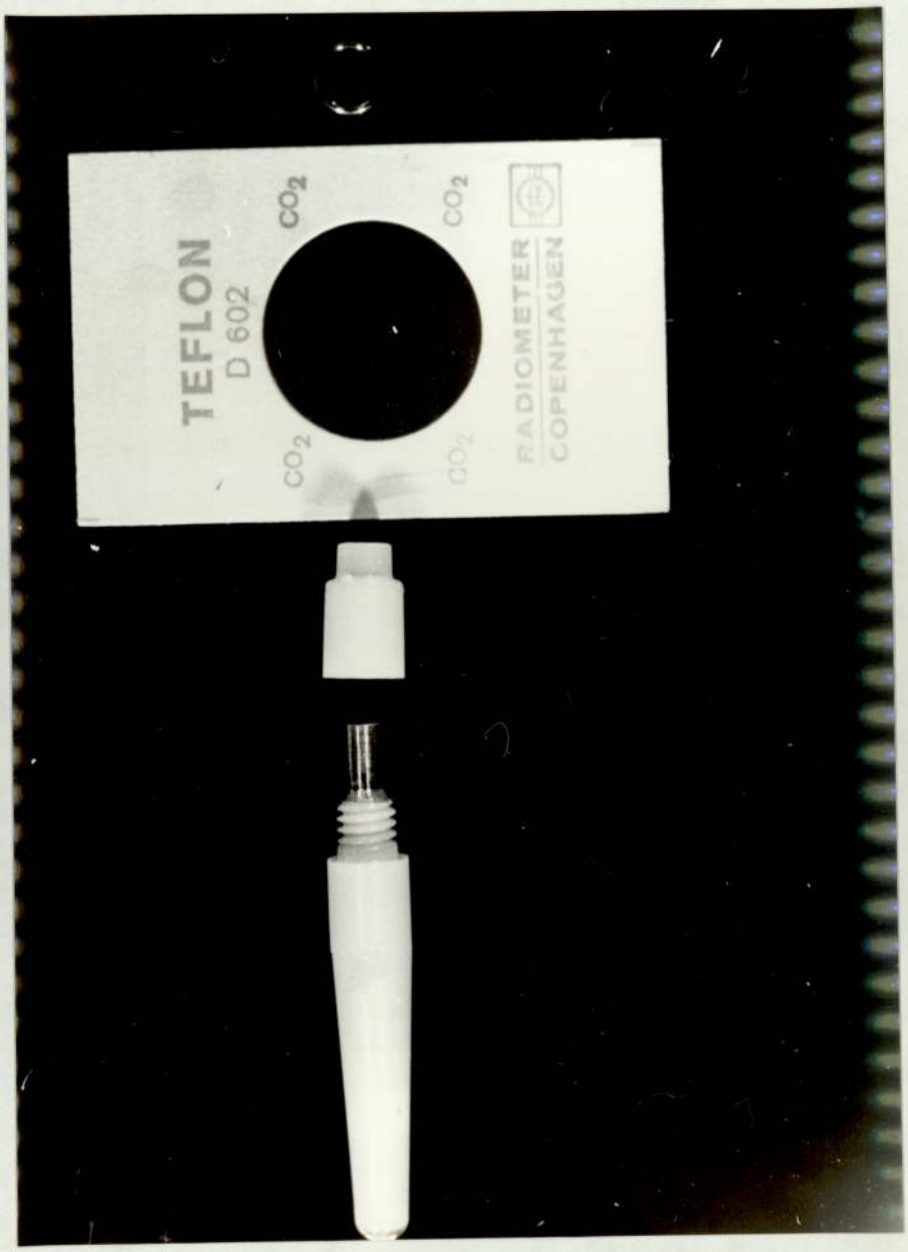
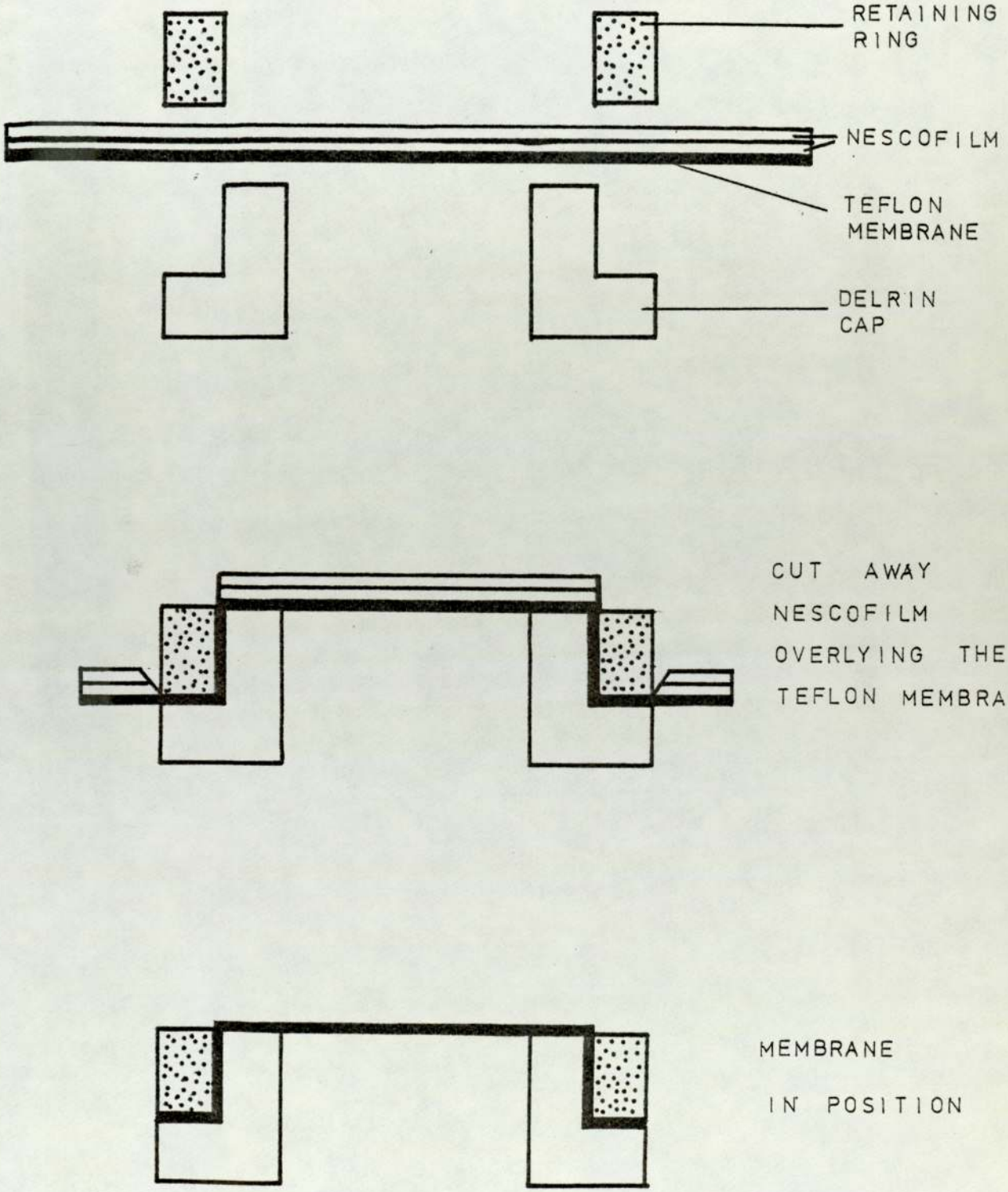


Figure 6 The MkII oxygen sensor assembled and ready for use.

Figure 7 A schematic of the membrane mounting procedure.



Electrode Properties Mark II

Response time, mechanical pressure and cathode oxygen consumption are all as for the Mark I sensor. The electrode is more stable in the short-term, calibration checks being necessary only after the sensor has been placed on the cornea or a test block.

As a protection against long-term instability the sensor is kept in a desiccator when not in use. No instability is apparent after eighteen months of use.

Oxygen Sensor Sleeve

Placing the oxygen sensor directly on to the cornea created instabilities due to movements of the eye relative to the sensor tip. This movement introduces "fresh" oxygen to the space between the corneal epithelium and sensor, that is acting as the oxygen reservoir. The problem of eye movement can be partly solved by mounting the sensor in a sleeve. The requirements of the sleeve are that it should:-

(a) Fit comfortably to the cornea.

(b) Be of a biocompatible material.

(c) Have a low oxygen permeability.

(d) Be able to withstand repeated sterilisation.

A hydrophilic polymer, Itek 107 (Itek Corporation), with a water content of 60% and linear swell of 1.338 is used to make the sensor sleeves. This material is acceptable to the patient and repeatedly steriliseable. The sleeve has a concave curve cut into one end to increase comfort and reduce instabilities. A compromise had to be

made between patient acceptability and oxygen permeability, Itek 107 proved to be the most acceptable since it reduces lateral diffusion of oxygen into the oxygen reservoir beneath the sensor.

The sleeves are cut from dehydrated rods of Itek 107. A hole is drilled in the centre using a succession of drills to produce a taper to match that of the sensor casing. A concave curve is cut into one end using a Nissel lathe. The sleeves are hydrated in 0.9% sodium chloride solution and boiled twice before use. It is found that after approximately 20 sterilisations small cracks appear in the sleeves, the sleeves are discarded as soon as these cracks appear. Figure 8 is a scale drawing of a hydrated sensor sleeve. Figure 9 shows a hydrated sleeve. Figure 10 shows the Mark II oxygen sensor mounted in a sleeve, ready for use.

2.4 METHODOLOGY

Electrode Preparation

The experimental procedure for the determination of oxygen consumption rate entails placing the oxygen sensor mounted in a hydrogel sleeve directly in contact with the corneal epithelium, and holding it in place while a trace representative of the fall in oxygen tension from the reservoir below the sensor is obtained.

The oxygen sensor is prepared afresh each day, using a new membrane, fresh electrolyte and a sterile sleeve. The oxygen sensor is calibrated at 34^{o}C in air and nitrogen saturated 0.9% sodium chloride solution. Following calibration the oxygen sensor assembly is maintained at 34^{o}C in air saturated 0.9% sodium chloride solution until

Figure 8 A scale drawing of the oxygen sensor hydrogel sleeve.

Radius of curve = 6.2. Central hole is drilled using a succession of drills, Numbers 27, 25, 23, 21, 19. Dehydrated dimensions are obtained by reducing the measured value by 1.338, this being the linear swell factor for Itek 107.

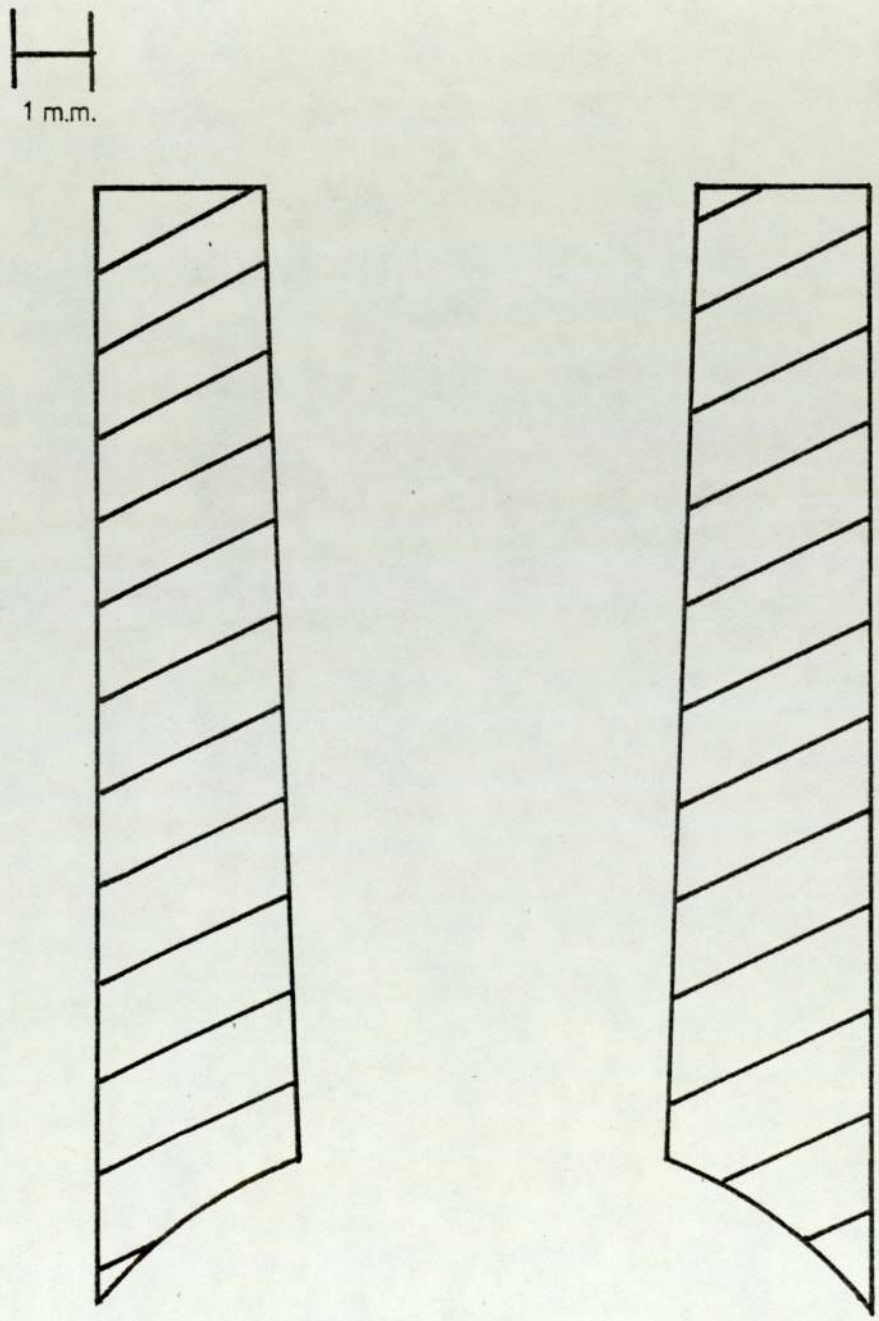


Figure 9 An oxygen sensor hydrogel sleeve, hydrated and ready for use.

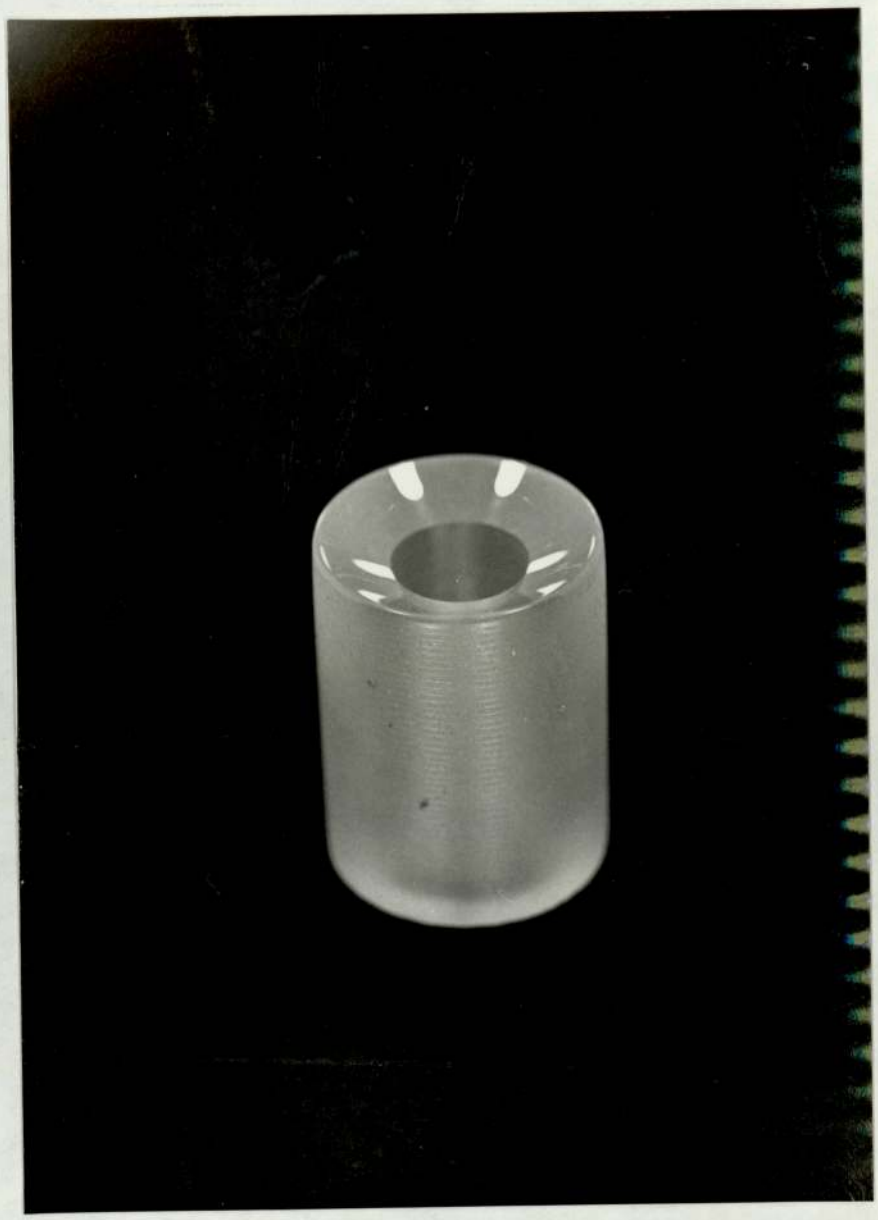
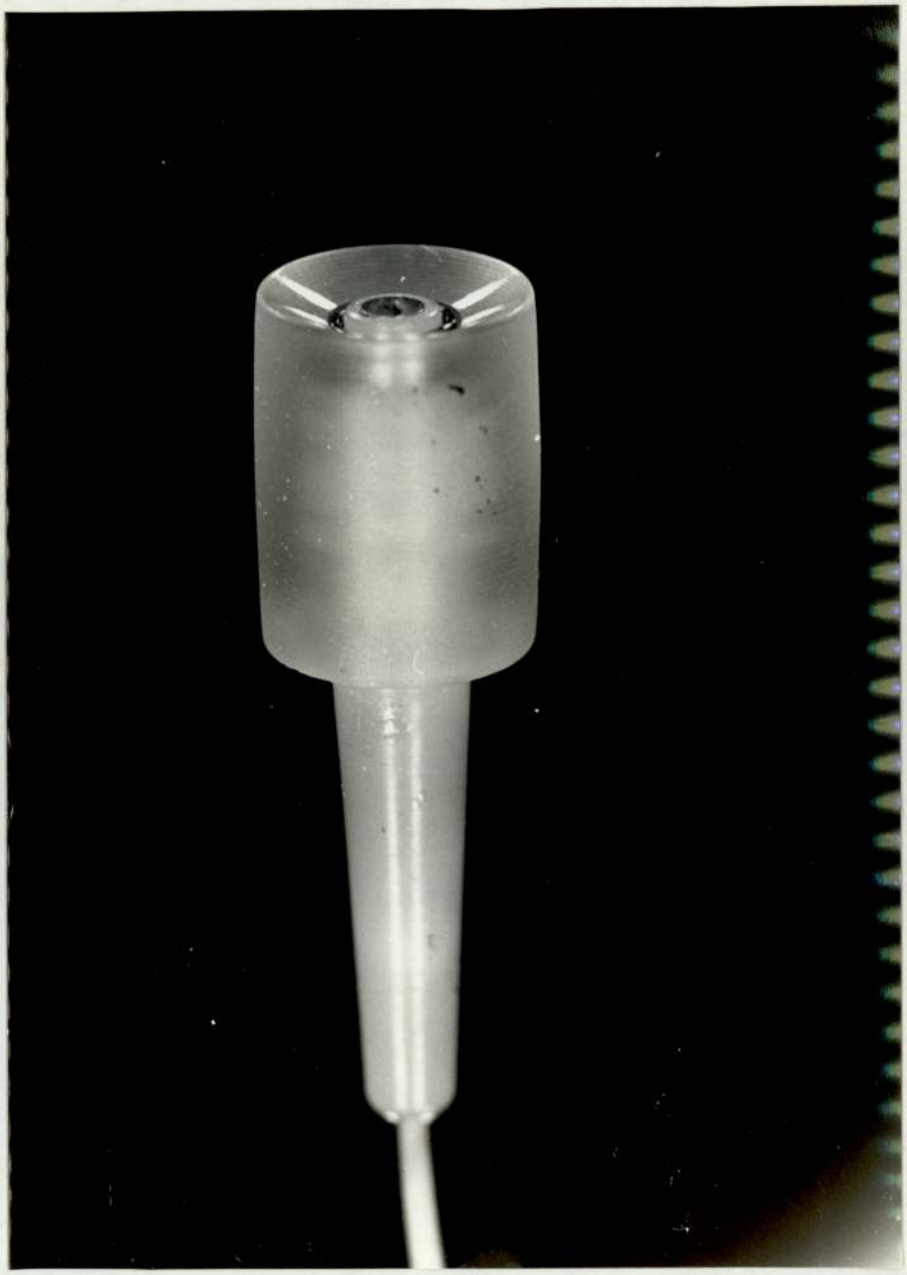


Figure 10 The MkII oxygen sensor mounted in a hydrogel sleeve, ready for use.



In operation the sensor lies "flush" to the curve of the sleeve. The sensor is shown protruding for illustrative purposes only.

required. When the subject is prepared the sensor is thoroughly rinsed using sterile 0.9% sodium chloride solution maintained at 34°C. After the experiment the sleeve is removed and sterilised and the sensor rinsed in sterile 0.9% sodium chloride solution and placed in the maintaining bath.

After use the sensor calibration is checked and any necessary adjustments made. Calibration checks are necessary since placing the sensor on the cornea may cause stretching and slipping of the membrane affecting sensor current output (ALBANESE, 1973).

Patient Selection and Instruction - Human

Patients used in this study are all male volunteers between 18 and 35 years of age, taken from the staff and students of The University of Aston.

Before any experimentation takes place each patient is given a written description of the experiment in which he is taking part. The patient is then requested to sign a declaration and complete a patient history. Appendix 1.

Each patient is given a thorough corneal examination using a Nikon slit lamp, without the use of vital stains. The ocular examination is always carried out by a registered Ophthalmic Optician. Only in the absence of damage and disease is the patient suitable. The patient is laid prostrate on a reclining ophthalmic chair and instructed to fixate a distant object and to move his eye as little as possible once the sensor is in position.

The sensor is placed on the cornea for a period not exceeding one minute. Figure 11 illustrates the positioning of the complete sensor on the cornea. After the experiment each patient is given a second slit lamp examination using Flourescein and Rose Bengal stains. If the examiner suspects any kind of trauma the patient is treated accordingly and instructed to return for further examinations.

Determination of Mean and Diurnal Oxygen Consumption Rate in the in vivo Human Cornea

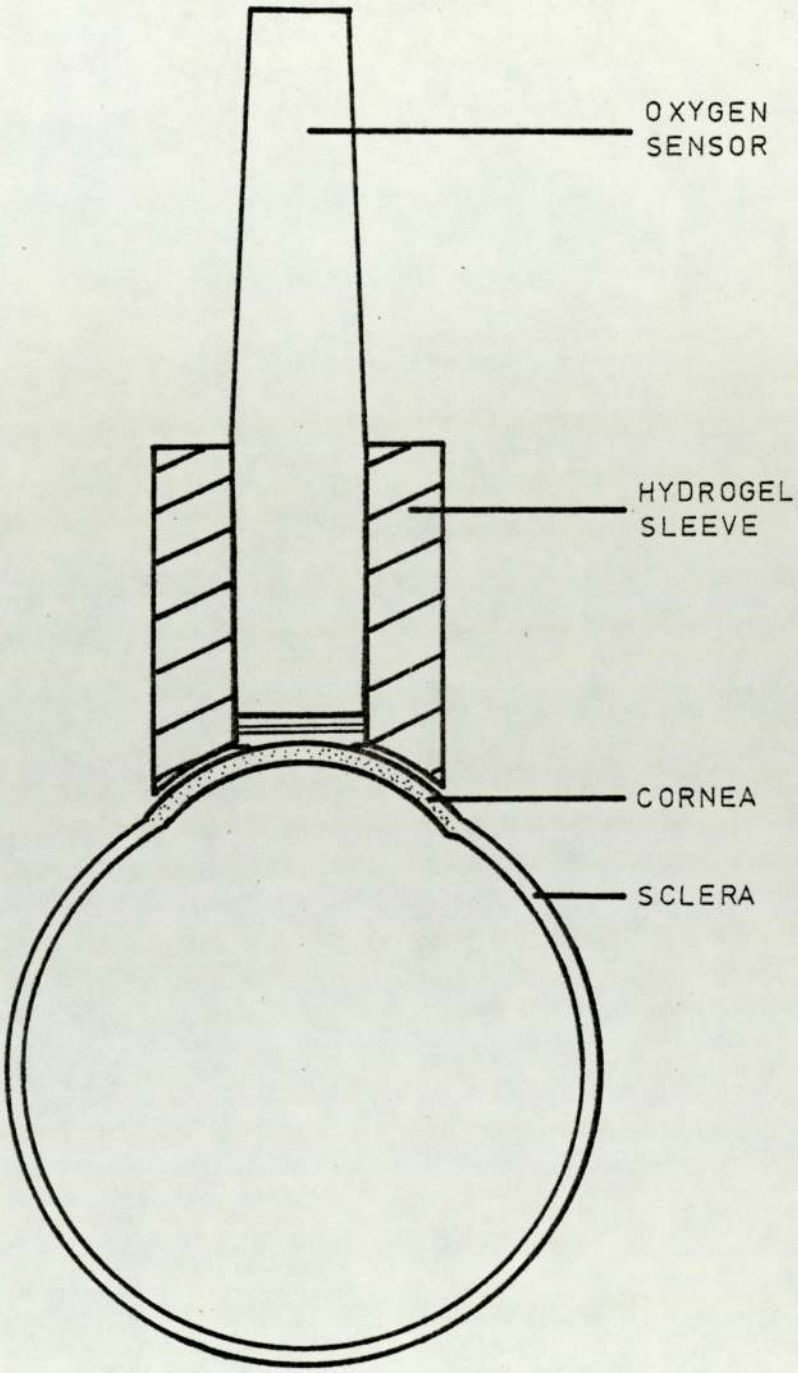
Measurements of the rate of oxygen consumption are taken at random and the results pooled. Mean values are determined for both the Mark I and Mark II sensors. Measurements were taken over a period of six months for the Mark I sensor and eighteen months for the Mark II. Diurnal variation in oxygen consumption rate is determined by comparing values for oxygen consumption rate taken at six intervals during the day:- 9-00, 11-00, 15-00, 17-00, 19-00 hours. No patient is used more than once in seven days to reduce corneal trauma to a minimum.

Subject Preparation - Rabbit

Male New Zealand White rabbits (Hyline Rabbits Ltd.), weighing between 2 and 3 kilograms (Kg.) are used in these experiments.

Initial experiments to determine oxygen consumption rate were performed without anaesthetics. However the procedure produced rapid eye movements in the animal preventing the production of a satisfactory trace. Thus in subsequent experiments anaesthetised animals were used.

Figure 11 A schematic of the oxygen sensor in position on the cornea.



Topical anaesthetics have been shown to affect corneal respiratory activity (AUGSBURGER and HILL, 1972, 1973; GREEN, 1976). However systemic anaesthetics may be employed since they have been shown to have little or no effect on corneal metabolism (HERRMANN et al, 1942; AUGSBURGER and HILL, 1973). General anaesthesia is induced by intraperitoneal injection of sodium pentobarbital, "Sagatal", (May and Baker Ltd.). The dosage used is 0.5 ml. Kg^{-1} body weight of 60 mg. ml^{-1} solution in 0.9% sodium chloride solution. One injection is sufficient to maintain anaesthesia for up to two hours.

While one hand holds the prepared sensor, mounted in a hydrophilic sleeve, in contact with the cornea, the eyelids and nictitating membrane are retracted by the other.

Determination of Diurnal Oxygen Consumption Rate in the Rabbit

Experimental details are the same as given previously for the human. All rabbit experiments are performed using the Mark II sensor. No animal is used more than once in any seven days.

2.5 INTERPRETATION OF DATA

The aim of the experimental procedure is to produce a trace from which corneal oxygen consumption rate can be calculated.

Early experimental methods for the determination of corneal oxygen consumption rate used a Clark type oxygen sensor to measure the fall of oxygen in a large reservoir of solution in contact with the corneal epithelium (HILL and FATT, 1963 (a)). The time taken to produce a

result is grossly unacceptable to a patient, 30 minutes being the average time required. Reduction of reservoir volume is successful in reducing experimental time, however inaccuracies arise due to the difficulties of accurately calculating reservoir volume. HILL and FATT (1963 (b)) overcame this problem by taking the oxygen reservoir to be the membrane covering the sensor.

The use of a small cathode and 12 μ membrane ensures that when the sensor is placed on the cornea the observed drop in current is almost entirely controlled by the decrease in oxygen tension within the membrane and tissue immediately below the cathode.

Unfortunately new problems arise when the membrane is taken as the oxygen reservoir:-

(a) The electrolyte layer between membrane and electrode surface, together with the tear film between the membrane and cornea must also be considered as part of the oxygen reservoir. The thickness of these two layers is unknown and likely to be variable depending on the force used to press the sensor to the cornea.

(b) The small volume of the membrane oxygen reservoir leads to a very rapid depletion of oxygen. The depletion is so rapid, taking approximately 60 seconds, that steady-state uptake of oxygen can no longer be assumed.

Interpreting the record of oxygen depletion in terms of non-steady state diffusion of oxygen from sensor to cornea is very difficult. A

reasonable estimate of oxygen transfer rate from sensor to cornea can be made from the initial portion of the record. The results from such an analysis are useful only where qualitative information is required.

JAUREGUI and FATT (1971, 1972) pointed out that if the logarithm of sensor output is plotted as a function of time after the sensor is placed on the cornea then a linear relationship is obtained. The slope of this line can be used to calculate the rate of oxygen depletion from the membrane which is considered to be equal to the oxygen consumption of the cornea. By this method the oxygen consumption rate is largely that of the corneal epithelium since the sensor is only in place for a few seconds and oxygen will not have reached the deeper stroma and endothelium.

Such a treatment eliminates the errors stated earlier since the contribution of tears and electrolyte are constant once the sensor is in place and a rate of change is being measured.

FARRIS et al., (1965) noted that to obtain a trace worthy of treatment in the manner of JAUREGUI and FATT (1971, 1972), care must be taken when placing the sensor on the cornea.

Moderately steady pressure must be used to maintain complete contact between the membrane surface and corneal epithelium. Inadequate pressure and eye movements are most frequently responsible for artifacts such as wide mechanical spikes, absence of initial precipitous drop and transient rises and dips in recorded current.

Figure 12 shows a typical record for the depletion of oxygen tension in the sensor membrane when placed on an in vivo human cornea. The upper base-line is set with the sensor placed in air saturated 0.9% sodium chloride solution at 34°C. A slight fall in measured oxygen tension occurs as the sensor is transferred from the maintaining solution to air. The calibration level is restored as the sensor is placed on the cornea. A ⟶ B.

An exponential decline in oxygen tension is obtained, B ⟶ C, that is representative of the fall in oxygen tension within the membrane as the sensor is placed on the oxygen consuming cornea.

At C-D, a mechanical spike interrupts the fall in oxygen tension and the logarithm (log) of oxygen tension (pO_2) versus time curve is no longer linear. Table 1 shows the values for oxygen tension with time, taken at three second intervals from Figure 12. Table 1 also shows the log. of oxygen tension with time, these figures are used to plot a graph of log pO_2 versus time, Figure 13.

The calculation of oxygen consumption is based on the work of JAUREGUI and FATT (1971). The graph depicting the decline in oxygen tension as a function of time in the area between points B and C (Figure 12) reveals that there is an exponential decrease in oxygen tension. A semi-logarithmic plot of oxygen tension with time, Figure 13, reveals a linear relationship between B and C.

The straight line of log pO_2 versus time satisfies the relationship:-

Figure 12 A typical trace of oxygen tension (chart reading) with time. Taken from the right cornea of a male human.

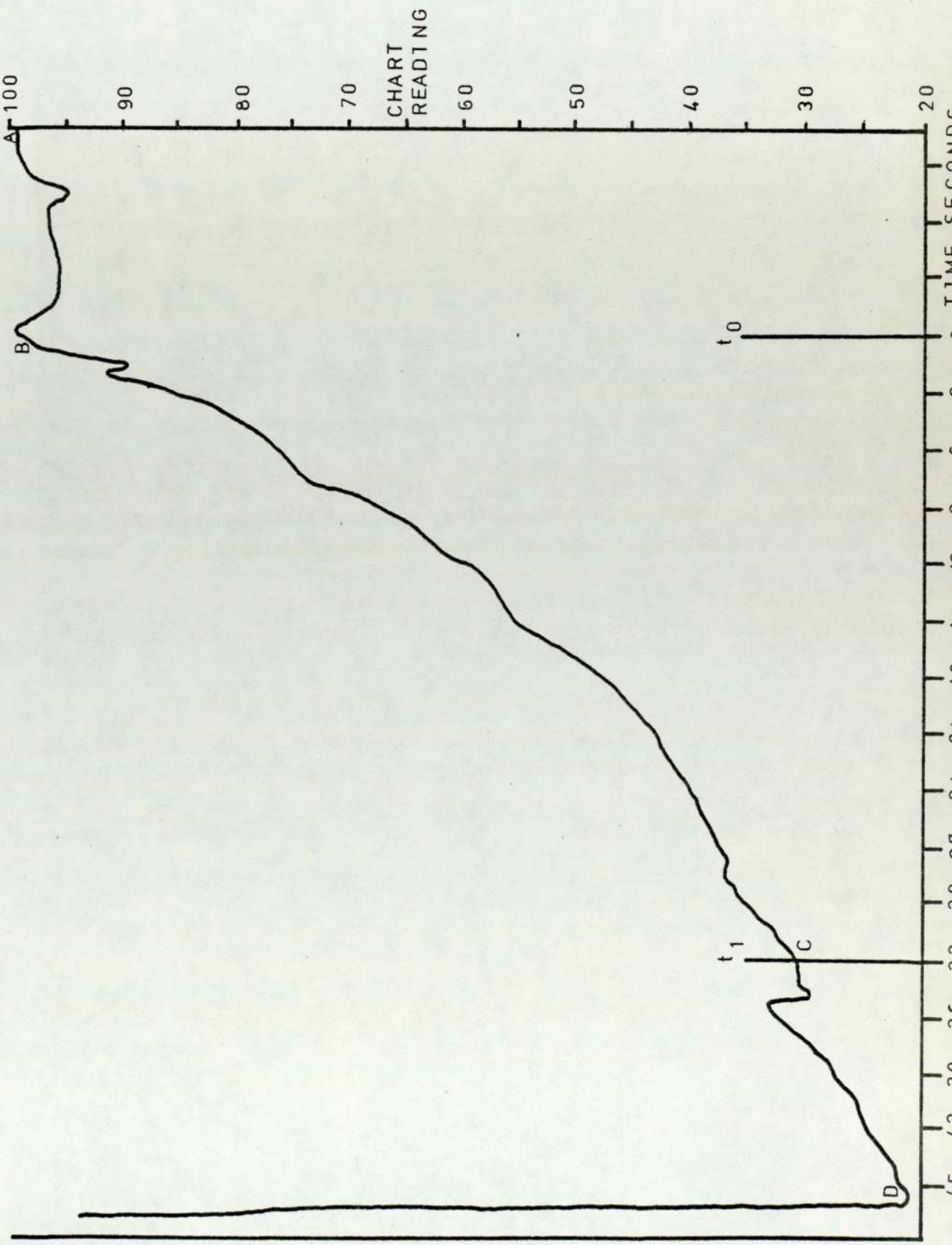
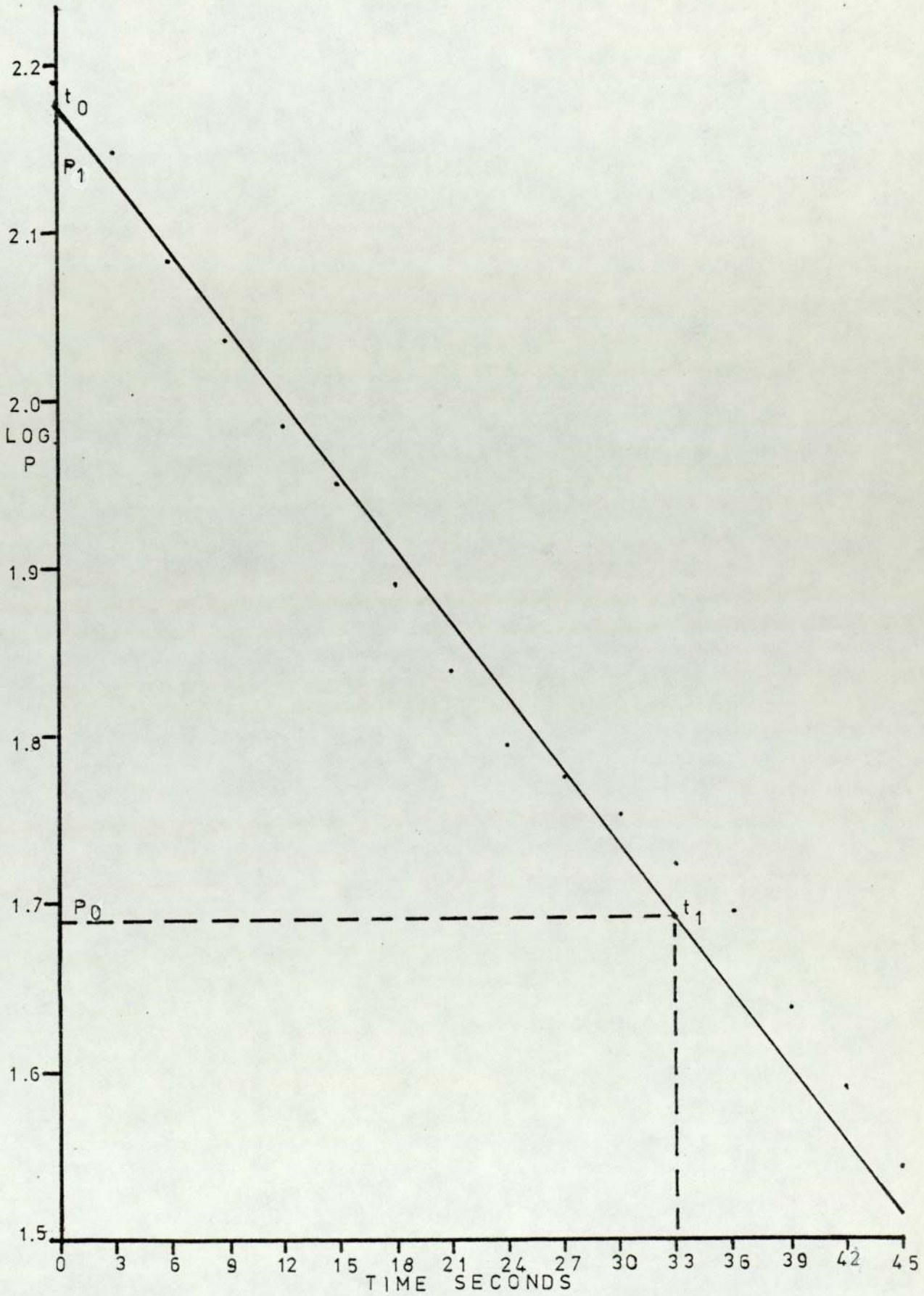


Table 1

Raw data taken from Figure 12, representing the fall in measured oxygen tension with time, due to the uptake of oxygen by the cornea.

TIME SECONDS	CHART READING	po_2 mm.Hg.	Log po_2
0	100	155	2.1903
3	90.5	140.275	2.1470
6	78	120.9	2.0824
9	70	108.5	2.0354
12	62.5	96.875	1.9862
15	57.5	89.125	1.950
18	50	77.5	1.8893
21	44.5	68.975	1.8387
24	41.5	62.325	1.7947
27	38.5	59.675	1.7758
30	36.5	56.575	1.7526
33	32	49.6	1.6955
36	34	52.7	1.7218
39	28	43.4	1.6375
42	25	38.75	1.5883
45	22.5	34.875	1.5425

Figure 13 Log P against time taken from Figure 12 and Table 1.



$\frac{d \log_e P}{dt}$ = Constant = Rate of change of natural logarithm.

By calculus:

$\frac{d \log_e P}{dt}$ can be defined as $= \frac{1}{P} \cdot \frac{dP}{dt}$

Therefore $\frac{dP}{dt} = P. \frac{\log_e P}{dt}$ _____(1)

Using the assumption that d approximates to Δ equation 1 can be written:-

$$\frac{dP}{dt} = P. \frac{\Delta \log_e P}{dt}$$

OR

$$\frac{dP}{dt} = P. \frac{\log_e P_1 - \log_e P_o}{t_1 - t_o} \quad \text{_____(2)}$$

From JAUREGUI and FATT (1971) the rate at which oxygen leaves the membrane of the sensor is assumed to be equal to the oxygen consumption rate of the anterior surface of the cornea = Qo.

$Qo = b \frac{dP}{dt}$ Where the constant b is the product of membrane thickness (l) and HENRY's law coefficient for the solubility of oxygen in Teflon (k)

$$Qo = l.k. \frac{dP}{dt} \quad \text{_____(3)}$$

Combining equations (2) and (3) gives:-

$$Qo = P.l.k. \frac{\log_e P_1 - \log_e P_o}{t}$$

Where:-

Qo = Rate of oxygen consumption.

P = The oxygen tension at which the Qo value is required, in this study = 155 mm. Hg.

l = Thickness of Teflon membrane.

k = HENRY's law coefficient for the solubility of oxygen in Teflon.

P_1 and Po = Those values of oxygen tension between which the plot of log pO_2 against time is linear.

t = The time taken for the oxygen tension to fall from P_1 to Po i.e. $t = (t_1 - to)$.

A conversion factor is required to obtain Qo in the accepted terminology of microlitres of oxygen per square centimetre per hour ($\mu l\ O_2\ cm^{-2}.\ hr^{-1}$). The conversion factor = 3600 x 1000, this being obtained from converting seconds into hours, and microlitres to millilitres.

The final formula:-

$$Qo = P.l.k. \left| \frac{\log e\ P_1 - \log e\ Po}{t} \right| 3600.\ 1000.\ \mu l\ O_2\ cm^{-2}.\ hr^{-1}.$$

P = 155 mm. Hg.

$l = 12.0 \times 10^{-4}$ cm.

$k = 2.76 \times 10^{-4}\ cm^3.\ cm^{-3}\ mm.\ Hg^{-1}$ (PASTERNAK et al., 1970; CRANK and PARK, 1968; BRANDRUP and IMMERGUT, 1975).

Example

From Figure 13 of log pO_2 versus time.

P_1 = 155 mm. Hg.

Po = 49.6 mm. Hg.

t = 33 seconds.

$$Qo = (155.\ 12 \times 10^{-4}.\ 2.76 \times 10^{-4})\ \frac{5.0434 - 3.9040}{33}.\ 3600.\ 1000.$$

$\mu l\ O_2\ cm^{-2}\ hr^{-1}$.

$$Qo = (5.1336 \times 10^{-5}).\ (3600.\ 1000).\ \frac{5.0434 - 3.9040}{33}\ \mu l\ O_2\ cm^{-2}.\ hr^{-1}.$$

$$Qo = (184.81)\ \frac{1.1394}{33}\ \mu l\ O_2\ cm^{-2}\ hr^{-1}.$$

$$Qo = (184.81)\ (0.0345)\ \mu l\ O_2\ cm^{-2}\ hr^{-1}.$$

$$Qo = 6.3759\ \mu l\ O_2\ cm^{-2}\ hr^{-1}.$$

$$Qo = 6.38\ \mu l\ O_2\ cm^{-2}\ hr^{-1}.$$

2.6 RESULTS

Raw data for corneal oxygen consumption rate in the human using the Mark I and Mark II oxygen sensors is presented in Tables 2 and 3 respectively.

Similar data for the male New Zealand white rabbit under sodium pentobarbital anaesthesia using the Mark II oxygen sensor is presented in Table 4.

Mean, standard error of the mean and comparison of the sets of data by the Student t-test are all performed on an Olivetti Model 101 desk computer. Figure 14 is a histogram of the means of the data given in Tables 2, 3 and 4, plus or minus the standard error of the mean. The student t-tests performed on this data show no significant difference between the Mark I and Mark II oxygen sensors. There is a significant difference between the results taken from the human cornea and rabbit cornea using the Mark II oxygen sensor; $t = 2.4467$, 149 degrees of freedom (^{o}F), P less than ($<$) 0.02. (FISHER and YATES, 1963; BISHOP, 1971).

Table 2

Raw data for corneal oxygen consumption rate in human using the MkI oxygen sensor.

Values are in $\mu l.O_2.\ cm^{-2}.\ hr^{-1}$.

4.67	N = 21
6.70	Mean = 6.24
5.78	S.E.M. = 0.3116
7.40	
4.62	
7.83	
6.65	
7.12	
5.00	
9.20	
7.18	
6.0	
7.03	
6.21	
3.38	
8.38	
7.09	
5.76	
5.50	
4.67	
4.86	

Table 3

Raw data for corneal oxygen consumption rate in human using the MkII oxygen sensor.

Values are in $\mu l.O_2 . cm^{-2} . hr^{-1}$.

3.54	7.15	6.36
5.24	6.02	4.64
5.71	5.89	
5.02	8.15	
5.02	5.77	
4.45	9.20	
7.03	6.01	
4.60	6.38	
7.81	8.04	
7.78	6.01	
5.18	7.63	
5.54	8.70	
6.25	5.73	
8.69	6.02	
7.13	6.65	
8.33	6.51	
6.05	7.76	
6.66	7.65	
7.71	6.10	
6.56	7.43	
10.89	4.66	
6.56	6.0	
7.13	5.92	

N = 48
Mean = 6.55
S.E.M. = 0.2002

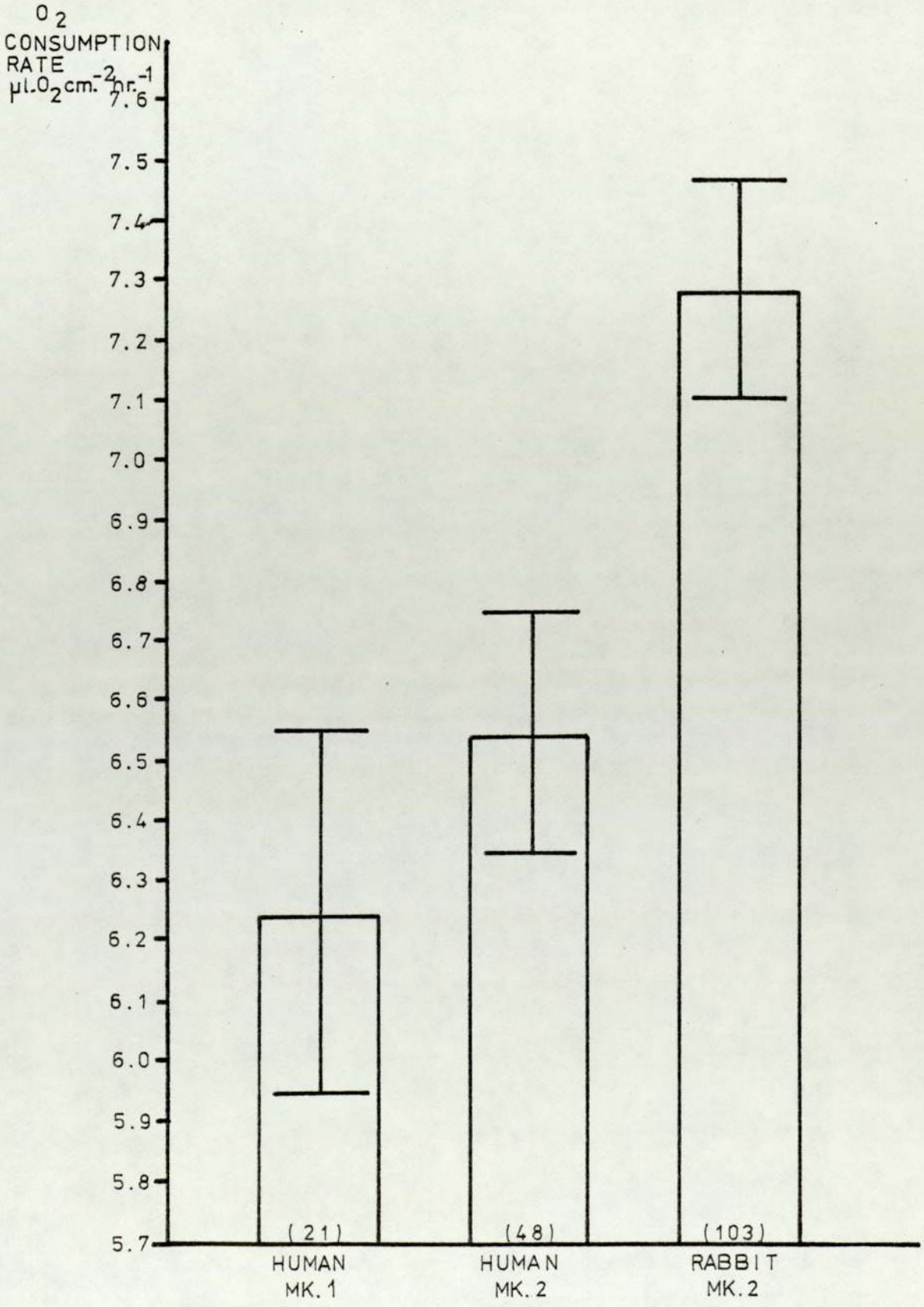
Table 4

Raw data for corneal oxygen consumption rate in male New Zealand white rabbit using the MkII oxygen sensor.

Values are in $\mu l.O_2.\ cm^{-2}.\ hr^{-1}$.

7.90	9.11	7.54	4.0	7.76
7.90	8.03	8.13	5.60	5.69
4.84	11.90	5.81	7.50	9.61
4.92	6.21	9.94	6.65	8.95
5.16	11.29	7.10	7.13	5.83
8.47	10.19	7.34	5.60	5.56
9.08	11.09	5.42	7.19	9.41
10.83	6.75	5.97	9.85	11.31
8.12	4.12	11.35	6.56	5.09
8.06	7.45	10.77	6.36	6.01
6.62	7.80	5.27	7.58	6.25
9.22	9.65	7.20	6.84	
6.81	6.93	7.60	6.71	N = 103
9.77	5.73	8.46	6.19	Mean = 7.29
6.76	6.77	6.19	9.98	S.E.M. = 0.1844
6.51	6.19	8.17	5.59	
8.0	5.49	8.58	8.26	
5.39	6.27	6.19	7.60	
6.60	11.35	8.22	4.33	
6.74	5.78	8.48	4.47	
5.41	4.17	5.17	5.67	
7.65	4.14	7.73	6.27	
8.51	8.96	4.88	6.28	

Figure 14 Oxygen consumption rate in the human cornea, using the MkI and MkII sensors, and rabbit cornea using the MkII oxygen sensor. Values are the mean ± SEM. Numbers of readings are given in parentheses.



Raw data for human diurnal variation in oxygen consumption rate is presented on Table 5. Figure 15 shows a histogram of the mean values for each time plus or minus the standard error of the mean (S.E.M.). A student t-test is used to compare the mean value for oxygen consumption rate taken from Table 3 with the means of data taken at each time of the day. In no case is there a significant difference between the grand mean and means at the different times of the day.

Raw data for diurnal variation in oxygen consumption rate in the rabbit is presented on Table 6. Mean values plus or minus the standard error of the mean for each time are plotted on Figure 16. As with the human data, a student t-test revealed no significant difference between the grand mean of Table 4 and the mean for each time of the day.

Table 7 summarises the mean and diurnal data obtained for human and rabbit corneal oxygen consumption rate.

2.7 DISCUSSION

In section 2.6 Tables 2 and 3 show the mean values for human corneal oxygen consumption rate using the Mark I and Mark II oxygen sensors to be 6.24 and 6.55 $\mu l\ O_2\ cm.^{-2}\ hr^{-1}$ respectively.

Also in section 2.6, Table 4 shows the mean value for corneal oxygen consumption rate in the rabbit using the Mark II sensor to be 7.29 $\mu l\ O_2\ cm^{-2}\ hr^{-1}$.

Table 5

Raw data for the diurnal variation in human corneal oxygen consumption rate.

Values are in $\mu l.O_2 . cm^{-2} . hr^{-1}$.

	TIME OF READING.HOURS.					
	9-00	11-00	13-00	15-00	17-00	19-00
	5.18	6.05	7.15	6.01	5.73	7.43
	5.54	6.66	6.02	6.38	6.02	4.66
	6.25	7.71	5.89	8.04	6.65	5.99
	8.69	6.56	8.15	6.01	6.51	5.91
	7.13	6.56	5.77	7.63	7.76	6.36
	8.33	7.13	9.20	8.70	7.65	4.64
N	6	6	6	6	6	6
Mean	6.85	6.78	7.03	7.13	6.72	5.83
SEM	0.5920	0.2328	0.5748	0.4706	0.3395	0.4346

Figure 15 Diurnal variation in corneal oxygen consumption rate in man. Values are the mean ± S.E.M. Number of readings is given in parentheses.

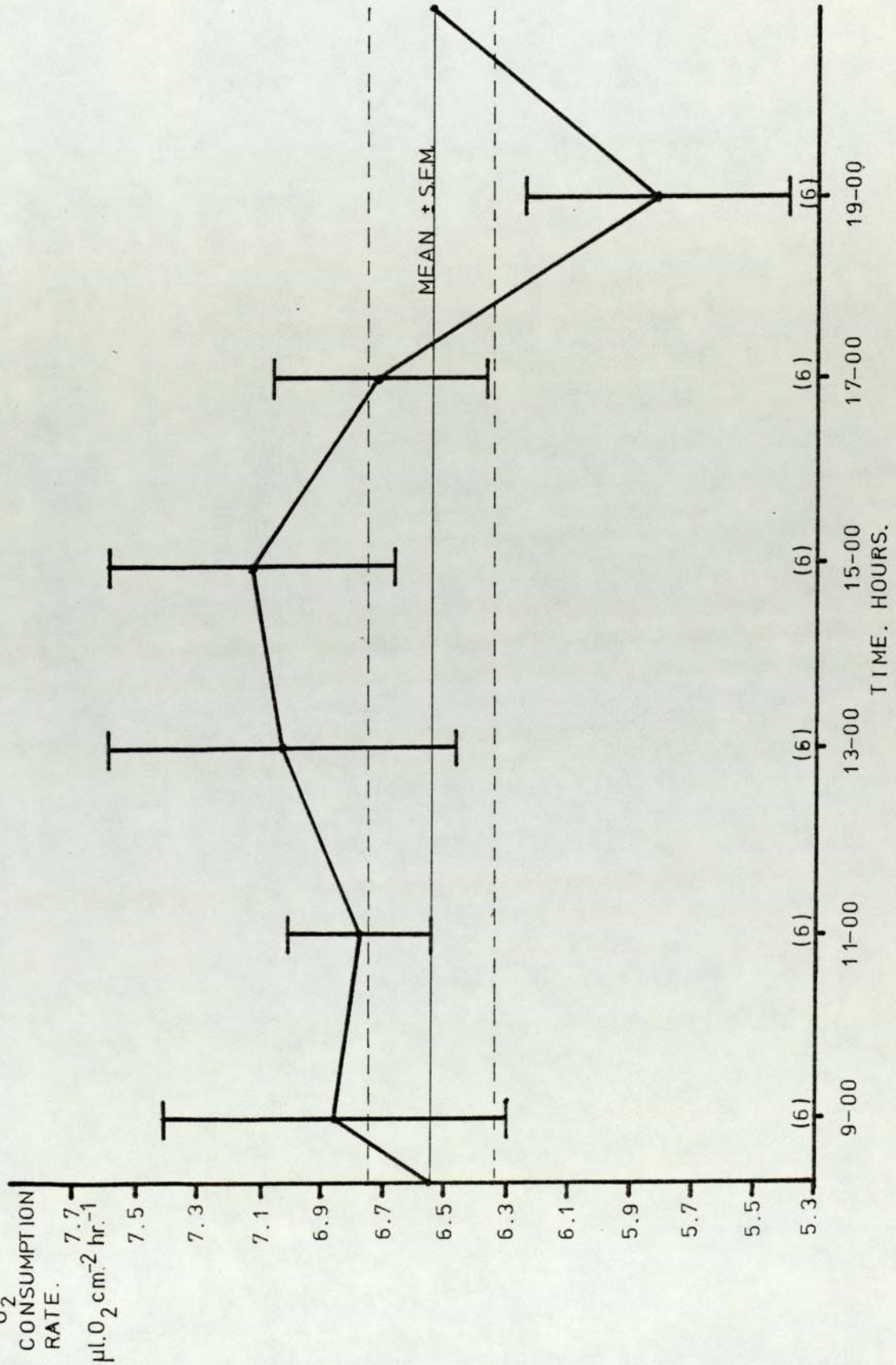


Table 6

Raw data for the diurnal variation in New Zealand white rabbit corneal oxygen consumption rate.

Values are in $\mu l\ O_2 \cdot\ cm^{-2} \cdot\ hr^{-1}$.

TIME OF READING. HOURS.

9-00	11-00	13-00	15-00	17-00	19-00
6.75	5.09	6.19	6.53	6.19	4.32
7.45	7.54	8.17	7.13	9.98	4.47
9.65	8.13	8.58	5.60	5.36	5.67
5.73	5.81	6.19	7.19	8.26	6.27
6.76	9.94	8.22	9.85	7.60	6.28
6.19	7.10	8.48	6.56		9.41
5.49	7.34	5.17	6.36		6.25
6.27	5.42	7.73	7.58		7.67
4.12	5.97	4.88	6.84		5.69
7.80	11.35	5.56	6.71		9.61
6.93	10.77	6.01			
11.35	5.27	3.99			
5.78	7.20	5.60			
4.17	7.60	7.46			
4.14	8.46				
8.96					
5.83					
N 17	15	14	10	5	10
Mean 6.67	7.53	6.59	7.05	7.48	6.56
SEM 0.4746	0.5045	0.3998	0.3541	0.8071	0.5754

Figure 16 Diurnal variation in oxygen consumption rate in the male New Zealand white rabbit. Values are the mean ± S.E.M. Number of readings is given in parentheses.

All animals are under sodium pentobarbital anaesthesia.

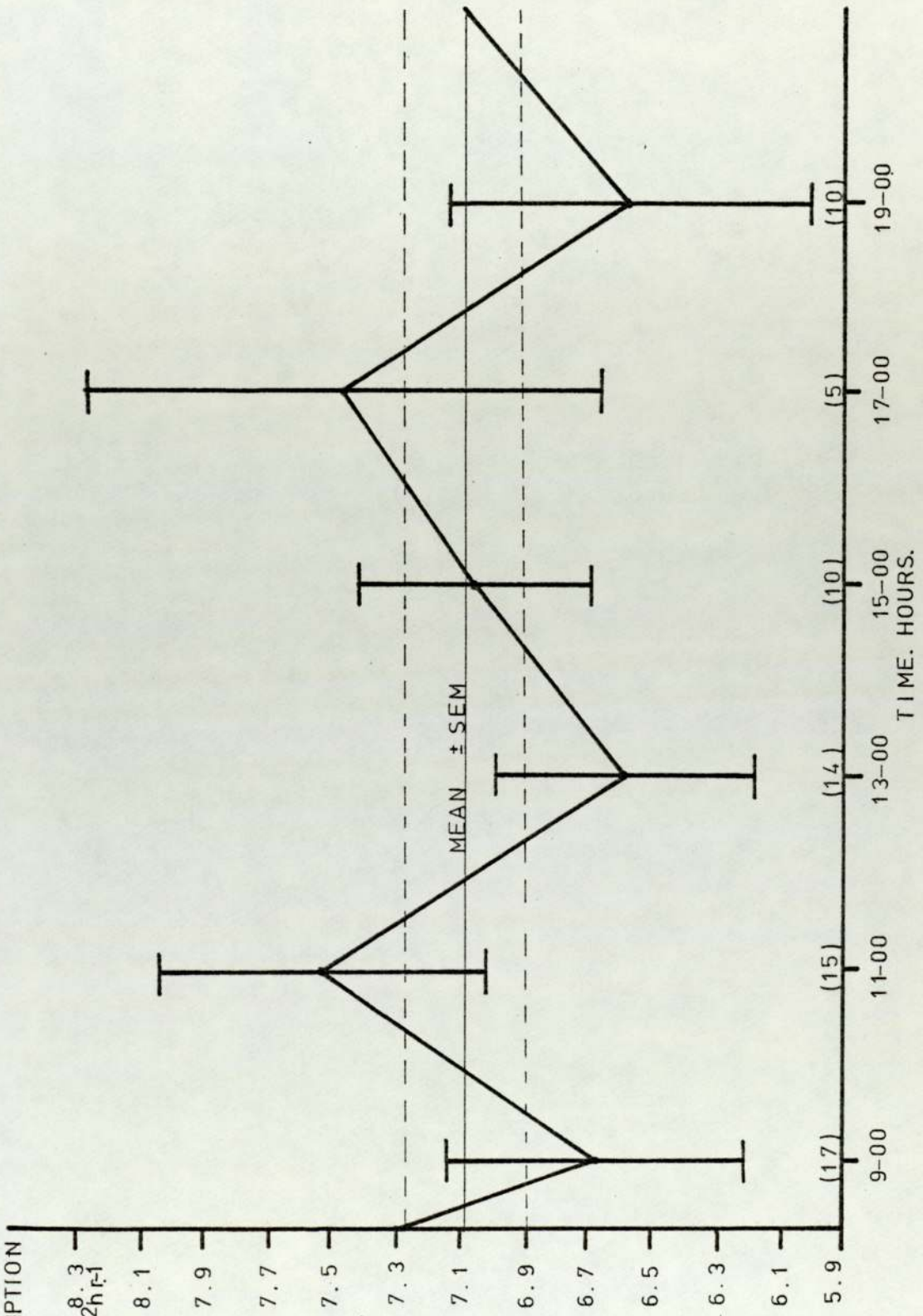


Table 7

Statistical data for variations in the mean between human and rabbit, and diurnal variation in both man and rabbit.

N	DATA	MEAN	SEM	COMPARISON	°FREEDOM	t	t for p=0.1	t for p=0.05	t for p=0.02	SIGNIFICANT
1	HUMAN MKI	6.24	0.3116	1 v 2	67	0.8381	1.67	2.0	2.39	NO
2	HUMAN MKII	6.55	0.2002	2 v 3	149	2.4467	1.65	1.96	2.36	YES
3	RABBIT MKII	7.29	0.1844							
	HUMAN DIURNAL									
4	9-00	6.85	0.5920	4 v 2	52	0.5094				
5	11-00	6.78	0.2328	5 v 2	52	0.4019				
6	13-00	7.03	0.5748	6 v 2	52	0.8036	1.675	2.01	2.45	NO
7	15-00	7.13	0.4706	7 v 2	52	0.9821				
8	17-00	6.72	0.3395	8 v 2	52	0.3008				
9	19-00	5.83	0.4346	9 v 2	52	1.6349				
	RABBIT DIURNAL									
10	9-00	6.67	0.4746	10 v 3	118	1.2555				
11	11-00	7.53	0.5045	11 v 3	116	0.4717				
12	13-00	6.59	0.3998	12 v 3	115	1.3406	1.66	1.98	2.36	NO
13	15-00	7.05	0.3541	13 v 3	111	0.3983				
14	17-00	7.48	0.8071	14 v 3	106	0.2225				
15	19-00	6.56	0.5740	15 v 3	111	1.1680				

One common feature to all three sets of data is the high range of values for oxygen consumption data:-

Mark I Human = 3.38 to 9.20 $\mu l\ O_2\ cm^{-2}.\ hr^{-1}$.

Mark II Human = 3.54 to 10.88 "

Mark II Rabbit = 3.99 to 11.90 "

The significance of this is discussed later.

Comparison of the mean human oxygen consumption rates obtained with the Mark I and Mark II sensors shows no significant difference. This is to be expected since there is no change in the critical components of the sensor namely the anode, cathode, electrolyte and membrane.

There are a number of factors to explain the significant ($P < 0.02$) difference between human and rabbit oxygen consumption rate:-

(a) Anaesthetic effect - AUGSBURGER and HILL (1972, 1973) have shown that topical anaesthetics have a significant effect on corneal oxygen consumption rate. The effect of topical anaesthetics is to reduce the corneal oxygen consumption rate. However in these experiments there is an increase in oxygen consumption rate in the rabbit and therefore the anaesthetic is unlikely to be responsible for the difference in oxygen consumption rate. It is noteworthy that in the experiments of AUGSBURGER and HILL (1972, 1973) the animals are all maintained under sodium pentobarbital/urethane general anaesthesia. These authors seem to have considered the effects of systemic general anaesthetics to be negligible or constant. HERRMANN et al., (1942) also found that topical anaesthetics reduced oxygen consumption and that the systemic anaesthetics sodium pentobarbitone and urethane have no effect. The effect of sodium pentobarbital in our system is unlikely to be responsible for the difference in oxygen consumption

rate between human and rabbit.

(b) The second and more plausible explanation is that of species difference. Most biological systems show species variation; corneal oxygen consumption rate seems also to possess this feature (HILL and FATT, 1963 (a); EDELHAUSER, 1974).

Comparison of the values for mean oxygen consumption rate in the human and rabbit with other authors highlights not only the large range between individual measurements, but also the differences between measuring systems:-

This study Mean = 6.55, Range = 3.39 to 9.20 $\mu l\ O_2\ cm^{-2}\ hr^{-1}$.

HILL and FATT (1963 (a)) Mean = 4.80, Range = 2.44 to 5.49 $\mu l\ O_2\ cm^{-2}\ hr^{-1}$.

HILL and FATT (1963 (b)) Mean = 1.90, Range = 1.6 to 3.7 $\mu l\ O_2\ cm^{-2}\ hr^{-1}$.

JAUREGUI and FATT (1971) Mean = 0.65 $\mu l\ O_2\ cm^{-2}\ hr^{-1}$.

JAUREGUI and FATT (1972) Mean = 2.8 $\mu l\ O_2\ cm^{-2}\ hr^{-1}$.

All of these systems use a 25 μ Platinum cathode and a 12 μ membrane.

A degree of variance is plainly due to the different methods of oxygen flux calculation. The results emphasize the inaccuracies that can occur within and between methods of oxygen flux determination.

Similar variability is found when comparing rabbit corneal oxygen consumption rates:-

This study - Mean = 7.29, Range = 3.99 to 11.90 $\mu l\ O_2\ cm^{-2}.\ hr^{-1}$.

Values (a) and (b) are converted from respirometer data by HILL and

FATT (1963 (a)).

(a) LANGHAM (1952) Mean = 8.6 $\mu l\ O_2\ cm^{-2}.\ hr^{-1}$.

(b) ROBBIE et al. (1947) Mean = 4.6 $\mu l\ O_2\ cm^{-2}.\ hr^{-1}$.

(c) FARRIS et al. (1965) Mean = 1.05 "

(d) FREEMAN (1969) Mean = 2.50 "

(e) EDELHAUSER (1974) Mean = 7.61 $\mu l\ O_2$ per cornea per hour, therefore assuming a corneal diameter of 13.0 mm., oxygen consumption on a per unit area basis = 5.73 $\mu l\ O_2\ cm^{-2}.\ hr^{-1}$.

Likewise there is a similar variation in data from studies, as with the human cornea, according to the method of calculation. Further variation may arise from the use of different subspecies of rabbit and different working temperatures.

Even allowing for these factors, in both the rabbit and human large discrepancies are evident. One possible explanation for the discrepancies between data is that the thickness of the electrolyte layer is not taken into account in the calculation. The thickness of the electrolyte layer is a function of sensor design and manufacture and is thus likely to vary considerably between sensor type and even between sensors of the same design specifications. The electrolyte layer is a component of the reservoir which is usually considered constant, however for each system the thickness of the electrolyte is constant but variation may occur between sensors. In some sensors the electrolyte thickness may be greater than the membrane thickness, as a result the value for membrane thickness that constitutes the reservoir volume may be an order of magnitude different from that actually taking part in the measurement.

Tables 5 and 6 show diurnal variation in corneal oxygen consumption in man and rabbit respectively. Comparison of the mean values of oxygen consumption rate for each of the six times during the day with the grand mean of corneal oxygen consumption rate shows no significant difference in either man or rabbit.

Visual analysis of Figure 14 indicates a peak of oxygen consumption rate in the mid-afternoon, 15-00 hours, and a fall in consumption rate as the afternoon progresses. A similar trend is found in whole body oxygen consumption rate (LACKEY et al., 1970) and in intraocular pressure in the rabbit (KIKKAWA, 1973, 1974). It is possible to infer from these results that diurnal variations in corneal oxygen consumption rate parallel those of general body functions.

Similar data for the rabbit reveals no discernable diurnal changes in the rate of oxygen consumption by the cornea. A possible explanation for this difference is that diurnal variations become negligible under anaesthesia.

It is well established that the cornea of the open eye obtains most of the oxygen required for its cellular respiration from the atmosphere. In the closed eye the capillaries of the Palpebral Conjunctiva and the Aqueous Humour are sufficiently rich in oxygen to adequately supply oxygen to the cornea. When a contact lens is worn, whether it be a hard or soft lens, there is a reduction of the oxygen tension at the corneal surface and within the corneal epithelium. The changes in the stroma, endothelium and Aqueous Humour are uncertain however it is

likely that the oxygen does not fall much below that of the Aqueous Humour, 55 mm Hg (FATT, 1968 (a), 1972 (b); HILL and CUCKLANZ, 1967; HILL and FATT, 1964; HILL and AUGSBURGER, 1971; HILL et al., 1973). If the oxygen tension at the epithelial surface is reduced to a level whereby insufficient oxygen is provided to supply the respiratory needs of the corneal cells, an oxygen debt is built up (HILL et al., 1972). If the oxygen tension is reduced still further or the oxygen debt not satisfied then the cornea will enter respiratory distress, the result being a number of traumatic conditions (SMELSER and OZANICS, 1952, 1953; LANGHAM and TAYLOR, 1956; DALLOS, 1946; KINSEY, 1952; MANDELL et al., 1970).

One critical factor, essential for the successful wearing of contact lenses is that the cornea obtains an adequate supply of oxygen. In the case of a hard contact lens, oxygen is provided by blink induced rocking which "pumps" tears saturated with air to the cornea at each blink (FATT, 1969; FATT and HILL, 1970; ROSENTHAL, 1968). In the soft gel lens no such rocking motion occurs and oxygen must be transmitted through the lens.

The quantity of oxygen that must be transmitted to the cornea is now known with reasonable precision. The cornea cannot consume oxygen at its normal rate if the oxygen tension at the epithelial surface falls below a critical value (POLSE and MANDELL, 1970 (b)). The successful contact lens will therefore transmit the required oxygen without allowing the oxygen tension to drop below the critical value at the lens cornea interface (FATT and ST. HELEN, 1971; FATT et al., 1969).

The ability of a gel contact lens to transmit the necessary oxygen to the cornea without an excessive drop in oxygen tension depends on the oxygen uptake rate of the cornea at its anterior surface, oxygen transmissibility, thickness and the oxygen tension at the anterior surface of the lens (FATT, 1972 (a), (b)). Measurement of oxygen transmissibility of a gel contact lens is possible (FATT and ST. HELEN, 1971; FATT, 1972 (a) (b); FATT et al., 1977). Both the oxygen tension of the atmosphere, and the oxygen tension of the palpebral conjunctiva are known (FATT and BEIBER, 1968) thus in principal it is possible to predict the success of a given contact lens providing the thickness and transmissibility have been measured.

The problem with such a prediction is that it is based upon the accuracy of the measurement of oxygen uptake rate by the cornea, and the critical oxygen tension required by the cornea for it to be able to consume oxygen at its normal rate and maintain normal function.

The oxygen consumption rate in this experiment has been shown to be highly variable. If the critical oxygen tension is as variable as oxygen consumption rate, then the success of any given contact lens will vary greatly from person to person. Eyes that have a high oxygen consumption rate and a high critical oxygen tension will either require a highly oxygen permeable lens or a very thin lens.

This experiment has shown that routine measurement of the rate of oxygen consumption is possible and has partially solved the problem of matching lens to patient requirements. There remains only the problem of determining the critical oxygen tension in individual patients.

No patient in this study suffered any major corneal damage. In a very small percentage of patients, traces of diffuse punctate stains were seen. Damage is usually a result of apprehension by the patient producing movements of the eye when the sensor is being placed on the cornea. Patients familiar with the techniques showed no such symptoms indicating that stress was the main cause of damage.

Once patient and operator are more experienced then the use of a polarographic oxygen sensor system becomes a simple procedure. The economy of equipment, accuracy and low stress factor of the system suggests that it is possible to develop a system for routine use suitable for determining the effect of contact lenses on corneal metabolism. Thus this oxygen sensor system provides a suitable diagnostic technique for use in the clinical environment.

2.8 CONCLUSION

Corneal oxygen consumption rates using a Clark type micropolarographic oxygen sensor are:-

Human Mark I = 6.24 ± 0.31. $\mu l\ O_2\ cm^{-2}.\ hr^{-1}$.

Human Mark II = 6.55 ± 0.20 "

Rabbit Mark II = 7.29 ± 0.18 "

There is no statistically significant diurnal variation in corneal oxygen consumption rate between the hours of 9-00 a.m. and 7-00 p.m. in human and New Zealand white rabbit males demonstrated in this study.

The description of individual rates of oxygen consumption by various patients has partially solved the problem of matching different contact lens properties to the physiological requirements of the individual patient.

Accuracy, low risk and low cost suggest that the use of a polarographic oxygen sensor system similar to that described in this work in assessing the affects of contact lenses on corneal respiration in the clinical environment is a realistic proposition.

SECTION 3

CORNEAL CARBON DIOXIDE EFFLUX

Mean epithelial carbon dioxide efflux rate in the *in vivo* New Zealand white rabbit cornea.

3.1 INTRODUCTION

To develop a comprehensive picture of the metabolism of the cornea, in addition to oxygen requirements, it is also necessary to monitor changes in end product metabolites.

Carbon dioxide efflux from the cornea is investigated with the view that such information may supply information regarding the nature of the respiratory mechanisms present in the cornea and how they are affected by the presence of a contact lens. Again with the hope that a suitable method for clinical use can be developed.

There are a number of investigations on the respiration of the cornea, however these methods all use *in vitro* techniques (LANGHAM, 1951; DE ROETTH, 1950, 1951; DUANE, 1949 (b); HERRMANN et al., 1942). HABERICH and DENNHARDT (1966) succeeded in comparing oxygen and carbon dioxide fluxes across the cornea by gas micro-analysis, however the method required enucleation and is therefore clinically unsuitable.

In vivo determination of carbon dioxide efflux as performed by FATT et al., (1964) involves the use of electrochemical analytical techniques. The method of FATT et al. (1964) forms the basis of the present study, the aim of which is to develop a carbon dioxide electrode with proportions small enough to enable the electrode to

fit directly into the palpebral aperture without discomfort, a response time that is acceptable to the patient and to use the system to determine a mean value for the in vivo carbon dioxide efflux rate of a rabbit cornea.

3.2 THEORY OF ELECTRODE OPERATION

STOW et al., (1957) first determined the partial pressure of carbon dioxide in liquids by measuring the pH of a film of water separated from the sample by a rubber membrane which is freely permeable to carbon dioxide but not to hydrogen or bicarbonate ions. Thus the pH of the sample is prevented from affecting the pH at the glass electrode surface.

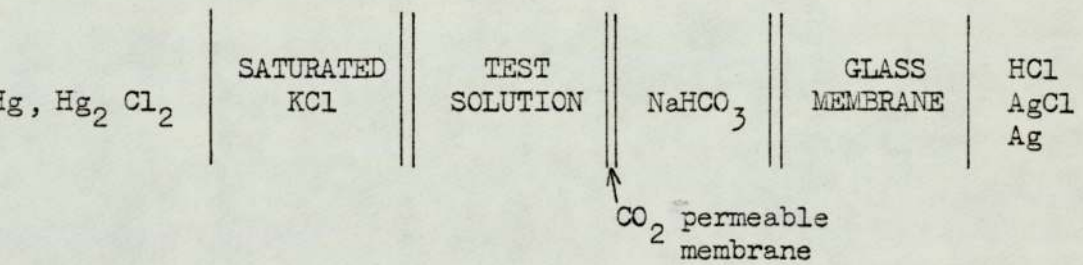
In the presence of pure water, carbon dioxide slowly forms carbonic acid which immediately dissociates into equal quantities of hydrogen and bicarbonate ions (SEVERINGHAUS and BRADLEY, 1971).

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-.$$

If excess bicarbonate ions are produced by adding sodium (or potassium) bicarbonate, the increment in bicarbonate ion concentration due to the dissociation of carbonic acid is negligible and all the increment occurs in the hydrogen ion. The product of the ions must equal the dissolved carbon dioxide multiplied by the apparent dissociation constant (SEVERINGHAUS and BRADLEY, 1958; SEVERINGHAUS, 1968).

The relationship of pH to pCO_2 is represented by the equation developed by SEVERINGHAUS (1968) to combine the equilibrium equations of the first and second order dissociations of carbonic acid. The sensitivity of each electrode system varies according to the concentration of sodium bicarbonate in the electrolyte and can be estimated when SEVERINGHAUS' equation is completed. Figure 1.

The electrolyte system consists of a pH electrode and a carbon dioxide permeable membrane separated by a layer of bicarbonate solution. This arrangement forms an electrochemical cell and schematically can be described as:-



(LAI et al., 1975) Figure 2.

3.3 APPARATUS

Design Requirements

Necessary specifications for a functional carbon dioxide electrode are:-

(a) Size: The electrode must be a suitable size to fit comfortably into the palpebral aperture of the patient.

(b) Safety: The electrode must be chemically and electrically isolated from the patient.

(c) Stability: No variations in potential difference during the experimental period.

Figure 1 The effect of bicarbonate ion concentration on CO_2 electrode sensitivity. After Severinghaus (1968).

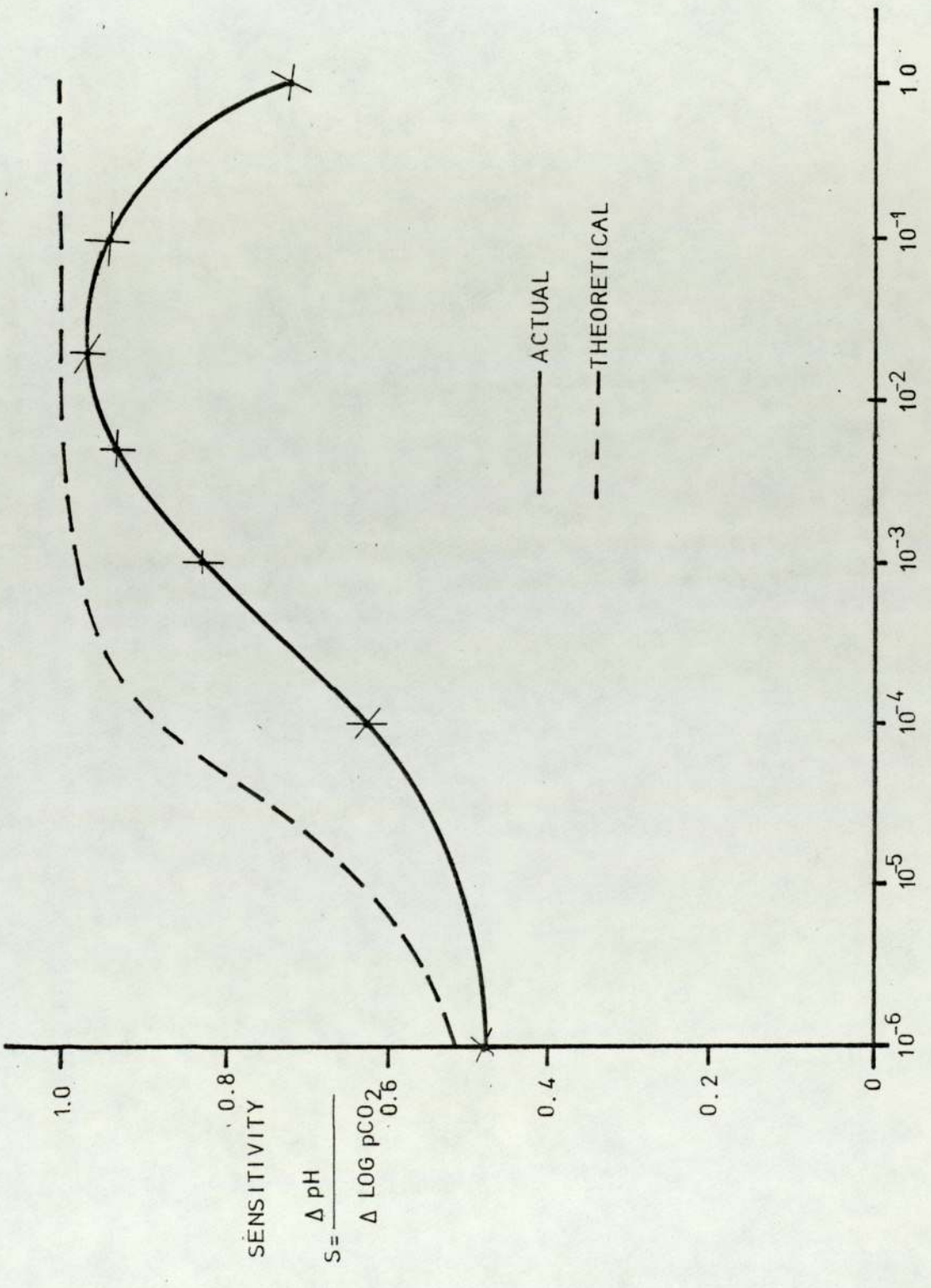
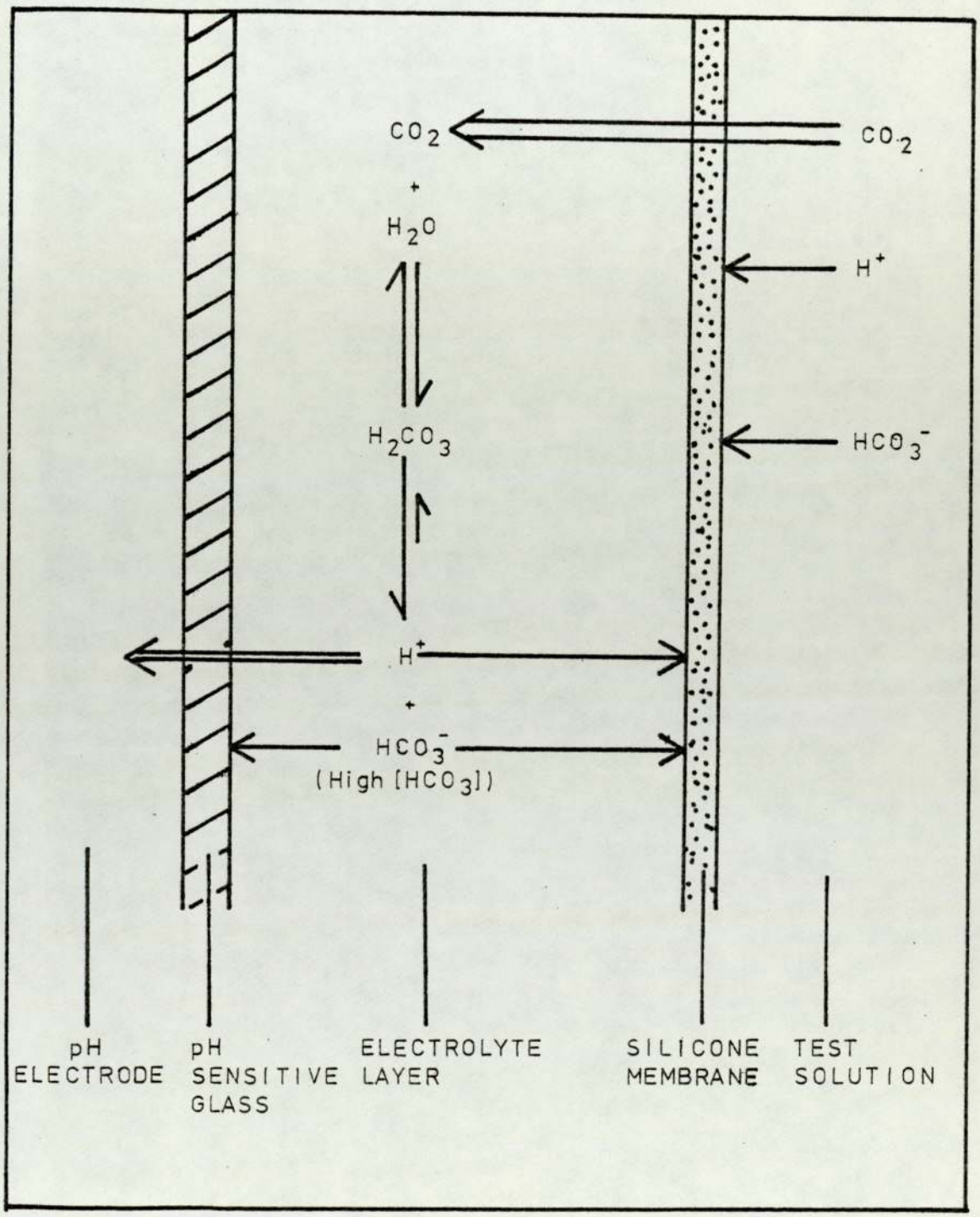


Figure 2 A schematic of CO_2 flux at the electrode membrane and the reactions of CO_2 with the electrolyte.



(d) Response time: Minimal.

(e) Accuracy: Maximum.

The only commercial electrode with a flat tip and a diameter of the required size is that of Radiometer, Model E5038-0 (Radiometer, V A Howe Ltd.). Figure 3 shows the E5038-0 carbon dioxide electrode.

The E5038-0 has a tip diameter of 10mm., by turning down the extreme tip of the electrode casing on a lathe it is possible to reduce this to 6.263mm. The electrode consists of a pH electrode that has a flat tip of pH sensitive glass attached to a glass column. The reference electrode consists of a strip of silver coated with silver chloride wound around the top of the glass column. Electrical contact is made via the electrolyte that surrounds the reference electrode and covers the pH sensitive tip. The electrolyte is held in place by a membrane that fits over the end of the electrode. A layer of electrolyte is maintained over the sensitive tip by a nylon spacer positioned between the membrane and electrode tip. The Electrolyte S43116, (Radiometer Ltd) consists of 0.005 M $NaHCO_3$ and 0.02 M NaCl. The membranes used are 25 μ Silicon rubber, D606, (Radiometer Ltd.). The mesh spacers are nylon, 0.050mm. thick (Radiometer Ltd.). Figures 4 and 5 show photographs of the E5038-0 as used in this study.

Peripheral Equipment

Peripheral equipment used in the determination of pCO_2 consists of a high impedence amplifier, variable potentiometer and chart recorder. Figure 6.

Figure 3 Scale drawing of the Radiometer E5038-0 CO_2 electrode modified for use in this study.

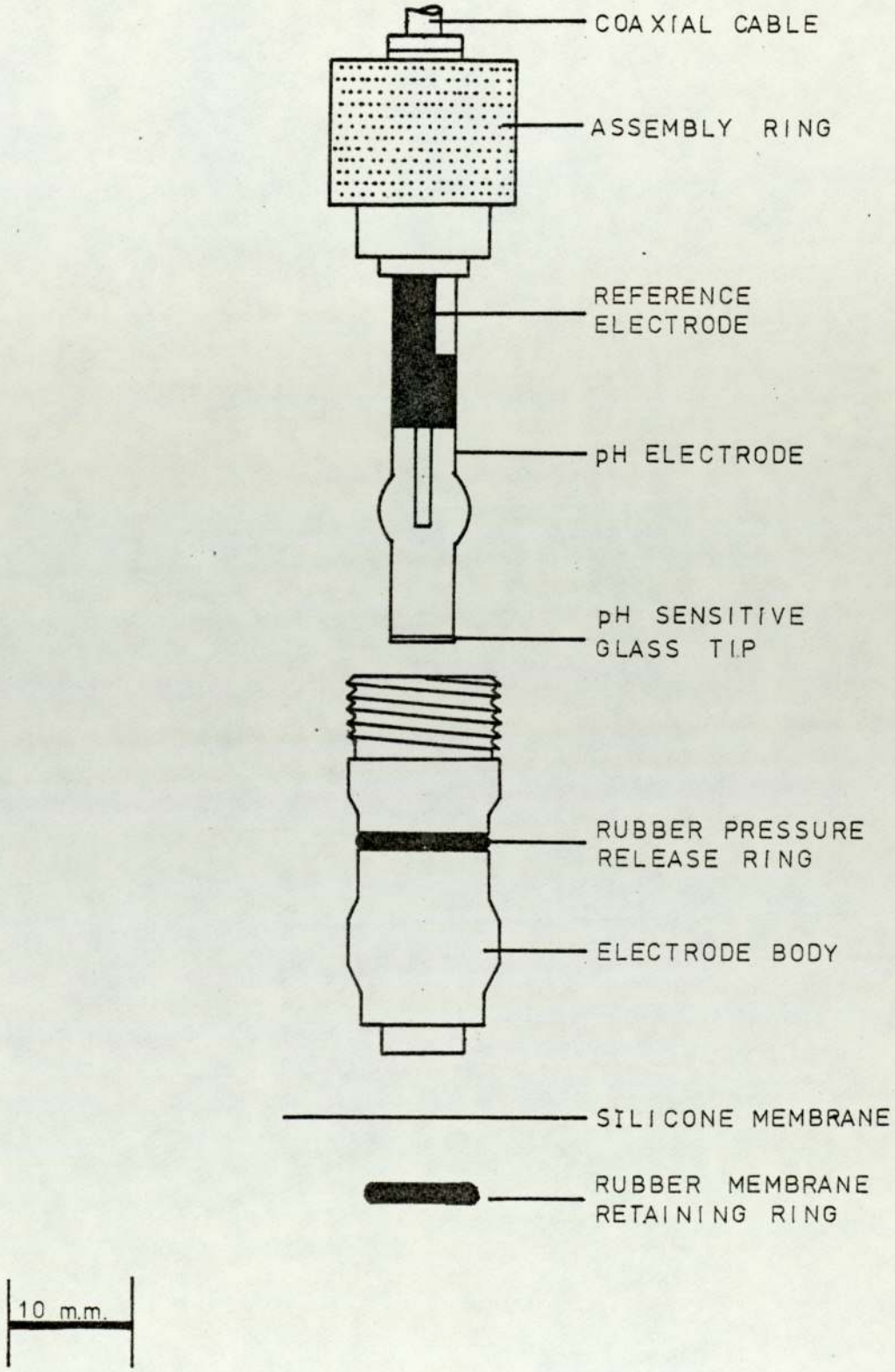


Figure 4 An exploded view of the Radiometer Model E5038-0 CO_2 electrode.

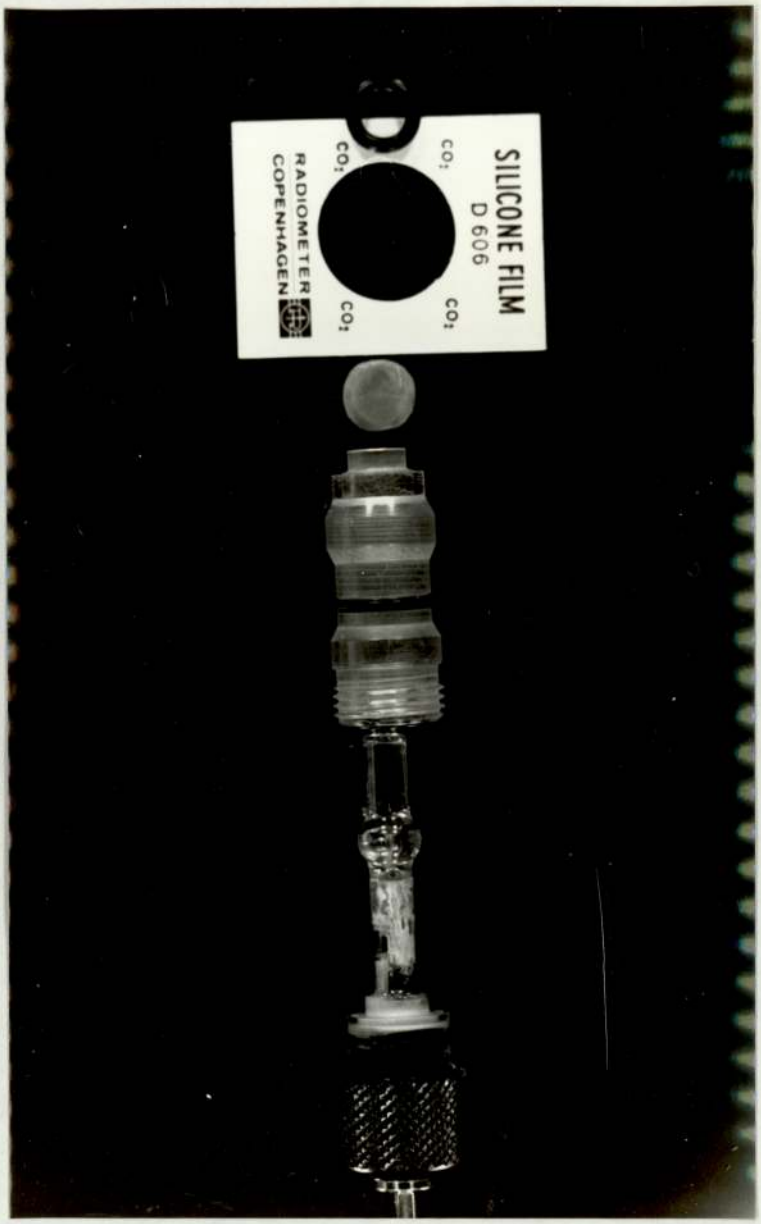


Figure 5 The Radiometer Model E5038-0 CO_2 electrode assembled and ready for use.

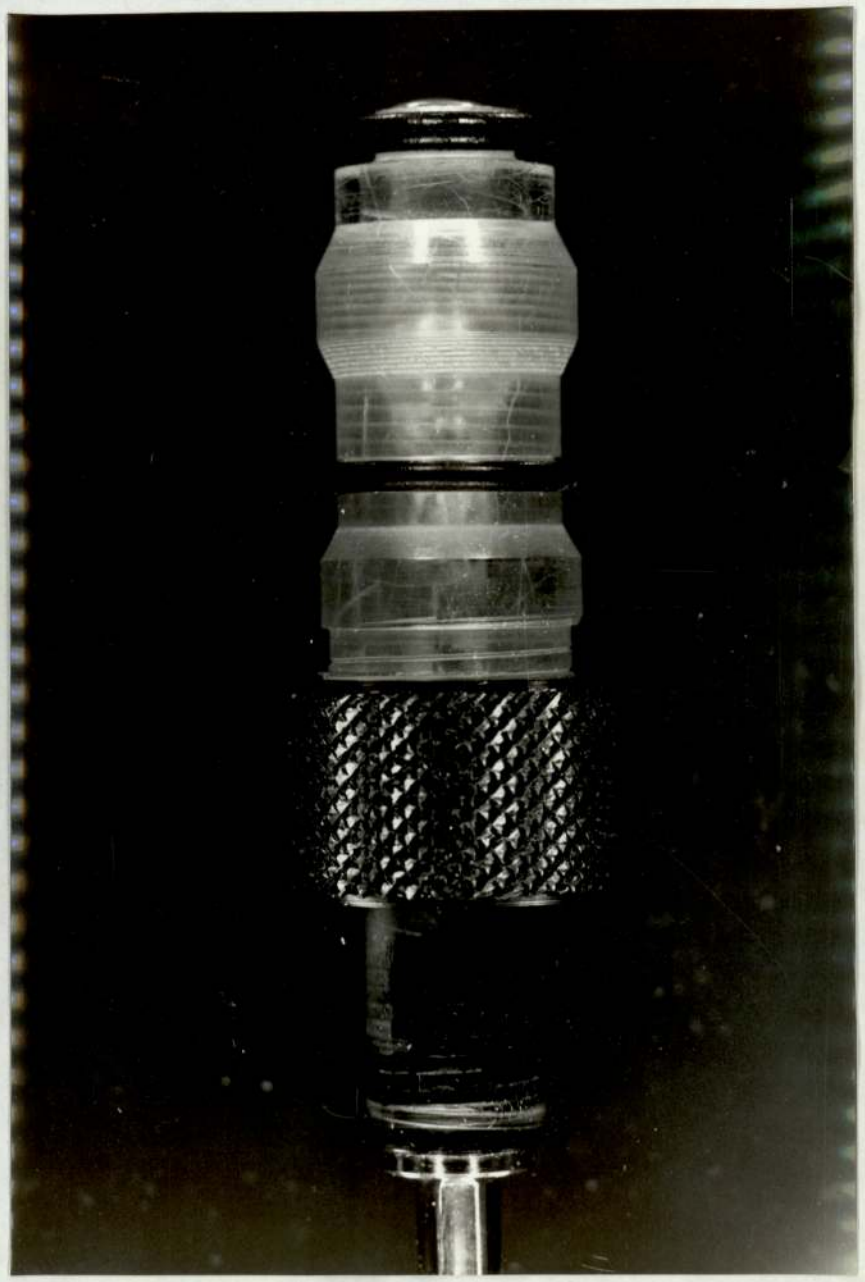


Figure 6 Peripheral equipment to the Radiometer E5038-0 CO_2 electrode.

A = High impedance amplifier

B = Potentiometer box

C = Chart recorder

The amplifier is a high impedence "Electrometer" amplifier (Z (IN) = $10^{15}\Omega$ minimum), Electronic Instrumentation Laboratories Ltd.

The potentiometer box is similar to the instrument used for the oxygen electrode, section 2.3, and performs the same function, that is matching amplifier output to chart recorder input.

The chart recorder is the Vitatron Model UR40 (Vitatron U.K. Ltd.) as used previously.

Electrode Properties

Temperature: Both pCO_2 electrodes and the dissolved carbon dioxide tension vary with temperature (BRADLEY et al., 1956), however it is possible by calculation to correct these errors (KELMAN and NUNN, 1966). To avoid lengthy calculations the electrode is calibrated at 34°C. When not in use the electrode is maintained in air saturated 0.9% sodium chloride solution.

Pressure: Variations in potential difference due to pressure effects can be determined in the same manner as that used for the oxygen sensor, section 2.3. The results show that pressure has a negligible effect on the carbon dioxide electrode.

Stability: Stability is checked by placing the electrode in air saturated 0.9% sodium chloride solution at 34°C and observing the potential difference hourly for eight hours. The response of the electrode varied over the first hour after which the output is constant.

Fluctuations during the first hour are considered to be due to settling of the silicon rubber membrane over the spacer.

Response time: This is taken as the time taken for a 95% response as the electrode is moved from a nitrogen saturated 0.9% sodium chloride solution to a similar air saturated solution, and is found to be two minutes. Later 10% and 20% carbon dioxide in nitrogen gas mixtures became available and these replaced the nitrogen and air saturated solutions when measuring the response time. To minimise the response time a number of electrode modifications are made:-

(a) Spacer: The spacer placed between membrane and electrode tip traps a layer of electrolyte into which the carbon dioxide molecules diffuse and react to produce H^+ ions. Response time varies according to the thickness of the spacer. Thus reducing spacer thickness is an effective method of reducing the response time. Substituting fibres of lens cleaning paper (Joseph paper) for the nylon mesh spacer reduces the spacer thickness. However despite the slight decrease in response time obtained it is difficult to control the number of fibres lying at the interface. As a result the electrode Potential Difference varies between experiments and calibration is difficult. Thus it is considered that no advantage can be obtained from this procedure.

(b) Electrolyte: Maximum electrode sensitivity is achieved by adjusting the bicarbonate concentration in the electrolyte as shown in Figure 1 (SEVERINGHAUS, 1968). By adjusting the electrolyte from 0.005M $NaHCO_3$, 0.02M NaCl to 0.001M $NaHCO_3$ and 0.02M NaCl, the decrease in bicarbonate concentration reduced the response time of the electrode

by approximately 50% from two minutes to one minute.

A further modification is the addition of carbonic anhydrase at a concentration of 1 mg ml^{-1} (SEVERINGHAUS and BRADLEY, 1971) to the electrolyte solution. Carbonic anhydrase catalyses the rate limiting step in the reaction, that is, the formation of carbonic acid from carbon dioxide and water. Thus, this will increase the rate of ion production since carbonic acid immediately dissociates to H^+ and HCO_3^- ions.

$$H_2O + CO_2 \xrightleftharpoons{\text{Carbonic Anhydrase}} H_2CO_3 \rightleftharpoons H^+ + HCO_3^-.$$

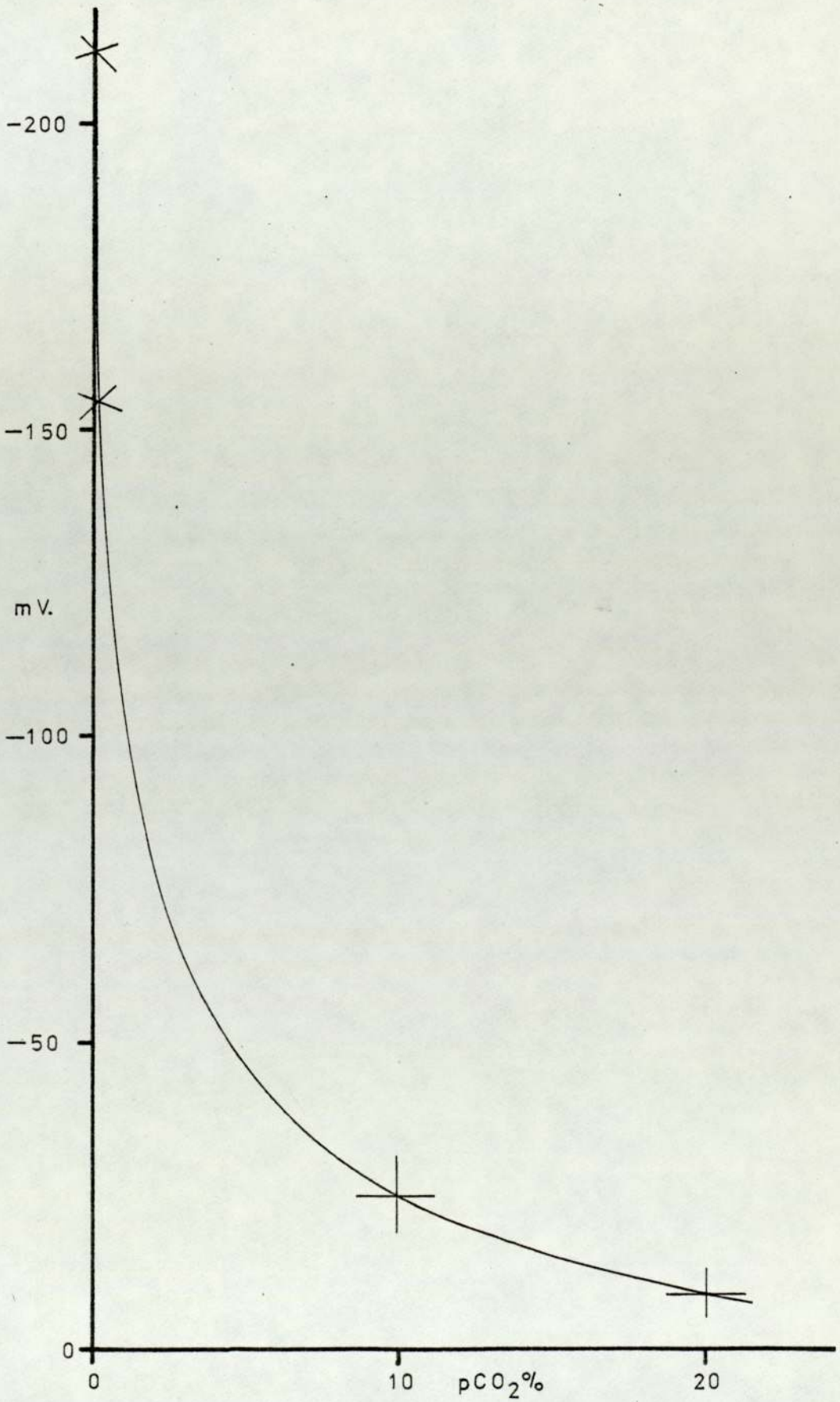
Carbonic anhydrase is added to both the standard electrolyte and the reduced bicarbonate electrolyte. The most rapid response time, equivalent to approximately fifteen seconds is obtained with the standard electrolyte-carbonic anhydrase mixture.

Calibration

The system is calibrated by immersing the electrode in 0.9% sodium chloride solutions at 34°C saturated with gases of varying carbon dioxide concentration and measuring the changes in millivolt (mV.) output, Figure 7.

The concentrations of CO_2 used are, 0.0 mm Hg (N_2), 0.3 mm Hg (Air), 76 mm Hg (10% CO_2, 90% N_2), 152 mm Hg (20% CO_2, 80% N_2). Using electrolyte system III (0.005M $NaHCO_3$ + 1 mg ml^{-1} carbonic anhydrase) the potential difference readings are:-

Figure 7 A calibration curve for the Radiometer E5038-0 CO_2 electrode.



N_2 = -212 mV

Air = -155 mV

10% CO_2 = -25 mV

20% CO_2 = -9 mV

Figure 8 shows the linear relationship between mV. and logarithm of pCO_2, as shown by FATT et al (1964) and SEVERINGHAUS (1968). Thus providing that the mV. reading is known, the pCO_2 concentration can be calculated and at any pCO_2 the pH, or in this case the potential difference reading, is a direct relation to the logarithm of pCO_2.

Carbon Dioxide Sleeve

Similar to the oxygen sensor system fluctuations in the electrode reading are reduced by improving comfort for the patient and thus diminishing the rate of eye movements. A hydrophilic polymer sleeve is made from Itek 107 (Itek Corporation). Figure 9. Figure 10 shows a sleeve hydrated and ready for use. The polymer has a 60% water content and is hydrated in 0.9% sodium chloride solution and boiled twice before use.

The electrode is pushed into the sleeve and the complete unit maintained in air saturated 0.9% sodium chloride solution until required. Figure 11.

3.4 METHODOLOGY

Electrode Properties

The experimental procedure is identical to that used for the oxygen

Figure 8 A semi-logarithmic plot of the calibration curve, Figure 7.

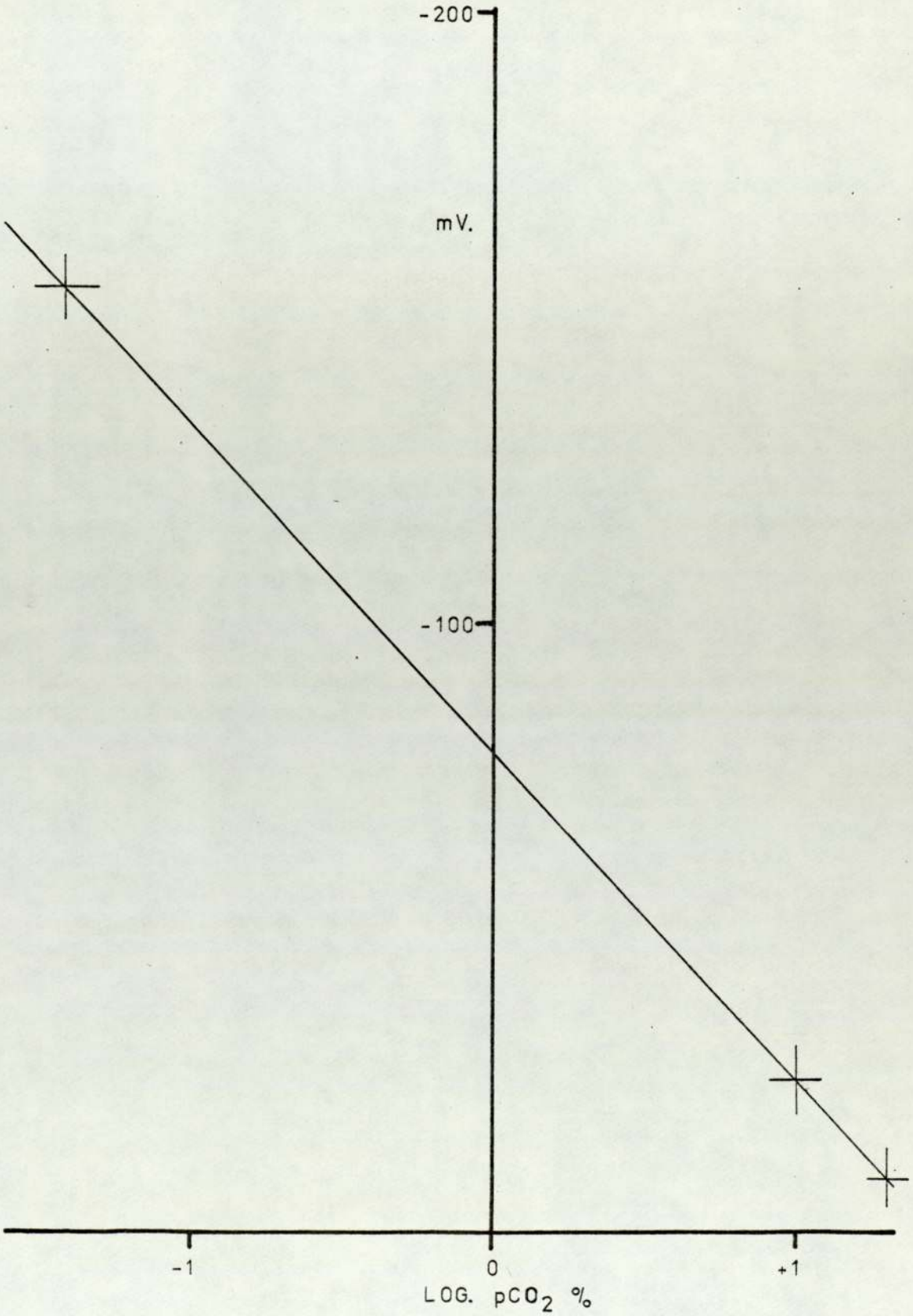
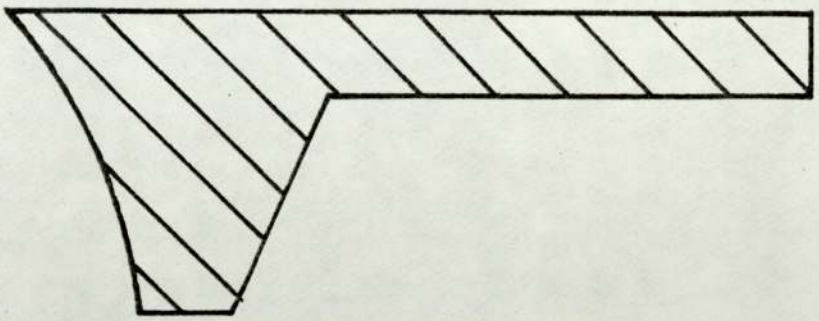


Figure 9 A scale drawing of the hydrogel sleeve to fit the Radiometer E5038-0 CO_2 electrode as modified for this study.

Dehydrated dimensions can be derived by dividing by the factor 1.338, this being the linear swell value for Itek 107.



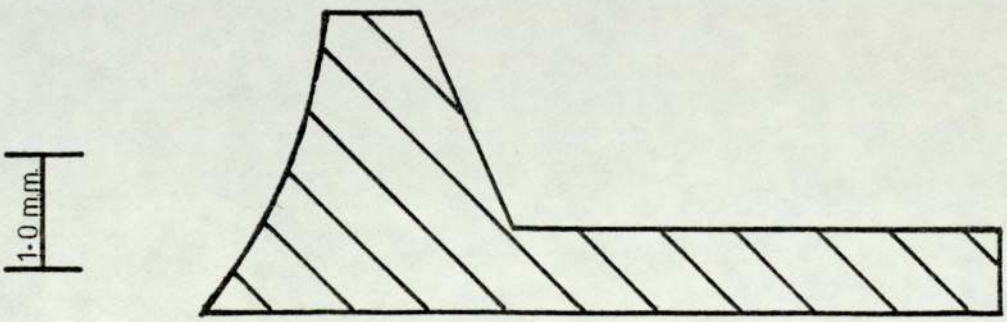


Figure 10 A CO_2 electrode sleeve, hydrated and ready for use.

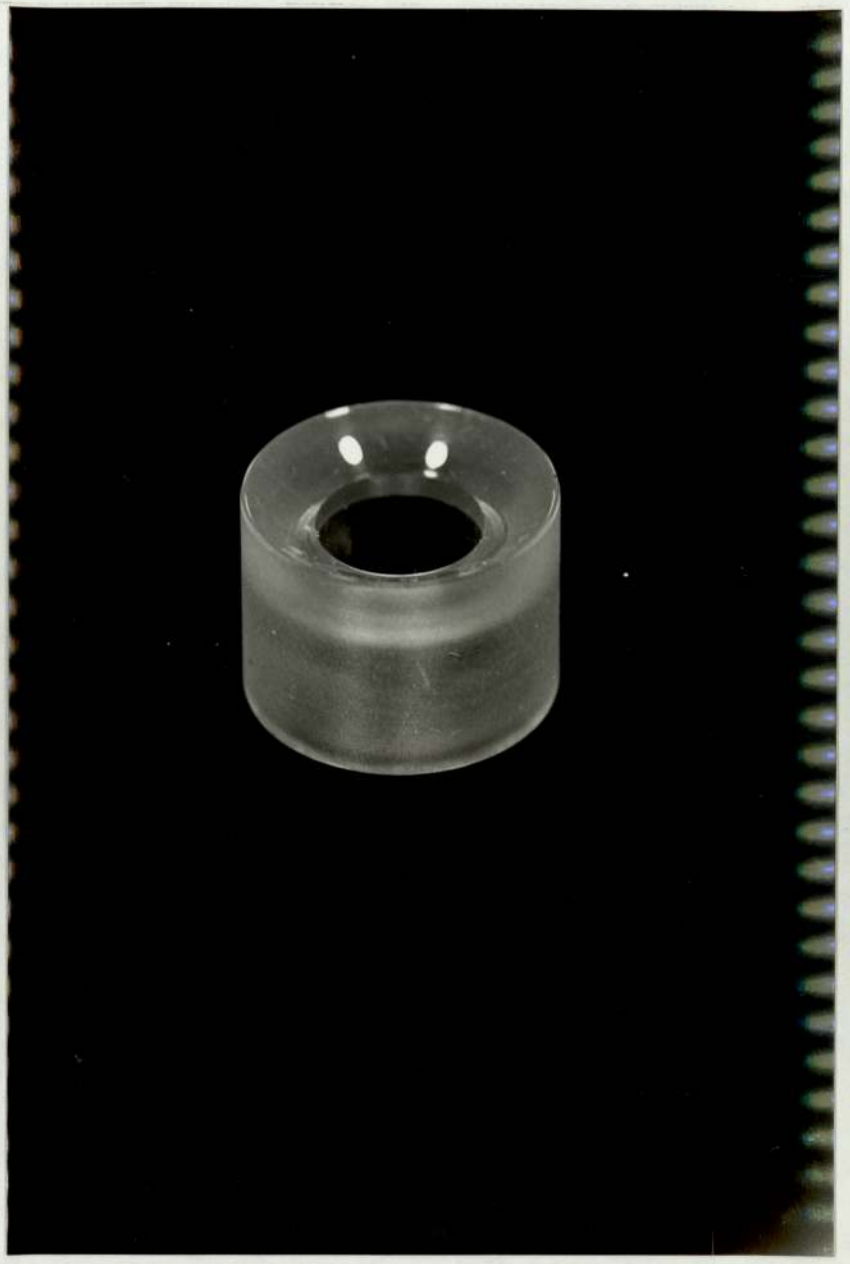
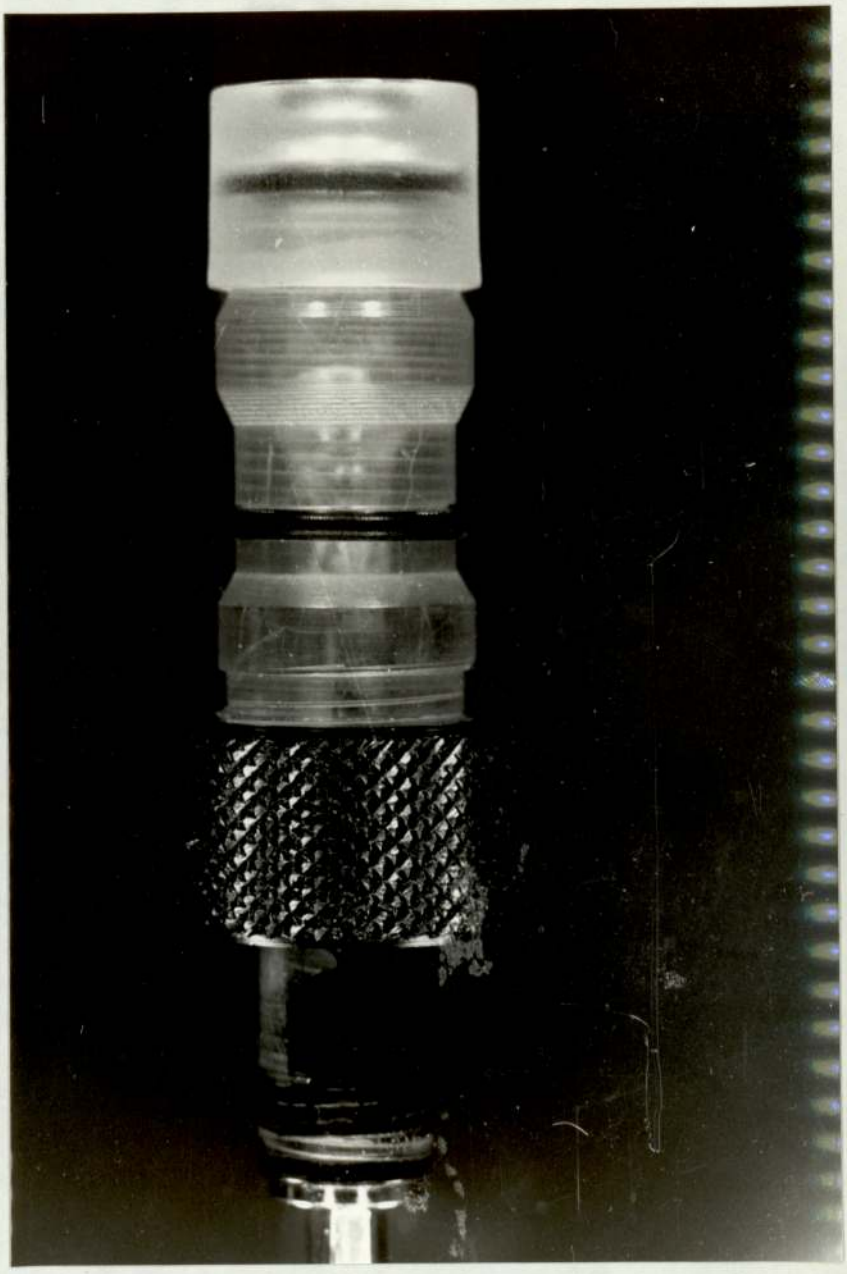


Figure 11 The modified Radiometer E5038-0 CO_2 electrode mounted in a hydrated hydrogel sleeve ready for use.



sensor, section 2.4, and entails placing the pCO_2 electrode unit directly into contact with the corneal epithelium and maintaining its position long enough for a representative trace of carbon dioxide efflux to be observed.

The pCO_2 electrode is freshly prepared as required using a new, membrane, spacer and electrolyte solution.

The electrode is calibrated as described in section 3.3 (Figure 7).

When the subject is prepared the electrode unit is rinsed thoroughly with sterile 0.9% sodium chloride solution at 34°C. Following the experiment the sleeve is again sterilised and the electrode rinsed in sterile 0.9% sodium chloride solution and returned to the maintaining bath.

Subject Preparation

Male New Zealand white rabbits 2 to 3Kg. body weight are used. They are given a general anaesthesic as described in section 2.4. The eye-lids and nictitating membrane are retracted with the fingers of one hand while the other holds the electrode unit to the cornea.

Determination of Mean Carbon Dioxide Efflux Rate

The experiment is performed on rabbits at intervals throughout the day. No rabbit being used more than once in any one week.

3.5 RESULTS

Many recordings of the rate of carbon dioxide efflux from the rabbit cornea are taken.

Figure 12 is a typical trace obtained from the left eye of a New Zealand white rabbit under sodium pentobarbital anaesthesia. Section 3.3. Figures 7 and 8 show the calibration curve and semi-log treated curve from this experiment.

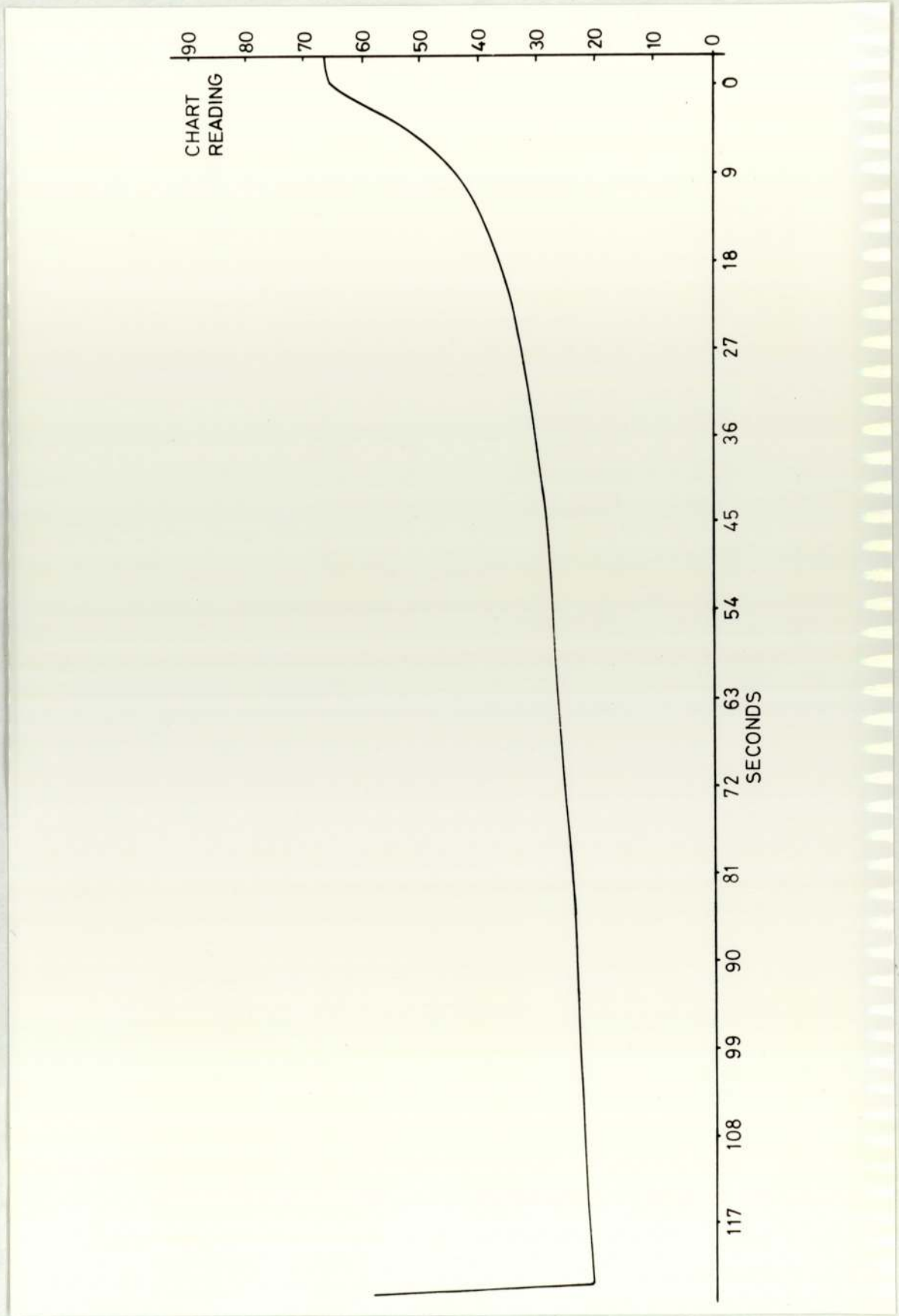
3.6 INTERPRETATION OF DATA

An electrode system is produced with a minimum response time. Calibration is performed in 0.9% sodium chloride solutions at 34°C saturated with varying gas mixtures, section 3.4. Figures 6 and 7.

Figure 12 is a typical trace. It is necessary to convert values of y on the trace to mV. This is performed by multiplying the chart reading by 2.12, derived from the 100% scale response on the chart recorder that is set at -212 mV., a 1% reading is equivalent to -2.12 mV. (-212 mV is the reading obtained when the electrode is placed in 0.9% sodium chloride solution saturated with nitrogen gas).

In order to analyse the data, the various carbon dioxide fluxes in the cornea must be considered. In the open eye the concentration of carbon dioxide at the corneal surface is small, approximately 0.3 mm Hg, depending on the atmospheric pressure, humidity and particular environment. For example the atmosphere in a room full of people will have a

Figure 12 A typical trace of pCO_2 (chart reading) with time taken from the left eye of a male New Zealand white rabbit under sodium pentobarbital anaesthesia.



higher carbon dioxide concentration than fresh air. In the Aqueous the carbon dioxide concentration is high, equal to that of the blood, that is, approximately 55 mm Hg (FATT and BEIBER, 1968). Since the cornea is freely permeable to carbon dioxide, there will be movement of carbon dioxide along a concentration gradient from the Aqueous into the tears. In the open eye this flux is happening continually, however when the cornea is occluded by the pCO_2 electrode the situation changes. The Aqueous, cornea and electrode create a closed system. In this system carbon dioxide will still move out of the Aqueous along the concentration gradient, but as the carbon dioxide concentration in the cornea and the electrode system increases, the flux of carbon dioxide will fall. Initially the carbon dioxide flux is high on account of the large carbon dioxide gradient involved, but as the difference is concentration between the components falls the flux will likewise decline. This produces an exponential decay in carbon dioxide flux from the cornea which is almost negligible as the carbon dioxide concentration in the two components nears equilibrium.

There is a second function that must be considered. This is the flux of carbon dioxide from the cornea itself, produced as an end product of metabolism. In the open eye situation this component is expected to be constant. In the closed system carbon dioxide is produced at a constant rate until corneal metabolism is disturbed. On the assumption that the electrode has no effect on metabolism, any carbon dioxide produced will diffuse out into the electrode. There is a small flux into the Aqueous, however the carbon dioxide concentration there is high, therefore a flux exists from Aqueous to tears and most of the

endogenously produced carbon dioxide produced will move to the tears. The size of the Aqueous flux is large enough to render the corneal component negligible in the open eye situation. If the epithelial carbon dioxide is produced at a steady rate then a linear change in carbon dioxide is expected at the electrode.

If the Aqueous and epithelial components are combined the combination of gas fluxes gives the curve obtained in Figure 12. If this curve is considered in the light of the above argument, the steep early section is seen as representative of the high rate of flux of carbon dioxide from the Aqueous to the tears. Coupled to this is the linear epithelial component, however at this early stage this component has little effect. Further along the trace, as the Aqueous-Tear concentrations approach equilibrium a linear portion of the curve attributable to the flux of carbon dioxide from the epithelium is seen. If this linear portion is considered as representative of the flux of carbon dioxide produced in the epithelium as an end product of metabolism it is possible to calculate the flux of carbon dioxide from the epithelium.

Linearity of the various sections of the curve, Figure 12, is checked by subjecting the co-ordinates taken from the curve to a curve fitting technique.

The values of y on the chart taken every three seconds converted into mV are given in Table 1. Table 1 also presents % pCO_2 calculated from measurements of mV.

Table 1

Raw data taken from Figure 12 converted to mV and pCO_2 using the formula given in Section 3.6.

T SECS	CHART READING	mV	pCO_2 %	T SECS	CHART READING	mV	pCO_2%
0	65	137.8	0.085	78	24.8	52.58	3.05
3	56.5	119.78	0.176	81	24.2	51.30	3.257
6	49	103.88	0.346	84	24	50.88	3.316
9	43	91.16	0.595	87	23.9	50.67	3.346
12	41	86.92	0.713	90	23.6	50.03	3.438
15	38.75	82.15	0.874	93	23.3	49.40	3.533
18	36.75	77.91	1.047	96	23.1	48.97	3.597
21	35.25	74.73	1.994	99	22.9	48.55	3.663
24	34	72.08	1.343	102	22.6	47.91	3.764
27	32.75	69.43	1.504	105	22.25	47.17	3.885
30	32	67.84	1.609	108	22	46.64	3.974
33	31	65.72	1.761	111	21.8	46.22	4.046
36	30.25	64.13	1.885	114	21.6	45.79	4.120
39	29.5	62.54	2.017	117	21.3	45.16	4.233
42	29.25	62	2.064	120	21.1	44.73	4.310
45	28.5	60.42	2.208				
48	28	59.36	2.310				
51	27.8	58.94	2.352				
54	27.7	58.72	2.374				
57	27.5	58.3	2.417				
60	27.1	57.45	2.506				
63	26.75	56.7	2.587				
66	26.3	55.76	2.694				
69	26	55.12	2.768				
72	25.5	54.06	2.896				
75	25.0	53	3.030				

Conversion of mV to pCO_2 in % is calculated as follows:

From Figure 8

Log pCO_2	mV
0	-79

From data

pCO_2%	mV
0.0395	-155
10	-25
20	-9

Given that $\log pCO_2$ is proportional to mV

$$\log pCO_2 = A + BV \qquad V = mV.$$

At $\log pCO_2 = 0$, $V = 79$.

Therefore $A + B(-79) = 0$

$$A = B.79 \qquad \text{(1)}$$

Therefore $\log pCO_2 = B79 + B.V.$

$$\log pCO_2 = B(79 + V) \qquad \text{(2)}$$

At $\log pCO_2 = 1$, (i.e. 10%), $V = -25$mV.

From (2) $B(79 - 25) = 1.$

Therefore $B = \frac{1}{54}$

$$B = 0.01852$$

From (1) $A = 0.01852 \times 79$

$$A = 1.4630$$

Therefore $\log pCO_2 = 1.4630 + 0.01852.\ V.$

Therefore $$pCO_2 = 10^{(1.4630 + 0.01852.V)} \quad \%$$

The curve obtained in this manner did not appear to fit any of the equations tested. However certain regions of Figure 12 (from 60 seconds onwards) produce a highly significant match to the linear curve $y = A + (B.\ x.)$. Values for A and B obtained from the data at

60 seconds onwards are:-

A = 0.7542

B = 2.9748×10^{-2}.

Figure 13 represents a plot of pCO_2% against time. The initial part of the curve represents the carbon dioxide flux from the Aqueous. The later values for pCO_2 against time form a straight line. Using the value of A obtained from the regression line for the curve of best fit as one value and the calculated value of pCO_2 at 120 seconds as the second point a line of best fit can be drawn through the data.

pCO_2 = A = 0.7542 pCO_2 = A = 4.3240

t = 0 seconds t = 120 seconds.

From the line of best fit drawn on Figure 13 it is seen that the linear portion extends from 50 seconds to 120 seconds. Using these points as the change in pCO_2 with time, representative of the epithelial carbon dioxide flux, the rate of carbon dioxide efflux can be calculated in absolute terms as follows.

The change in potential difference from 51 to 111 seconds = 58.94 mV to 46.22 mV.

From Figure 8 the difference in log pCO_2 = 0.23.

Therefore the difference in pCO_2 = 1.6982% min^{-1}.

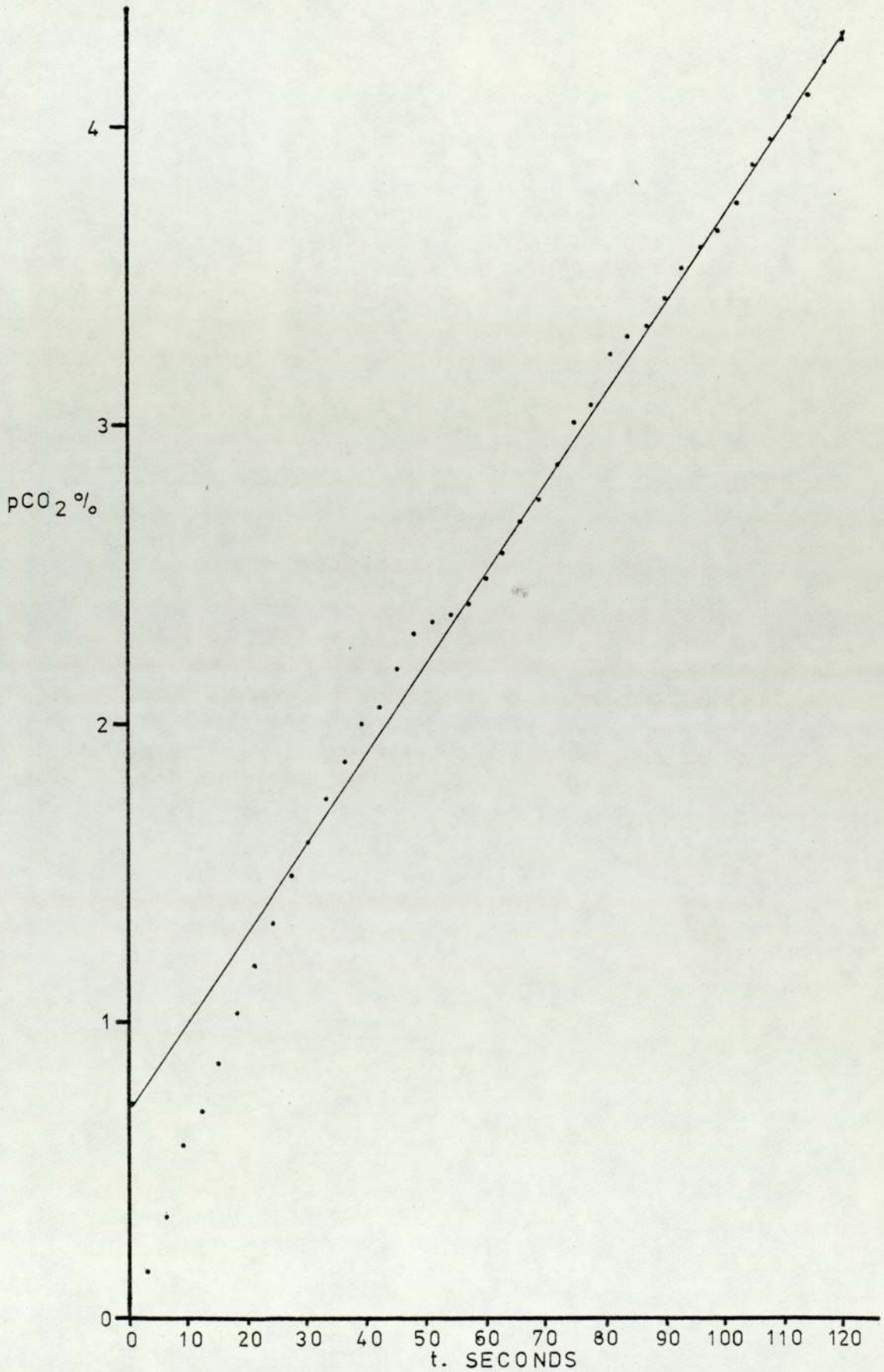
The solubility of carbon dioxide in water at 35°C = 0.1105 g $100ml^{-1}$

Density of carbon dioxide = 1.977 g l^{-1}

Radius of the electrode = 0.325 cm

Volume of electrolyte = 1.655×10^{-3} cm^3

Figure 13 pCO_2 concentration percent against time in seconds using the data from Table 1, taken from Figure 12.



100% scale reading in terms of $\mu l\ CO_2\ l^{-1} = 0.1105\ g\ 100\ ml^{-1}$

Therefore a 100% saturated solution contains $= \frac{0.1105.\ 10.\ 10^6}{1.997}\ \mu l\ l^{-1}$

$= 5.5893 \times 10^5\ \mu l\ l^{-1}$

Therefore a 1% saturated solution contains $= 5.5893 \times 10^3\ \mu l\ l^{-1}$

The rate of change of pCO_2 from the trace $= 1.6982\%\ min^{-1}$

Therefore the rate of change per volume of electrolyte

$= (5.5893 \times 10^3).\ (1.6982).\ \frac{1.6588 \times 10^{-3}}{100}\ \mu l\ min^{-1}$

For a unit area of tissue per hour

$= \frac{(5.5893 \times 10^3.\ 1.6982.\ 1.6588 \times 10^{-3}.\ 1.0.\ 60)}{1000\ (0.0325)^2 \pi}\ \mu l\ hr^{-1}\ cm^{-2}$

Therefore the carbon dioxide efflux $= \frac{944.6948}{331.8307}\ \mu l\ cm^{-2}\ hr^{-1}$

Therefore the carbon dioxide efflux rate from the corneal epithelium calculated from the trace shown in Figure 12 = $2.8469\ \mu l\ CO_2\ cm^{-2}\ hr^{-1}$

Therefore the efflux rate per area of epithelium = $3.2198\ \mu l\ CO_2\ cm^{-2}\ hr^{-1}$

(For a cornea of average dimensions e.g. 12 mm. diameter).

Values for carbon dioxide efflux rate from rabbit corneal epithelium are shown below:-

2.48 $\mu l\ CO_2\ cm^{-2}\ hr^{-1}$

2.48 "

2.28 "

2.85 "

2.85 "

N = 5

Mean = 2.5873

S.E.M. = 0.1126.

3.7 DISCUSSION

Results for carbon dioxide efflux rate as shown in section 3.6 are very inaccurate, since a number of assumptions must be taken into consideration:-

(a) In solution carbon dioxide acts as a gas. Calibration of carbon dioxide electrodes is usually performed in the gas phase where carbon dioxide concentrations are real. In aqueous solutions carbon dioxide gas dissolves, as a result carbon dioxide dissolved in the solution will only act as a real gas if the solution is saturated. In this system the electrode is calibrated in aqueous solutions bubbled through with various gas mixes where a representative carbon dioxide concentration is obtained, due to the effect of the inert gas contributing to maintain the same carbon dioxide concentration in solution as in the gas. In the experimental system there are no molecules of inert gas to maintain a carbon dioxide concentration is solution representative of the carbon dioxide being produced. The result is that the electrode only "sees" that carbon dioxide present in the tears, which may be representative of the gas being produced, but which could also be higher than the "real" concentration due to the carbon dioxide being free to dissolve in a non-saturated solution.

(b) When calculating the mass of gas dissolved in solution at $35^{o}C$ the result is expressed in g 100 cc^{-1} of water. However, obviously this differs when considering the mass of carbon dioxide dissolved in a saturated solution of electrolyte.

(c) The calculation assumes that the response of the electrode is linear up to 100% carbon dioxide. The electrode is linear in the measured range, however at high carbon dioxide concentrations linearity cannot be guaranteed (SEVERINGHAUS and BRADLEY, 1958).

The value for carbon dioxide efflux from the whole cornea is given as 21 µl carbon dioxide per hour by FATT et al., (1964). This is a factor of approximately seven times that value calculated in this system. The system used by FATT et al., (1964) includes that flux of carbon dioxide from the Aqueous down the diffusion gradient to the tears whereas in the present system this has been eliminated.

Studies on corneal respiration in vitro have shown that the respiratory coefficient is equal to one, in the undisturbed cornea (DE ROETTH, 1950; HABERICH and DENNHARDT, 1966). From section 2.6 the oxygen consumption rate of the rabbit corneal epithelium = 7.287 µl O_2 cm^{-2} hr^{-1}. If the respiratory quotient (R.Q) is equal to one a flux of carbon dioxide of 7.287 µl cm^{-2} hr^{-1} is expected. This figure is only a factor of three times greater than the measured value. Given the assumptions laid out earlier, the movement of carbon dioxide into the Aqueous, and that component dissolving in the tissue, the flux of carbon dioxide from the epithelium in this system seems reasonable. LANGHAM (1954) calculated carbon dioxide efflux from the cornea, on a unit weight basis, to be 3 µl per cornea. This corresponds closely to the value calculated in this study, therefore it may be possible that the system is more accurate than supposed.

The aim of this experiment is to produce a new system for monitoring the respiration and metabolism of the cornea. The results show that it is possible to monitor in vivo carbon dioxide efflux from the corneal epithelium. Unfortunately this system is wholly inadequate for use in a clinical environment. The size of the electrode makes measurement painful and tedious, and as can be seen from Figure 12 the time taken to obtain a useful trace is in the order of three minutes. A time of three minutes is much too long to be acceptable to the patient even with an anaesthetised eye.

A second reason for the unsuitability of this system is that the primary effect of contact lenses is the inducement of anoxia at the corneal surface. It has been shown that in the cornea a high percentage of glucose is metabolised to lactic acid and the remainder oxidised to carbon dioxide and water (RILEY, 1969 (a); KINOSHITA, 1962; OLSEN et al., 1970; LANGHAM, 1960). The carbon dioxide produced derives from the glucose and lactic acid oxidised by the aerobic pathways, the glycolytic Hexose Monophosphate Shunt and Krebs Citric Acid Cycle (KINOSHITA, 1962). Reducing the oxygen available to the cornea by contact lens wear will only reduce the carbon dioxide produced from the aerobic pathways which are the principal pathways producing carbon dioxide. Therefore in anoxia the aerobic pathways will cease to function and carbon dioxide production will fall. Given the current insensitive technique it will be difficult to identify what will be a reduced carbon dioxide efflux.

The only effect carbon dioxide can have in the contact lens wear situation is if the production of carbon dioxide continues when the

contact lens is worn, in which case a build up of carbon dioxide in the tear layer beneath the contact lens could lead to pH changes. These changes could effect the osmotic and metabolic balance of the epithelium. If an increase in carbon dioxide were to occur then the carbon dioxide will of course be free to move back into the stroma, endothelium and Aqueous by diffusion and could have a detrimental effect on the mechanisms responsible for the maintenance of corneal hydration located at the endothelium.

3.8 CONCLUSION

Using a micro-carbon dioxide electrode the mean carbon dioxide efflux from the corneal epithelium of male New Zealand white rabbits is found to be 2.5873 $\mu l\ CO_2\ cm^{-2}\ hr^{-1} \pm 0.1126$ ($N = 5$).

Inaccuracies in the method of calculation and slow response time of the electrode, eliminate the use of this system as a method for the quantitative determination of carbon dioxide efflux from the cornea. Use of this system in the clinical environment for the early detection of corneal trauma due to the effects of contact lens wear on corneal physiology is not recommended.

SECTION 4

CORNEAL LACTIC ACID CONCENTRATION AND PRODUCTION

4.1 INTRODUCTION

The corneal epithelium can metabolise glucose by both aerobic and anaerobic respiration. Under aerobic conditions the Hexose Monophosphate Shunt, Krebs Citric Acid Cycle and anaerobic Embden Myerhoff Pathway all function (KINOSHITA et al., 1955; KINOSHITA and MASURAT, 1959). In the absence of oxygen only anaerobic glycolysis via the Embden Myerhoff Pathway occurs. The rate of anaerobic glycolysis increases in all layers of the cornea in response to oxygen deficiency (DE ROETTH, 1951), resulting in an increase in lactic acid, the end product of glycolysis, in the corneal tissues (LANGHAM, 1954; REIM et al., 1966; REIM, 1972; REIM et al., 1972).

Many investigations have been conducted on the effects of contact lenses on corneal respiration, involving studies on the relevant metabolic substrates or end products (SMELSER and CHEN, 1955; MORLEY and McCULLOCH, 1961; PRAUS et al., 1969; DREIFUS and PRAUS, 1970; BURNS et al., 1971; KILP, 1974). Unfortunately this work has involved removal of some part of the tissue and is therefore unsuitable for clinical use. LANGHAM (1954) suggested that diffusion of lactic acid away from the epithelium occurs during anoxia due to the high concentration of lactic acid produced in the epithelium. In addition he suggests that a probable route for the removal of excess lactic acid is along the existing concentration gradient to the Aqueous Humour and that the passage of lactic acid through the corneal epithelium is unlikely because of the cellular structure of the epithelium.

The aim of these experiments is to determine the quantity of lactic acid produced under anoxic conditions and to determine the fate of any excess lactic acid produced during contact lens wear. This is conducted with the view of developing a method for estimating concentrations of lactic acid *in vivo*, in order to monitor corneal metabolism in the presence of a contact lens.

EXPERIMENT 1

MEASUREMENT OF TEAR FLUID LACTIC ACID CONCENTRATION USING THIN-LAYER CHROMATOGRAPHY

4.2 INTRODUCTION

There is very little information regarding the lactic acid concentration of the tears. The major problem when analysing tear components is sample size. Tear flow is approximately 0.5 to 2.0 μl minute^{-1} (EHLERS, 1965; NORN, 1965; MISHIMA et al., 1966), as a result there are practical problems when attempting to obtain tear samples greater than 3 to 4 μl due to the induction of reflex tearing.

Any method used for analysing tear component concentrations must combine high sensitivity with small sample size. The method chosen here for the determination of lactic acid concentration in the tears is Thin-Layer Chromatography (T.L.C.). This technique has several advantages that make it suitable for tear analysis:-

(a) No pre-treatment of the sample is necessary.

(b) Small sample sizes, 2 to 5 μl are used typically to obtain optimum results.

(c) Speed

(d) Reproducibility

(e) Low cost of materials.

There are numerous reports describing the separation and isolation of lactic acid from other organic acids; some of which are sufficiently

accurate and reproducible for quantitative analysis (CARLES et al., 1958; PETROWITZ and PASTUSKA, 1962; TING and DUGGER, 1965; HIGGINS and VON BRAND, 1966; WEREAT et al., 1968; MÜLLER, 1971).

The aim of this experiment is to test the suitability of a number of solvents, sorbents and indicator systems in the separation, identification and quantitation of lactic acid in human tears.

4.3 APPARATUS AND METHODOLOGY

Three types of capillary tube are tested for use in the collection of tears:-

(a) Camlab Glass Micropipettes 2 µl

(b) Drummond Microcaps 2 µl

(c) Fine bore silicon rubber tubing - Silastic (dow corning). Internal diameter = 0.012 mm., outer diameter = 0.025mm.

The aim is to collect 3 to 4 µl of tears without inducing reflex tearing. In terms of patient comfort Silastic is the most acceptable, however on account of its hydrophobic nature, which prevents the movement of tears along the capillary except under suction, Silastic is unsuitable in tear collection.

Both "Microcaps" and Camlab micropipettes proved effective in the collection of tears. However Camlab micropipettes are less brittle and therefore less likely to break in the patients eye. Thus for safety reasons the Camlab micropipettes are preferred.

Tear collection is performed by reclining the patient and tilting the head such that the tears flow to the Temporal region. A capillary tube held in a suction test is held to the temporal canthus, care being taken not to touch the scleral surface, Figure 1. Tears are allowed to flow up the capillary by capillary rise, without any form of suction.

With care it is possible to collect 3 to 4 μl of tears in approximately 10 minutes, the precise time varying according to the "wetness" of the individual eye. Tears collected in the capillary are transferred to a plastic hard contact lens carrying case where they can be stored for short periods without evaporational loss.

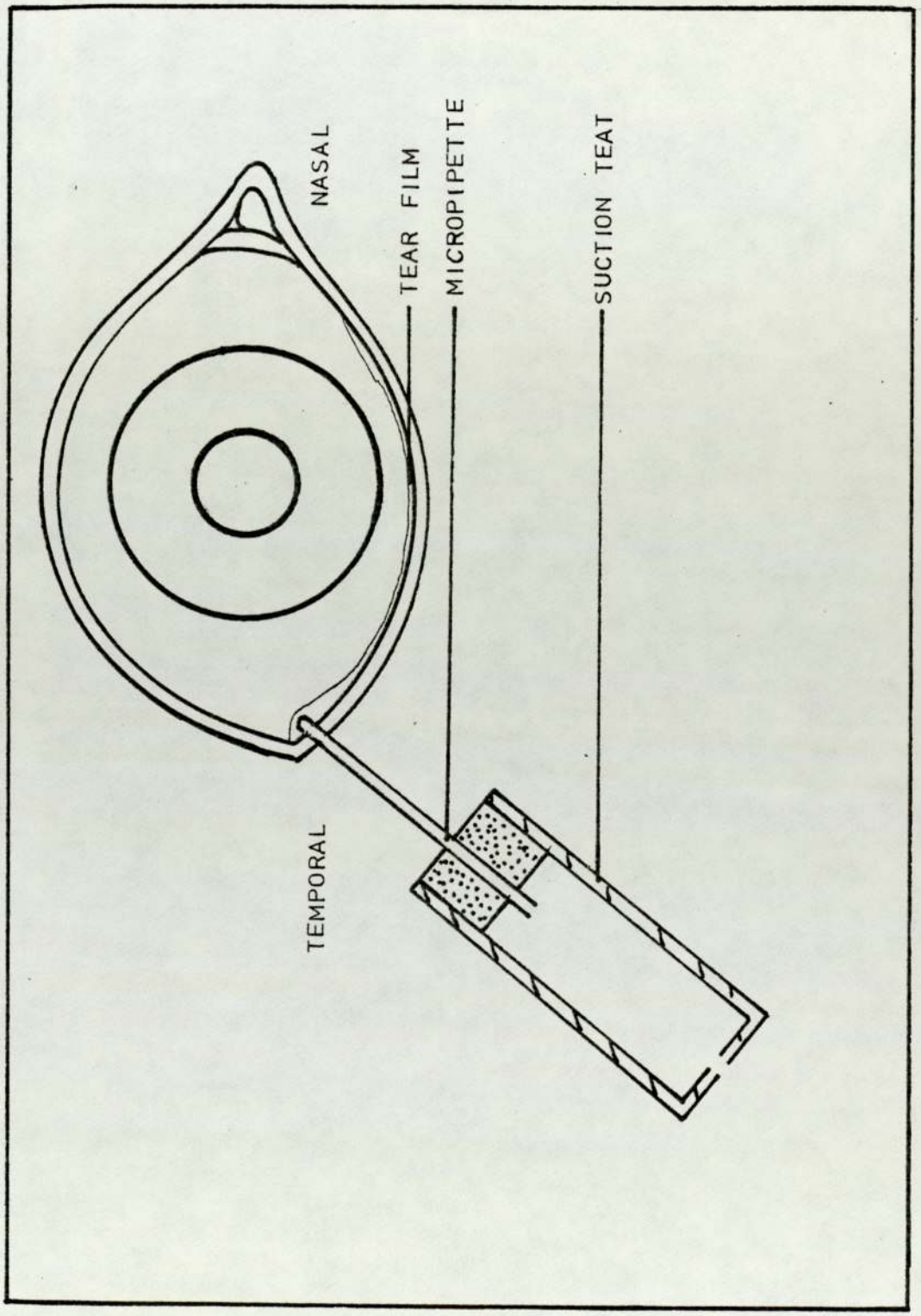
Solvents, Sorbents, Indicators and Sample Size

The solvents, sorbents and indicators tested are selected from those used by HIGGINS and VON BRAND (1966) and WEREAT et al., 1968).

Solvents:-

(1)	Propal - 1 - ol	(96)
	Formic Acid	(12)
	Water	(12)
(2)	Propan - 1 - ol	(50)
	Eucalyptol	(50)
	Formic Acid	(20)
	Water	(5)

Figure 1 Collection of tears using glass microcapillary tubes.



(3) Ethan - 1 - ol 90% in Water (100)

Water (12)

Ammonium Hydroxide 25% in water (16)

(Numbers in parentheses indicate parts by volume).

All reagents are analar grade obtained from B.D.H. Ltd. Before use solvents (1) and (3) require 24 hours equilibration, solvent (2) requires 48 hours.

Sorbents:-

(1) Cellulose MN300G 0.1 mm.

(2) Cellulose MN300G 0.25 mm.

(3) Silica Gel G25 0.25 mm.

All sorbents are on glass plates 200 mm. by 200 mm. pre-prepared by Machnerey Nagel and Co. Ltd., (Camlab Cambridge).

Indicators:-

(1) Bromocresol Green - 0.1% in Ethan - 1 - ol. Add 0.1 M NaOH till just blue-green. Dilute 1:3 with acetone immediately before use.

(2) Bromophenol Blue - 0.04% in 95% Ethan - 1 - ol in water. Add 0.1M NaOH till just purple.

(3) Aniline Ribose - 2ml. Aniline, 20ml. H_2O, 20ml 96% Ethan - 1 - ol in water, 60ml. Butan - 1 - ol, 2g. Ribose. Mix and leave for three days in the dark before use.

All reagents are analar quality, obtained from B.D.H. Ltd. Staining method also offers a choice between spraying and quenching. Quenching takes place in a shallow (25mm.) bath. Spraying uses a Camlab T.L.C. aerosol kit.

Sample size is determined by observing the spread of sample on each sorbent type and sorbent thickness. Samples of 0.5 to 5.0 µl are transferred to the plates using a Hamilton 0-10 µl microsyringe (V.A. Howe Ltd.).

To determine the most efficient system in terms of sensitivity and reproducibility, diluted samples of a 90% lactic acid (B.D.H. Ltd.) stock solution in 0.9% sodium chloride solution are used as the test substance.

After many combinations a method of separation taken from AKHREM and KUTZNETSOVA (1965) and TRUTER (1963) using solvent system (2), Silica Gel G25 sorbent plates, Aniline Ribose indicator with quench staining and a sample size of 2 µl is seen to be the most acceptable. The full method used for the determination of tear fluid lactic acid concentration is as follows.

A 5mm. band of sorbent is removed from two parallel edges of a 200mm. x 200mm. Silica Gel G25 T.L.C. plate. This allows the plates to be handled without risk of touching the surface and possibly contaminating the plate. Each plate is labelled in the top left hand corner with the plate and batch number. All plates are activated in an oven at 105°C for ten minutes.

Activated plates are placed in a Shandon Multiplate holding rack, SAB 2883 (Shandon Southern Instruments Ltd.). The holder plus plates is placed in a Shandon Multiplate Glass T.L.C. Tank, SA 2882. Pre-equilibrated solvent is added to the tank until the plates are covered to a depth of 5.0 mm. The tank is sealed and the plates left to "wash" overnight.

After washing the plates are removed from the tank and dried in a current of air. When dry each plate is removed from the rack and prepared for sample application. 2 µl samples are dispensed from a Hamilton Microsyringe, the spot position being accurately identified by the use of a Desaga Multipurpose Spotting Template (Camlab Ltd.). Use of the template also allows easy identification of the start and finish level of the solvent front.

After application each spot is dried in a current of air. After drying the plates are again placed in the rack and placed into the developing tank which already contains a small quantity of equilibrated solvent, the depth of solvent being less than that required to touch the plates. The tank is sealed and the atmosphere inside the tank allowed to equilibrate with the solvent for 20 minutes. After equilibration the remainder of the solvent is added through a small hole to maintain the atmosphere within the tank. The solvent is added until the plates are covered to a depth of 5.0 mm.

The plates are "developed" in a dark cupboard for 60 minutes. The plates are then removed and air dried for 10 minutes. Following the

drying period the plates are placed in an oven for 20 minutes at 75 to 80°C to ensure full evaporation of the formic acid component of the solvent. The plates are then removed and allowed to cool in air.

When cool each plate is submerged for ten seconds in a shallow bath containing pre-equilibrated aniline ribose. After staining the plates are drained and placed in an oven at 115 to 120°C for 5 to 6 minutes.

Lactic acid shows as a dark brown spot on a yellow/brown background. In darkness the spot will remain stable for two weeks. After this time the stain will fade.

Quantification of lactic acid is by visual comparison of spot size with standard dilutions run on the same plate. Attempted quantification using a thin layer chromatography densitometer (Joyce-Loebl Ltd.) proved unsuccessful due to the lack of contrast and faintness of spot. Figure 2 shows the equipment used for the determination of lactic acid concentration by T.L.C.

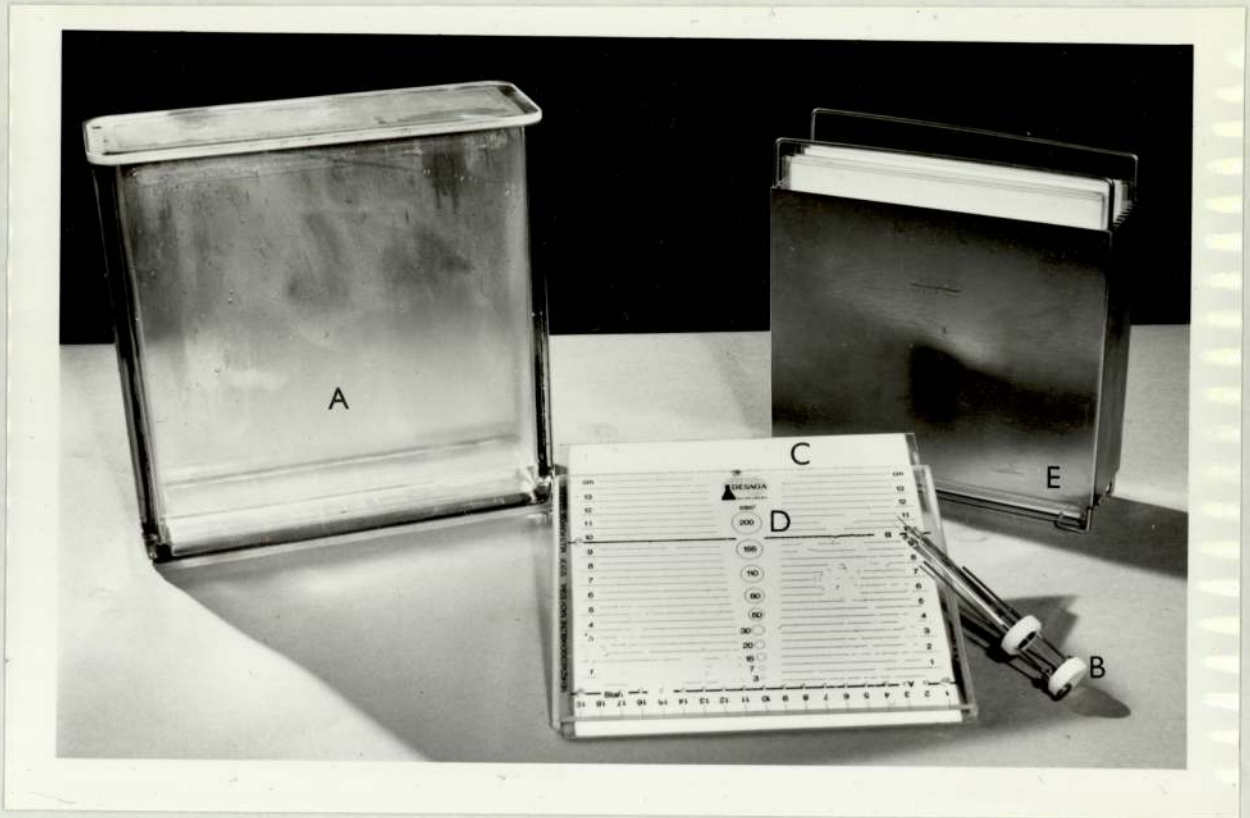
Patient Selection

All patients are colleagues and volunteer subjects from the Department of Ophthalmic Optics of The University of Aston.

4.4 RESULTS

Using the system described it is possible to identify a spot of 0.002 µl of the standard lactic acid solution applied as a 2 µl sample. This

Figure 2 Thin layer chromatography apparatus.



A = Multiplate T.L.C. tank.

B = Hamilton microlitre syringe.

C = Thin layer chromatography plate.

D = Desaga multipurpose T.L.C. template.

E = Multiplate T.L.C. rack.

represents a 0.1% lactic acid concentration in a 2 μl sample. Therefore if lactic acid is present in the tears at a 0.1% concentration then it should be identifiable. A 0.1% concentration contains 1.621×10^{-8} Moles of lactic acid.

Tear samples taken from both eyes of the volunteer patients failed to show a recognisable spot. One volunteer, who is at present a hard contact lens wearer would have been expected to show a positive result, however even in this case the results proved negative.

In one patient a very faint spot could be seen, however because of its diffuse nature and lack of definition it proved impossible to quantify. Furthermore since it is the only sample to exhibit any trace, the possibility of contamination cannot be eliminated.

4.5 DISCUSSION

Two inferences can be made from the failure to identify lactic acid in the tears by this method:-

(1) That the method is not sensitive enough to detect any lactic acid that may be present in human tears. If this is the case then a more sensitive method is required.

(2) Lactic acid is absent from the tears. Only the work of RUBEN and CARRUTHERS (1972) and KILP and HEISIG (1975) has shown the presence of lactic acid in the tears.

It is unlikely that lactic acid should be totally absent from the tears since the cornea and surrounding areas have been shown to produce lactic acid (LANGHAM, 1954; DE ROETTH, 1950). The ability of lactic acid to pass across the epithelial cell membranes has been questioned (LANGHAM, 1954) and it has been suggested that lactic acid from the corneal epithelium passes into the Aqueous Humour along a concentration gradient which would explain the apparent absence of lactic acid in the tears of corneal origin. However cell breakdown is occurring continually and it is not unreasonable to expect to find a small concentration of lactic acid derived from the contents of these broken cells. An additional factor is that the high concentrations of lactate dehydrogenase in the tears (AUGSBURGER et al., 1972; HILL, 1976) catalyse the conversion of lactic acid to pyruvate and thus remove lactic acid from the measuring system. If this catalytic conversion is occurring in the tears then this could explain the inability to detect lactic acid in the tears. Fixing of the tears before the experiment could possibly answer this question.

4.6 CONCLUSION

A system is described capable of identifying lactic acid in the tears. The sensitivity of the system allows positive identification of lactic acid in a 2 μl aliquot of tears providing the lactic acid concentration is greater than 0.1% of the sample. However by the method described no lactic acid could be identified in human tears and is thus unsuitable for both monitoring corneal metabolic activity and as an indicator of the possible effects of contact lens wear on corneal metabolism in the clinical environment.

EXPERIMENT 2

Lactic acid concentration in corneal tissues with anoxia.

4.7 INTRODUCTION

The glycolytic pathway, metabolising glucose to lactic acid is present in the cornea as reported by several workers (HERRMANN and HICKMAN, 1948 (b); MORLEY and TOTH, 1961; RILEY, 1969 (a), (b); BURNS et al., 1971). Furthermore, there are reports of increased glycolytic activity if the cornea becomes anoxic by either reducing the oxygen tension at the epithelial surface or placing a contact lens on the cornea (FRIEDENWALD, 1948; MORLEY and McCULLOCH, 1961; DREIFUS and PRAUS, 1970).

The failure of Experiment 1, section 4, to identify and quantify lactic acid production in human tear fluid suggests that other methods of analysis are required to study corneal lactic acid concentrations.

Many attempts have been made to assay lactic acid concentration (MÜLLER, 1971). The principal means of assaying corneal lactic acid has been the photometric method developed by BARKER and SUMMERSON (1941), (SMELSER and CHEN, 1955; LANGHAM, 1952; PRAUS et al., 1969; DREIFUS et al., 1969). However since the 1950's enzymatic procedures have become more important (RUBEN and CARRUTHERS, 1972; BRUUN-LARSON and LORENZEN, 1975). The advantages of analysing lactic acid enzymatically are:-

(a) High specificity

(b) High sensitivity allowing the use of micro-techniques

(c) Small demand on capital equipment

(d) The stereospecificity of the enzymes

(e) Simplicity and small sample size making them suitable for routine clinical work.

(ROSENBERG and RUSH, 1966; WESTEGARD et al., 1972).

This experiment is designed to measure, by enzymatic methods, the distribution of lactic acid in the component tissues of the anterior segment of the eye namely the tear fluid, epithelium, stroma, endothelium and Aqueous Humour. Lactic acid concentrations will be determined under normal and anoxic conditions in order to simulate the most severe effect of contact lens wear.

4.8 APPARATUS AND METHODOLOGY

Animal Preparation and Treatment

Rabbits used in these experiments are male New Zealand whites weighing between 2 to 3 kg. body weight. The treatments undergone by the rabbits are:-

(a) Non-aneasthetised control - the results from these animals are used as a control to allow comparison and determination of any change caused either by anaesthesia or anoxia. After the tear sample is taken the rabbits are killed by a firm blow to the neck as described in the Universities Federation for Animal Welfare (1966) handbook.

(b) Anaesthetised anoxia - all these animals are placed under general anaesthesia before being subjected to any experimental procedures. Each animal is given a subcutaneous injection of Atropine Sulphate

(B.D.H. Ltd.), 2.5mg. suspended in 1ml of 0.9% sodium chloride solution (UFAW, 1966), 30 minutes before administering any anaesthetic. The Atropine sulphate is necessary since over a period of three to four hours most rabbits develop serious breathing difficulties frequently resulting in suffocation. The Atropine sulphate is given as a "pre-med" treatment because of its drying effect on mucous membranes.

Initial anaesthesia is induced by an intraperitoneal injection of sodium pentobarbital, 60 mg ml^{-1}, (May and Baker Ltd.), at a dose of 0.5 ml kg^{-1} body weight. This is usually sufficient to keep the animal under anaesthesia for up to two hours. Response to sodium pentobarbital varies between rabbits, a dose such as this is rarely sufficient to kill the animal however frequently incomplete anaesthesia results. If complete anaesthesia is not obtained within fifteen minutes, a further 0.5 ml may be given. It is advisable to use a different animal if there is no response, since further administration invariably results in overdose and subsequent death.

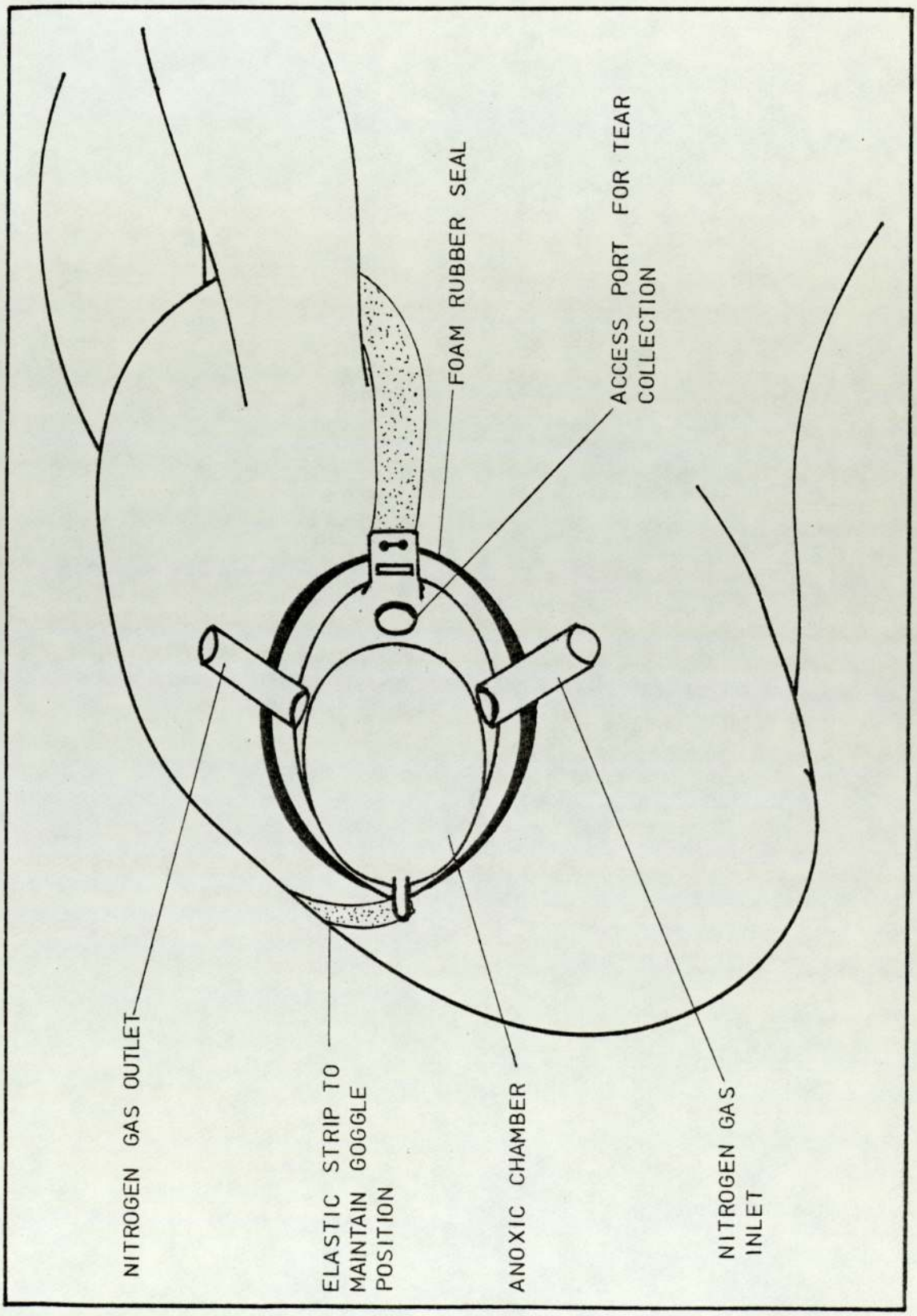
Once the animal is anaesthetised an area of the rabbits ear is shaved. A peripheral ear vein is exposed and dilated by wiping with a tissue damp with Benzene (B.D.H. Ltd.). A plugged syringe needle size 25G is pushed into the vein and held in position using adhesive tape. The ear is washed using Ethan - 1 - ol (B.D.H. Ltd.) and allowed to dry. When dry the plug is removed from the needle, a 10 ml syringe (Plastipak, Fisons Ltd.) is attached and 0.25 ml of Heparinised 0.9% sodium chloride solution, 100,000 U mg^{-1}, 1 mg ml^{-1}, injected. The syringe is removed and replaced by a similar syringe containing Ethyl Carbamate (Urethane, B.D.H. Ltd.), 20% W/V in 0.9% sodium chloride solution. Ethyl carbamate is used to maintain the anaesthesia over

longer periods because of its less deleterious effect on the rabbit respiratory system as compared to sodium pentobarbital (HERRMANN et al., 1942; U.F.A.W., 1966). Initially 6ml of Ethyl Carbamate is injected followed by 5ml every 45 minutes for the first three hours. Later the dose depends on the depth of anaesthesia of the particular animal which is determined by observing the "pedal-reflex" and muscle tone. The syringe containing Ethyl Carbamate is removed and replaced by the syringe containing Heparinised sodium chloride solution in order to inject 0.05 ml of this solution. Subsequently this syringe is also detached and the plug replaced, to prevent blood loss, until further anaesthetic is required.

When the animal is fully anaesthetised the apparatus for reducing the oxygen tension at the epithelium is fitted. This consists of a swimming goggle (Speedo Ltd.) modified by fixing inlet and outlet tubes, for the passage of nitrogen gas, and a small side hole for tear collection. The side hole is plugged until required. The goggle is fastened securely over one eye using elastic ribbon and adhesive tape. Figure 3.

Nitrogen gas (B.O.C. Ltd.) is humidified by bubbling through 0.9% sodium chloride solution kept at 34^{o}C in a student water bath (Gallenkamp Ltd.). The gas passes through a manometric flow gauge, which allows constant monitoring of flow rate, before flowing via 2.5 cm. polyethylene tubing (Gallenkamp Ltd.) to the goggle. The flow rate is always between 1.5 and 2.0 litres minute^{-1} (l. min^{-1}). This is sufficient to maintain a positive pressure at the goggle outlet

Figure 3 "SPEEDO" swimming goggle modified for use in passing nitrogen gas over rabbit eyes.



which prevents any air from entering the chamber.

The effect of anoxia on the cornea is determined by passing nitrogen gas over the corneal surface for 0, 3, 5 and 8 hour periods.

Sample Preparation

After the experimental treatment the tissue must be prepared for assay. The main aspect of the sample preparation is the "fixing" of the sample by Perchloric acid. As discussed in the preceding experiment in order to accurately measure lactic acid concentrations in the cornea it is essential that lactic acid is not enzymatically converted to pyruvate by lactate dehydrogenases present in the tissue (REIM et al., 1971; BAUM, 1963). This is effected by the addition of ice-cold Perchloric acid (B.D.H. Ltd.) which instantly prevents any further enzyme action by denaturing any protein present.

(a) Tears:

Tear samples are collected from the Temporal canthus using 10µl glass microcapillary tubes, (Micropipettes, Camlab Ltd.). It is possible with practise to collect up to 10 µl in a 15 to 20 minute period without inducing reflex tearing. Tears are always collected while the animal is alive. In the unanaesthetised group the animals require restraining in a specially designed box to prevent stress and possible damage due to rapid eye movements. When placed inside the box the animals offer little resistance and samples can be collected without inducing reflex tear flow. In the anaesthetised group the tears from the untreated experimental control eye are taken as for the control

group. Tears from the experimental eye are collected by inserting the capillary through a small hole in the side of the goggle.

In these experiments unless stated otherwise the right eye is the experimental eye with the left eye acting as an experimental control to determine treatment and anaesthetic effects. Due to the volumes of sample required by the assay procedure a minimum sample of 10 µl must be collected. It is impractical to collect 10 µl at one attempt, therefore smaller volumes are collected and pooled in a pre-cooled plastic hard lens carrying case. By keeping the tears in the case and sealing after each addition it is possible to collect the required volume and to keep evaporation to a minimum.

When sufficient tears are collected a precise volume is taken using a 10 µl Hamilton Microsyringe (V.A. Howe Ltd.) and placed in a clean plastic hard lens carrying case. To this is added twice the sample volume of ice-cold 0.6M Perchloric acid, and the solutions thoroughly mixed before placing on ice until the next stage of the procedure; see stroma plus endothelial sample.

(b) Aqueous Humour:

After the animal is killed, by administering an overdose of sodium pentobarbital, a size 25G needle is attached to a 1ml syringe

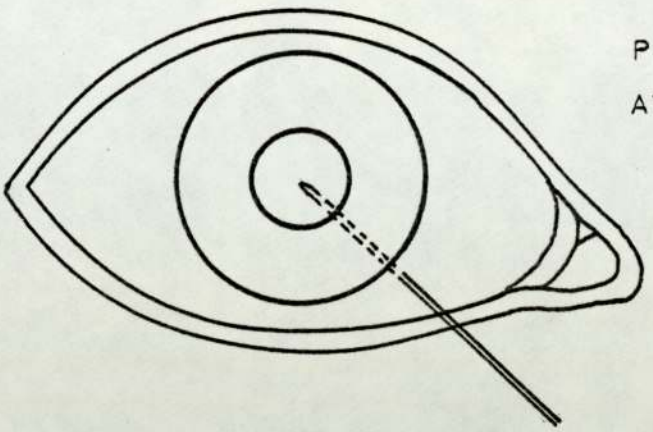
(Plastipak, Fisons Ltd.). This is then inserted into the Aqueous Humour from a point in the sclera just outside the limbus. The needle is positioned close to the posterior wall of the cornea and the Aqueous Humour sample removed. Figure 4. A maximum of 0.15ml is removed and placed in a plastic hard contact lens carrying case, no more is taken since the total volume is approximately 0.2ml (JONES and MAURICE, 1963) and some must remain to maintain corneal turgidity, allowing the epithelial sample to be taken. Immediately 100 µl (0.1ml) is removed using an automatic pipette (Gilson Pipetman, fully adjustable micropipette, 0-200 µl, Anachem Ltd.) and placed in a polycarbonate test tube, 63mm. x 10mm., (LP3, Baird and Tatlock Ltd) containing 0.2ml of ice-cold 0.6M Perchloric acid. The contents are thoroughly mixed using a vortex mixer (Gallenkamp Ltd.) and the tube plus contents kept on ice until the next procedure; see stroma plus endothelial sample.

(c) Epithelium:

Following removal of the Aqueous Humour the epithelium is removed. This is performed by making a shallow circular cut in the epithelium using a size 3 scalpel with a number 11 blade (Swann-Morton Ltd.), such that the knife enters Descemets membrane or the anterior layers of the stroma. Using the edge of the scalpel it is possible to raise a small flap of epithelium which can be gripped with fine forceps. Having gripped the small flap of epithelium it is possible to strip the whole epithelium from the stroma by pulling the flap back on itself with a peeling motion. With practise it is possible to strip the whole epithelium in one piece by this method. Figure 5 and 6 show transverse sections of rabbit cornea before and after stripping of the

Figure 4 Collection of Aqueous Humour from rabbit eye.

1

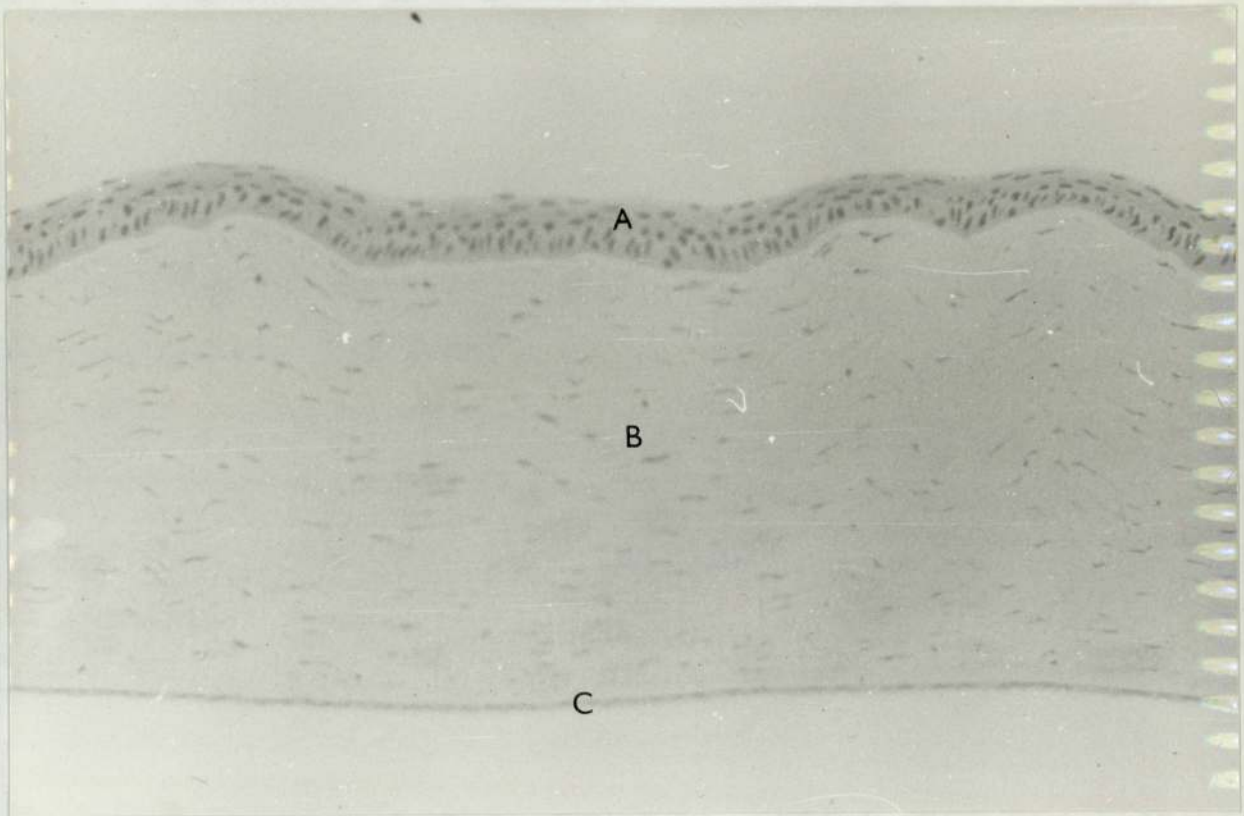


PIERCE SCLERA
AT THE LIMBUS

2

POSITION THE NEEDLE
TIP AT THE POSTERIOR
CORNEAL SURFACE

Figure 5 Rabbit cornea. Transverse section, haematoxylin and eosin stain.

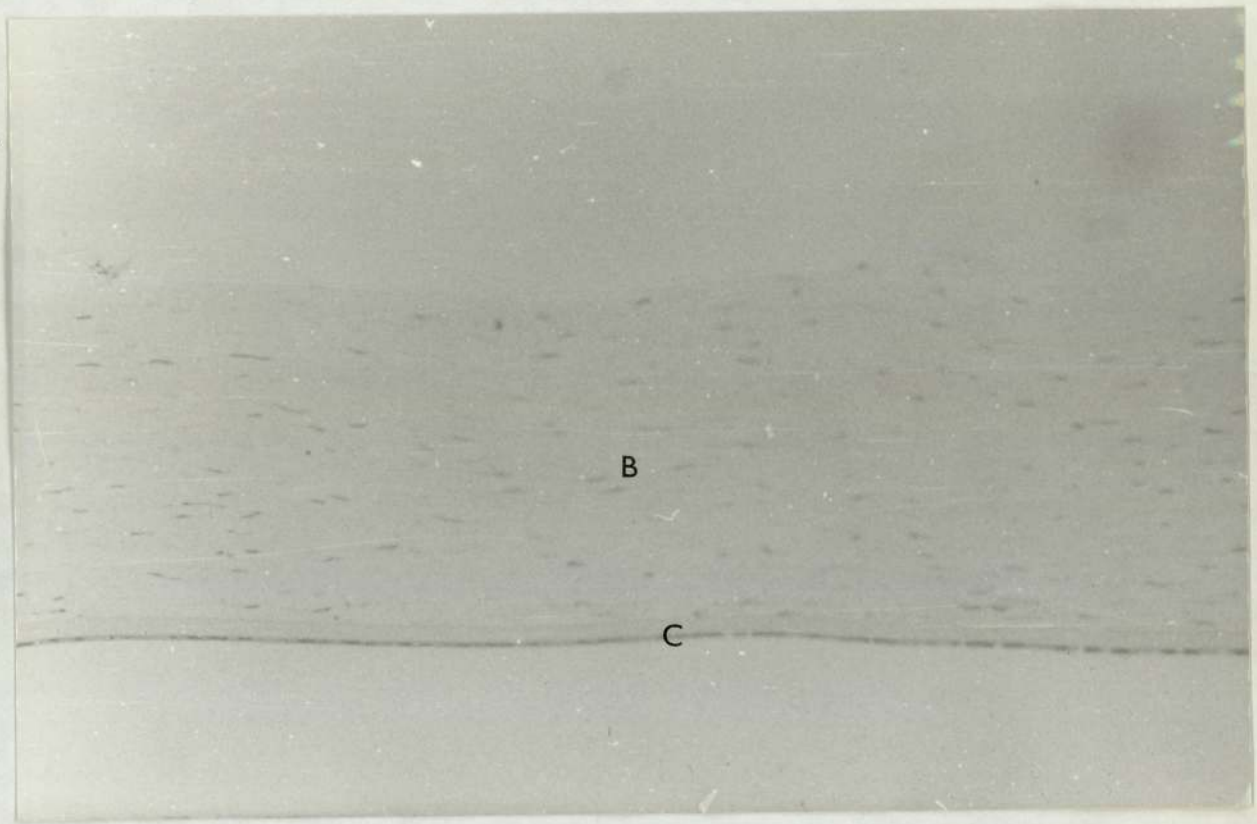


A = Epithelium

B = Stroma

C = Endothelium

Figure 6 Rabbit cornea stripped of its epithelium. Transverse section, haematoxylin and eosin stain.



B = Stroma

C = Endothelium

epithelium. Comparison shows that no epithelium remains after the stripping procedure.

The epithelial sample is placed in a 5ml. beaker containing ice-cold 0.6 M Perchloric acid and rinsed thoroughly. The tissue sample is dabbed dry using Whatman Number 1 filter paper (Gallenkamp Ltd.), and placed in a pre-weighed 0.1ml microhomogeniser (Jencons Scientific Ltd.) containing 0.1 ml of ice-cold 0.6 M Perchloric acid. The homogeniser plus Perchloric acid and sample is weighed on a Mettler H31 precision balance (Gallenkamp Ltd.).

The epithelial sample is homogenised for 10 to 15 minutes after which another 0.1 ml of 0.6 M Perchloric acid is added. The tissue is further homogenised until completely disintigrated. Care must be taken to keep the homogenate ice cold during the homogenising and until the next procedure; see stroma plus endothelial sample.

(d) Stroma and endothelium:

The stroma and endothelium are taken as one tissue since the endothelium is extremely difficult to separate from the stroma and the volume of endothelial tissue is so small that handling and sample preparation are impractical.

The stroma and endothelium are cut away from the limbus using a pair of fine bow-scissors and placed in a 5ml beaker containing ice-cold 0.6M Perchloric acid. The tissue is trimmed of any extraneous limbal tissue. The tissue is thoroughly rinsed to remove any Aqueous Humour that may be covering the endothelial surface, and dabbed dry.

It is then placed in a pre-weighed 0.1 ml microhomogeniser containing 0.1 ml of ice-cold 0.6M Perchloric acid and weighed before homogenising as described for the epithelial sample earlier. The sample is then kept on ice ready for the next procedure.

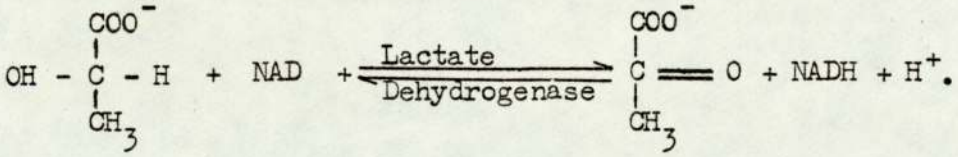
After isolation and preparation the various tissue samples are removed from iced storage and allowed to run into individual non-heparinised micro-haematocrit capillary tubes (Hawkesly Number 1604, Gallenkamp Ltd.) which are all labelled. Once the tubes are full, one end is sealed by pushing into a block of Cristaseal sealing compound (Hawkesly A808, Gallenkamp Ltd.). They are then centrifuged at 11,000 revolutions per minute (RPM), (12,000g) in a micro-haematocrit centrifuge (Hawkesly, Gallenkamp Ltd.) for six minutes to separate any debris and protein precipitates from the supernatant containing the lactic acid. The samples are retained intact at 0°C until required. Measurement of lactic acid is always performed within seven days of preparation as BERGMEYER (1970) has shown that lactic acid is stable in Perchloric acid filtrates for up to seven days at 30°C.

It is possible with practise to kill the animal and fix the tissues in Perchloric acid within two minutes, and centrifuge within one hour.

Assay

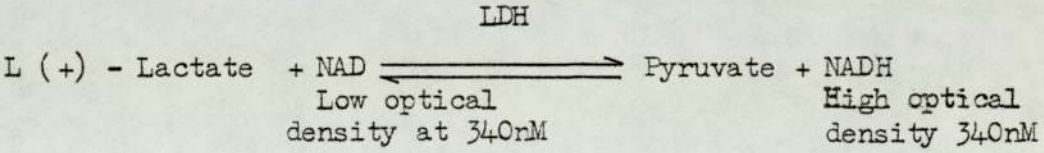
(a) Theory:

The basis of all enzymatic methods of lactic acid analysis is the oxidation of lactic acid to pyruvic acid, catalysed by lactate dehydrogenase (LDH), in the presence of nicotinamide adenine dinucleotide (NAD).



L - lactate Pyruvate

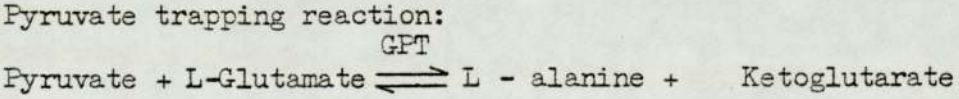
The shift of this reaction in favour of lactic acid depends on the proton concentration. In order to effect quantitative dehydrogenation the reaction products pyruvic acid and protons must be continually removed (WIELAND, 1962). NOLL (1966) describes a method whereby the pyruvic acid is converted by Glutamate-pyruvate-transaminase (G.P.T.) to L-alanine and is thereby removed from the reaction mixture. This has the nett result of favouring further pyruvic acid formation. When lactic acid is oxidised to pyruvic acid a molar equivalent of reduced nicotinamide-adenine dinucleotide (NADH) is produced. The lactic acid (lactate) concentration in the test solution is proportional to the amount of NADH produced, which can be determined spectrophotometrically by the change in absorbance at 340 Nanometres (nM).



Low optical density at 340nM

High optical density 340nM

Pyruvate trapping reaction:



Measurement of lactate concentration in the corneal sample is similar to the method described in document number L03053 published by Calbiochem Ltd., 1975, U.S. Patent 3,573,171 which explains the operation of their "Rapid-Lactate" kit. The "Rapid-Lactate" kit used, described in the Calbiochem Catalogue, Number 869218, requires a sample volume of 0.1ml. However since it is impossible to collect 0.1ml samples of tears, the working volume of the system is reduced.

The smallest ultra-violet (U.V.) cuvette available has a working volume of 0.4 to 0.5 ml. This implies a six-fold reduction in the working volume of the system which gives a sample volume of 0.4833 ml. and a reagent volume of 16.667 ml. The volumes are impossible to reproduce accurately, therefore each volume is reduced by a further factor of 0.9311 giving a reagent volume of 0.45 ml. and a sample volume of 15.18 µl. 15 µl is used as the sample volume since 15.18 µl is difficult to dispense accurately and secondly a 0.18 µl volume has a very slight effect on observed lactic acid concentration; see section 4.9.

(b) Method:

A Pye Unicam Model SP400, spectrophotometer is set to scan at a fixed wavelength of 340nM, and to record the absorbance on a paper chart. It is essential that the cuvette chamber of the spectrophotometer be kept at 30°C (OLSON, 1962). This is achieved by linking a water bath set at 30°C to the spectrophotometer via a reciprocating pump and rubber tubing.

The "Rapid-Lactate" kit reagent, vial B, containing 3.1×10^{-3} Ml^{-1} of NAD and a non-reactive stabiliser is reconstituted by injecting 15ml. of distilled water into the vial and inverting gently. The contents of vial B are then used to reconstitute vial A, which contains:

TRIS buffer	$0.22\ Ml^{-1}$
Glutamate	$2.2 \times 10^{-2}\ Ml^{-1}$
GPT	$2400\ IU.\ l^{-1}$
LDH	$21{,}000\ IU\ l^{-1}$

Care is taken to invert vial A gently after adding the contents of

vial B to ensure that all contents are thoroughly mixed. After which 0.45 ml. of the fully reconstituted reagent is transferred to labelled polycarbonate reaction tubes, 63mm. by 10mm. (Baird and Tatlock Ltd.), using an automatic pipette (Gilson Pipetman, 0 to 200 µl, Anachem Ltd.). When ready the tubes are placed in a 30°C water bath until the reagent reaches the working temperature of 30°C. A sample of reagent is then taken to zero the spectrophotometer. A quartz microcuvette (Pye-Unicam, self masking, black walled, 10mm. light path, reference number 681440) is filled with distilled water and placed in the blank compartment of the spectrophotometer. A second microcuvette similar to the first, shown to have no significant difference in absorption to the blank, is filled with reagent at 30°C and placed in the sample compartment. If the initial reading is greater than 0.4 absorbance units the reagent is considered unsuitable and discarded. Any value lower than 0.4 is considered acceptable and forms the baseline for further measurements. The sample curvette is removed and thoroughly washed and dried.

The capillary tubes containing the samples are removed from the refrigerator and placed in a beaker of water at 30°C to reach working temperature. The sealed end of the tube is then broken off and the sample blown out on to a sheet of Nescofilm (Fisons Ltd.). An automatic pipette, (Gilson Pipetman 0 to 20 µl, Anachem Ltd.), is used to pipette 15.0 µl of sample into the reaction tube. The tube is sealed, shaken and placed in the water bath for the reaction to take place. Usually a number of samples, up to a maximum of 36 are run at one time. Each sample is transferred into the appropriately labelled

reaction tube, a clean pipette is used for each sample to prevent cross-contamination.

The reaction tubes are incubated for a minimum of 20 minutes, during which time they are shaken gently, twice, to ensure complete mixing and completeness of reaction. After 20 minutes each tube is removed from the bath shaken quickly and the contents immediately placed in the sample cuvette which is quickly placed in the sample compartment. The absorbance is measured and noted on the chart and then the sample cuvette is thoroughly washed and dried ready for the next sample. Using this method it is possible to assay 30 samples in 90 minutes.

Standard solutions of Lithium lactate are used to check for variability within the system and between sets of reagent. No significant differences were observed between batches of reagent.

Figure 7 shows a schematic illustration of the assay procedure.

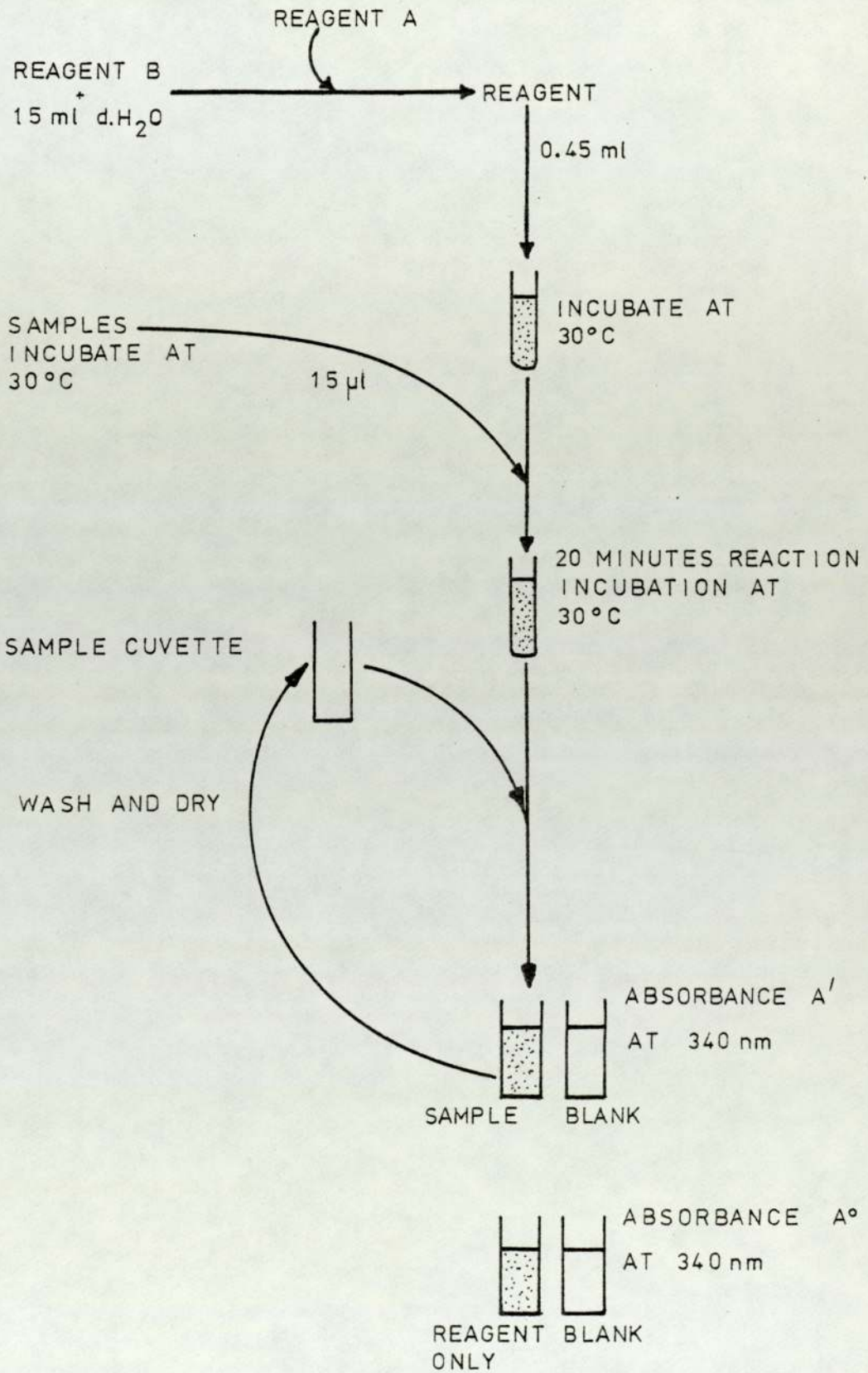
4.9 DATA TREATMENT

Data obtained from the spectrophotometer represents the difference in absorbance at 340nM between the sample and blank, (A^1), and reagent and blank (A^o). These values can be converted into lactic acid concentrations by the following equation.

$\Delta A. (C. D)$ = Lactic acid concentration in milligrams per decilitre ($mg\ dl^{-1}$)

Where $\Delta A = A^1 - A^o$ which is the observed change in absorbance after the reaction has proceeded to completion

Figure 7 Lactic acid assay procedure using the Calbiochem "Rapid-Lactate Stat-Pack .



C = Conversion factor

D = Dilution factor

Reagent volume = 0.45ml.

Sample volume = 15.0 µl.

All samples are protein free filtrates in 0.6M Perchloric acid.

In substrate determination the rate of change is not important. All the substrate must however be converted to product. The conversion factor for deriving lactate concentration is determined as follows:

The millimolar extinction coefficient of NADH (the absorbance of one micromole per millilitre at 340nM in a 10 mm. light path) is 6.22. The formation of one micromole of NADH in the 0.4515 ml. of reaction mixture corresponds to an absorbance change of $\frac{6.22}{0.4515} = 13.7763$.

The number of micromoles of NADH formed by 0.015 ml. of sample =

$$\frac{\Delta A}{13.7763} \cdot \frac{1}{0.015}$$

One millimole of lactic acid weighs 90.1 mg. To convert micromoles into millimoles divide by 1000. Therefore:

$$\frac{\Delta A}{13.7763} \cdot \frac{1}{0.015} \cdot \frac{90.1}{1} \cdot \frac{1}{1000} = \text{mg of lactic acid per ml. of sample.}$$

To convert into mg dl^{-1} multiply by 100.

Therefore $C = \frac{\Delta A}{13.7763} \cdot \frac{90.1}{0.015} \cdot \frac{100}{1000}$ mg lactic acid dl^{-1} of sample

The dilution factor D depends on the sample preparation. Both tears and Aqueous Humour are diluted by twice their own volume of Perchloric acid therefore a 1:3 dilution occurs. In the formula D = 3. The concentration of lactic acid in these samples:-

$$\frac{\Delta A}{13.7763} \cdot \frac{90.1}{0.015} \cdot \frac{100}{1000} \cdot 3 \text{ mg dl}^{-1}$$

$$= \Delta A .131 \text{ mg dl}^{-1}.$$

The solid tissue samples, epithelium, and stroma plus endothelium are suspended in 0.2 ml. of Perchloric acid. The lactic acid dissolves in the Perchloric acid and therefore does not constitute an additional dilution factor, which is therefore zero. The concentration of lactic acid in the tissue suspension:-

$$\frac{\Delta A}{13.7763} \cdot \frac{90.1}{0.015} \cdot \frac{100}{1000} \text{ mg dl}^{-1}$$

$$= \Delta A . 43.5 \text{ mg dl}^{-1}.$$

With whole tissue it is usual to express the results in terms of mg of lactic acid per g. of tissue wet weight rather than mg dl^{-1}. If the concentration of lactic acid in 0.2 ml of sample is represented by α mg dl^{-1} then the concentration of lactic acid = $\frac{\alpha}{500}$ mg of lactic acid in the 0.2 ml of homogenate. All the tissue is homogenised in the 0.2ml of Perchloric acid therefore by inference the concentration of lactic acid in the tissue sample = $\frac{\alpha}{500}$ mg.

If the tissue weighs β g. then the concentration of lactic acid per g. of tissue (θ) = $\frac{\alpha}{500} \frac{1}{\beta}$ mg g^{-1} wet weight.

Therefore the tissue lactic acid concentration in mg per g. wet weight

$$\theta = \frac{\alpha}{500 \times \beta} \text{ mg g}^{-1}.$$

4.10 RESULTS

Raw data for lactic acid concentration in the component tissues of the cornea is given in Tables 1 to 4.

Table 1

Tear fluid lactic acid concentration in normal and anoxic eyes. Values are $mgdl^{-1}$.

HOURS OF ANOXIA							
0		3		5		8	
R	L	R	L	R	L	R	L
7.86	11.79	6.55	6.55	9.17	3.93	15.72	10.48
9.17	10.48	6.55	7.86	5.24	5.24	17.03	7.86
9.17	15.72		10.48	7.86	6.55	2.62	5.24
7.86	18.34		6.55	13.1	3.93		5.24
5.24	15.72		3.93	7.86	1.31		2.62
2.62	7.86		5.24	6.55	1.31		6.55
2.62	10.48			7.86			9.17
	3.93			6.55			9.17
				15.72			9.17
				15.72			
				17.03			
N	7	8	2	6	11	6	3	9
MEAN	6.36	11.79	6.55	6.77	10.24	3.71	11.79	7.28
SEM	1.086	1.661	0	0.921	1.296	0.857	4.601	0.849

Table 2

Aqueous humour lactic acid concentration in normal and anoxic rabbit eyes. Values are mg dl^{-1}.

HOURS OF ANOXIA							
0		3		5		8	
R	L	R	L	R	L	R	L
102.18	108.73	89.08	83.84	106.11	96.94	150.65	133.62
104.80	107.42	104.80	81.22	107.42	96.94	153.27	133.62
111.35	108.73	148.03	132.31	103.49	75.98	96.94	82.53
110.04	108.73	149.34	133.62	106.11	83.84	104.80	81.22
106.11	115.25	129.69	111.35	110.04	98.25	179.47	158.51
121.83	96.94	128.34	112.66	111.35	103.49	182.09	161.13
95.63	96.94	112.66	103.49	128.38	115.28	167.68	153.27
86.46	98.25	125.76	103.49	127.07	110.04	168.99	154.58
81.22	98.25	125.76	103.49	123.14	100.87	133.62	122.31
83.84	96.94	104.80	103.49	121.83	104.80	134.93	123.62
91.7	99.56	104.80	93.01	115.25	104.80	159.82	124.45
87.79	108.73	145.41	86.46	112.65	104.80	162.44	125.76
		142.79	125.76	103.49	106.11		
		116.59	128.38	110.04	87.77		
				98.25	92.25		
				103.49	94.32		
N	12	12	14	14	16	16	12	12
MEAN	98.58	103.71	123.42	107.33	111.76	98.53	149.56	129.53
SEM	3.674	1.873	5.023	4.732	2.265	2.517	7.875	7.654

Table 3

Epithelial lactic acid concentration in normal and anoxic rabbit corneae. Values are mg g^{-1} wet weight.

HOURS OF ANOXIA							
0		3		5		8	
R	L	R	L	R	L	R	L
0.86	0.99	0.44	1.73	0.46	0.54	1.41	0.95
0.97	1.16	0.44	1.80	0.35	0.46	1.54	0.92
1.22	1.32	1.23	0.90	1.09	0.58	0.97	0.88
1.06	1.32	1.23	1.11	1.27	0.54	0.97	0.88
1.30	1.32	0.96	1.03	0.85	1.02	1.43	0.97
1.06	1.18	0.89	0.75	1.53	1.79	1.40	0.97
1.06	1.12	0.98	0.79	1.54	2.24	1.29	1.14
0.69	0.68	0.94	0.82	1.39	0.80	1.22	1.09
0.69	0.68	1.10	0.78	1.35	1.14	1.21	1.11
1.47	1.41	1.01	0.82	1.01	0.89	1.14	1.14
		1.65	0.78	1.01	0.98	1.84	1.13
		1.70	0.61	0.72	0.57	1.87	1.13
		0.78	0.65	0.63	0.59		
		0.78		1.14	0.53		
				1.14	0.53		
N	10	10	14	13	15	15	12	12
MEAN	1.04	1.12	1.01	0.97	1.03	0.88	1.36	1.03
SEM	0.078	0.082	0.098	0.105	0.095	0.133	0.084	0.030

Table 4

Stroma + endothelium lactic acid concentration in normal and anoxic rabbit corneae. Values are mg g^{-1} wet weight.

HOURS OF ANOXIA							
0		3		5		8	
R	L	R	L	R	L	R	L
0.69	0.59	0.55	0.68	0.44	0.52	0.64	0.54
0.69	0.45	0.63	0.66	0.44	0.52	0.70	0.41
0.67	0.80	0.50	0.44	0.33	0.43	0.54	0.52
0.68	0.55	0.45	0.52	0.28	0.43	0.64	0.49
0.38	0.51	0.42	0.41	0.18	0.47	0.51	0.80
0.35	0.36	0.47	0.41	0.17	0.59	0.56	0.67
0.42	0.36	0.42	0.44	0.51	0.45	0.62	0.40
0.36	0.86	0.44	0.47	0.48	0.40	0.75	0.40
0.82	1.11	0.50	0.52	0.35	0.42	0.54	0.52
0.31	0.28	0.50	0.52	0.41	0.50	0.54	0.45
		0.26	0.34	0.54	0.68	0.51	
		0.67	0.48	0.57	0.53	0.51	
		0.49	0.41	0.29	0.35		
		0.26		0.31	0.28		
				0.26	0.39		
				0.36	0.30		
N	10	10	14	13	16	16	12	10
MEAN	0.54	0.59	0.47	0.49	0.37	0.45	0.59	0.52
SEM	0.591	0.0818	0.030	0.026	0.030	0.024	0.022	0.040

The raw data gives values for component tissue lactic acid concentrations in the normal cornea and after 3, 5 and 8 hours anoxia. In all cases the right eye is the experimental eye, the left acting as a control for experimental procedures and anaesthetic effects.

Figures 8 to 11 show mean plus or minus the standard error of the mean ($\pm$ SEM) for right and left eyes at each time period for each component tissue; tears, Aqueous Humour, epithelium and stroma plus endothelium.

The effect of the experimental procedure is obtained by comparing the experimental control eye (E.C.) with the zero hour control (C). Statistical data is shown on Table 5. A student t-test is used to test the significance of any differences between the experimental (E) and the experimental control eyes at the various times. Results from the comparisons are shown on Table 6.

4.11 INTERPRETATION OF DATA

Tears

Tear collection using microcapillary tubes held to the conjunctival sac continues to be a most difficult and painstaking procedure. To collect a sample of more than 10 μl without inducing reflex tearing takes upwards of ten minutes, depending on the "wetness" of the individual eye. In some animals the sample is collected with little difficulty and with no stress to the animal. Alternatively tear collection has taken up to 30 minutes and there is the question of whether or not reflex tears have been induced.

Figure 8 Tear fluid lactic acid concentration in the right and left eyes of New Zealand white rabbits after varying periods of anoxia.

Values are the mean ± S.E.M. Number of readings is given in parentheses.

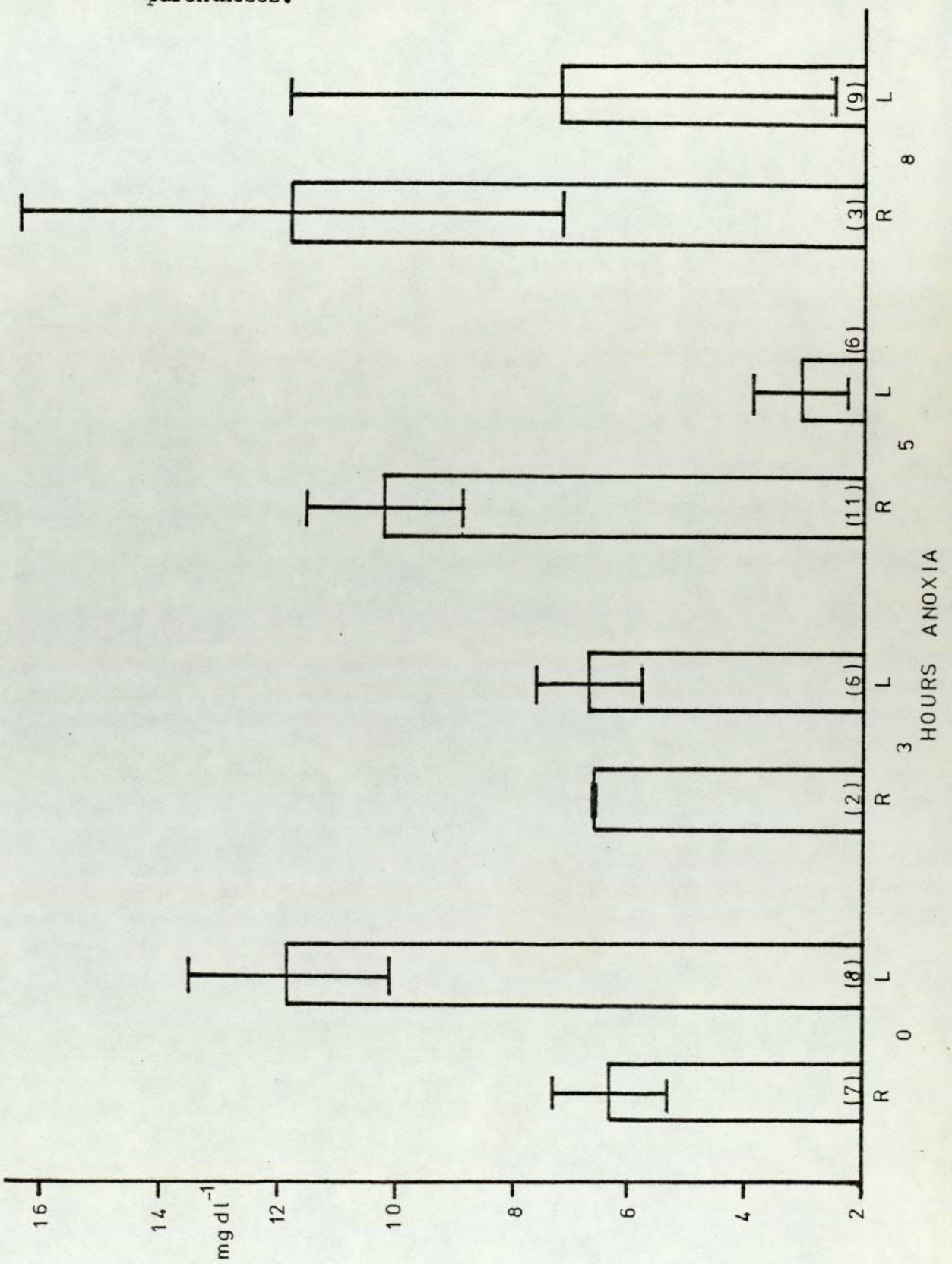


Figure 9 Aqueous humour lactic acid concentration in the right and left eyes of New Zealand white rabbits after varying periods of anoxia.

Values are the mean ± S.E.M. Number of readings is given in parentheses.

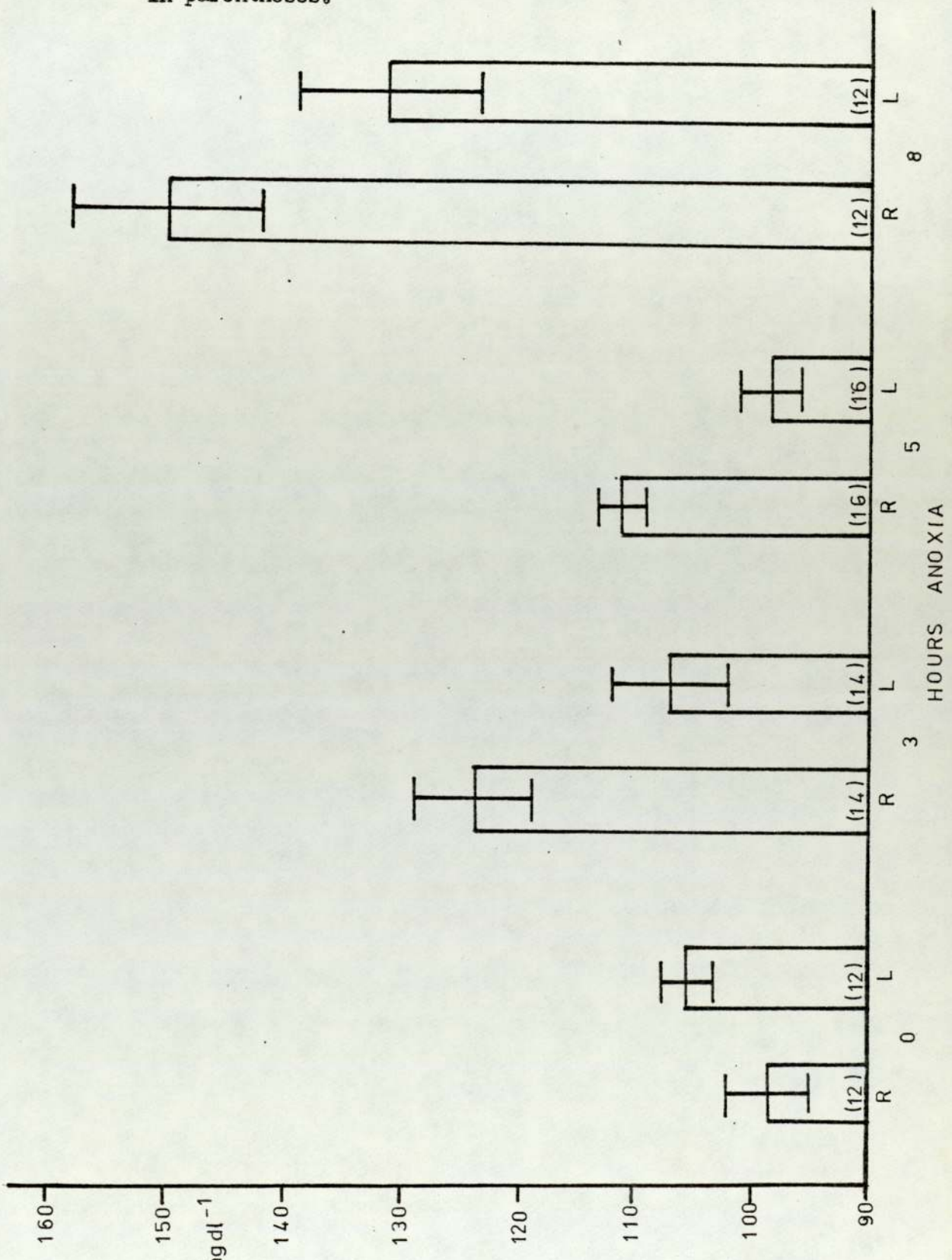


Figure 10 Epithelial lactic acid concentration in the right and left eyes of New Zealand white rabbits after varying periods of anoxia.

Values are the mean ± S.E.M. Number of readings is given in parentheses.

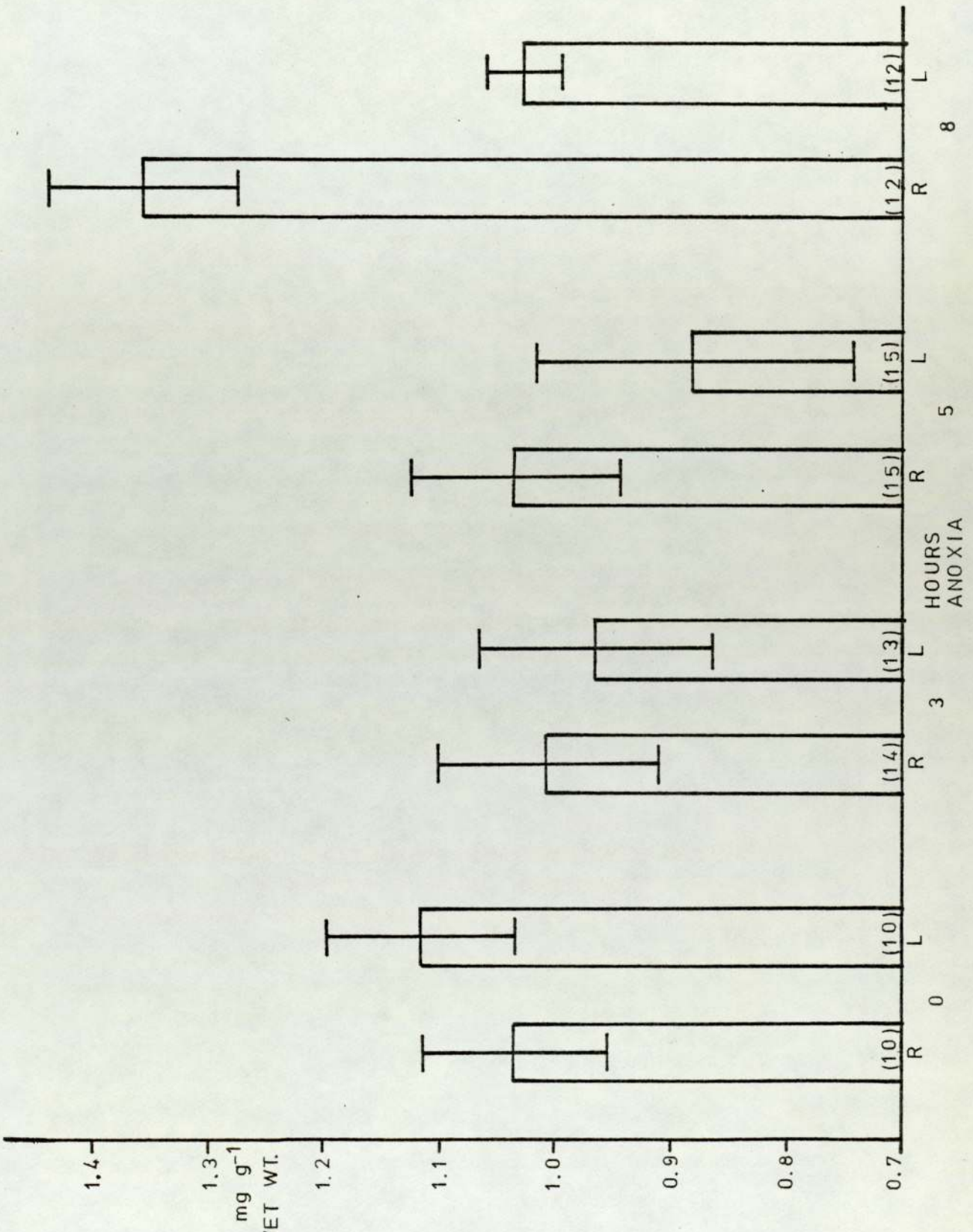


Figure 11 Stroma + endothelium lactic acid concentration in the right and left eyes of New Zealand white rabbits after varying periods of anoxia.

Values are the mean ± S.E.M. Number of readings is given in parentheses.

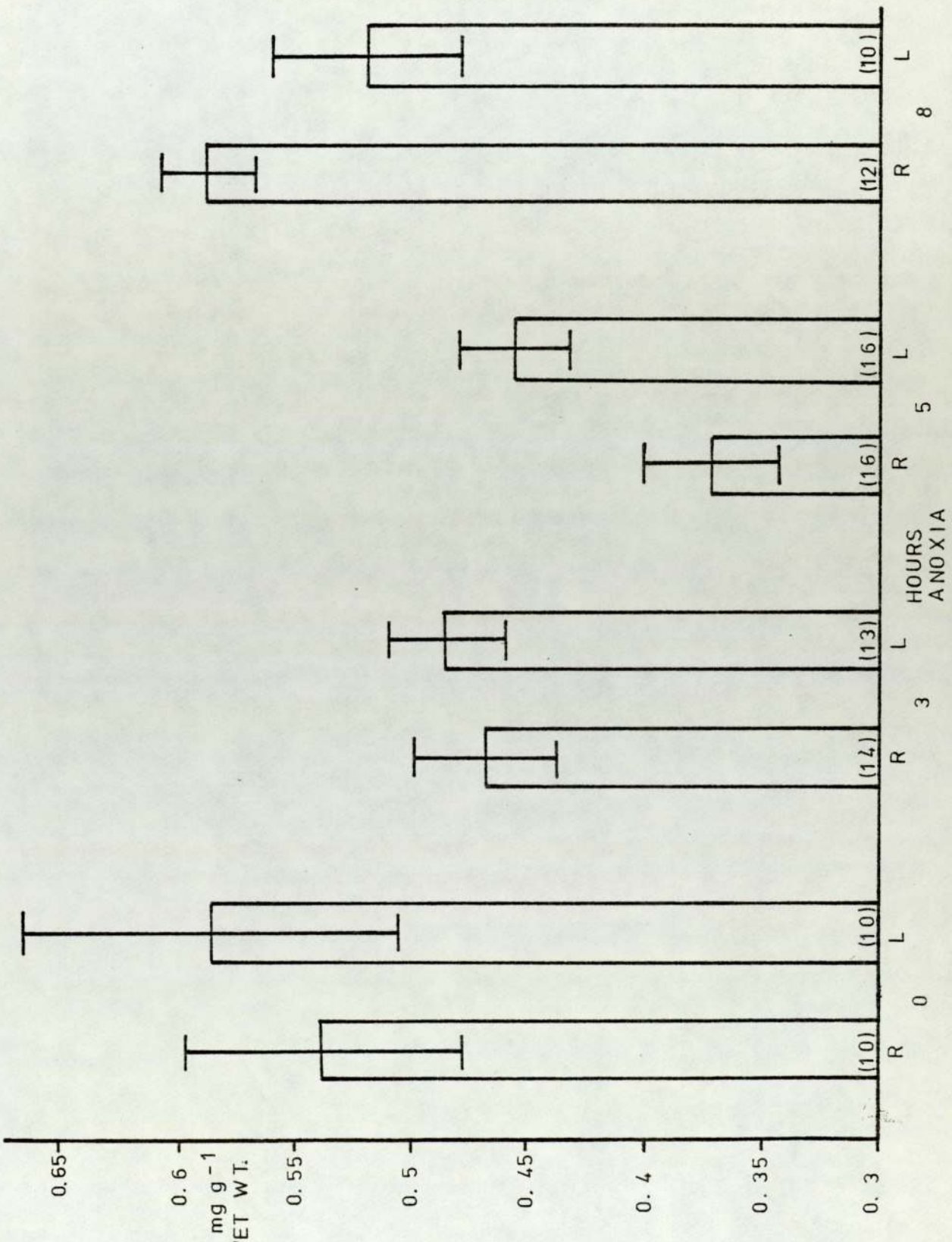


Table 5

Statistical data for the comparison of experimental and experimental control eyes with the zero hours control.

COMPARISON	t	°F	SIGNIFICANCE LEVEL p=0.1	p=0.05	p=0.02
RTs 0v3	0.08	7	NO	NO	NO
0v5	2.16	16	YES	YES	NO
0v8	1.70	9	NO	NO	NO
LTs 0v3	2.44	12	YES	YES	NO
0v5	3.99	12	YES	YES	YES
0v8	2.60	15	YES	YES	NO
RAq 0v3	4.01	24	YES	YES	YES
0v5	3.31	26	YES	YES	YES
0v8	5.99	22	YES	YES	YES
LAq 0v3	0.69	24	NO	NO	NO
0v5	1.6	26	NO	NO	NO
0v8	3.28	22	YES	YES	YES
REp 0v3	0.16	22	NO	NO	NO
0v5	0.0	23	NO	NO	NO
0v8	22.5	20	YES	YES	YES
LEp 0v3	1.25	21	NO	NO	NO
0v5	1.43	23	NO	NO	NO
0v8	1.28	20	NO	NO	NO
RSt + End 0v3	1.44	22	NO	NO	NO
0v5	2.78	24	YES	YES	YES
0v8	0.83	20	NO	NO	NO
LSt + End 0v3	1.30	21	NO	NO	NO
0v5	1.84	24	YES	NO	NO
0v8	0.74	20	NO	NO	NO

Table 6

Statistical data for the comparison of experimental eye and its experimental control eye.

COMPARISON	t	^{o}F	SIGNIFICANCE LEVEL p=0.1	p=0.05	p=0.02
TEARS					
C8 v E8	1.62	10	NO	NO	NO
C5 v E5	3.52	15	YES	YES	YES
C3 v E3	0.12	6	NO	NO	NO
C0 v E0	2.75	13	YES	YES	YES
AQUEOUS					
C8 v E8	1.82	22	YES	NO	NO
C5 v E5	4.06	30	YES	YES	YES
C3 v E3	2.38	26	YES	YES	NO
C0 v E0	1.25	22	NO	NO	NO
EPITHELIUM					
C8 v E8	4.0	22	YES	YES	YES
C5 v E5	1.0	28	NO	NO	NO
C3 v E3	0.3	25	NO	NO	NO
C0 v E0	0.8	18	NO	NO	NO
STROMA + ENDOTHELIUM					
C8 v E8	1.55	20	NO	NO	NO
C5 v E5	2.12	30	YES	YES	NO
C3 v E3	0.44	25	NO	NO	NO
C0 v E0	0.48	18	NO	NO	NO

The collection of tears from an anaesthetised animal wearing a goggle is even more tedious. As stated in section 4.8 tears are collected by placing the microcapillary through a small hole in the side of the goggle. Working through the small hole restricts movement of the capillary tube and some tears are lost, and sample collection time increases. Furthermore blinking is reduced in anaesthetised animals which results in the volume of tears in the conjuntival tear pool diminishing. However no drying of the corneal surface is observed.

The data of Table 1 shown diagramatically on Figure 8 reflect the difficulty in tear sample collection. The large difference between right and left eyes at zero hours ($P < 0.02$) indicates that even without the effects of experimental procedures, experimental variation and possible anaesthetic effect, large variations in tear lactic acid concentration occur. There is no reason why the lactic acid concentrations in the tears of the left eye should be significantly different from that of the right eye. In these unanaesthetised control eyes tears are collected from the right and left eye, alternating every two to three minutes.

The difference between the (C) and the (E.C.)'s are significant; 3 hours, $p < 0.05$; 5 hours, $p < 0.02$; 8 hours $p < 0.05$. To what degree the effect of anaesthesia, experimental procedure and tear collection is causing this significant difference between (C) and (E.C.) is difficult to assess. It is possible that the value for lactic acid concentration in the left eye at zero hours is in error, however this is unlikely since more readings are taken for this sample than the right eye (C). The similarity of values for (C) right eye,

3 and 8 hours (E.C.) indicate that the value for lactic acid concentration in the 5 hours (E.C.) eye is a result of experimental error, however it is possible that anaesthesia is affecting lactic acid concentration at 5 hours in the (E.C.). Taking into account the above inferences it can be concluded that experimental procedure and anaesthetic have little effect on tear lactic acid concentration.

Lactic acid concentration in the tears of eyes subjected to anoxia for periods up to 8 hours show a significant increase at 5 hours ($p < 0.05$) but no significant increase at 3 and 8 hours. Comparison of the (E) eyes with their (E.C.)'s show a significant difference at 5 hours ($p < 0.02$).

Visual analysis of the data presented in Figure 8 suggests that anoxia has no effect up to 3 hours but that after 3 hours tear fluid lactic acid concentration is increasing. This effect is significant at 5 hours however at 8 hours the large variation in the data masks the visually apparent continuation of the trend of increasing tear lactic acid concentration with anoxia.

RUBEN and CARRUTHERS (1972) have shown that lactic acid is present in human tears, however the method yields only purely qualitative information. These authors also suggest that high lactic acid levels in the precorneal tear film, present when contact lenses are worn are indicative of chronic anoxia. REIM et al., (1972) state that there is a quantity of lactic acid moving from the epithelium to the tears but that the size of this flux is constant. The data given in this work agree with the results of RUBEN and CARRUTHERS (1972) and REIM et al.,

(1972) in that lactic acid is present in the tears. The mean value for the lactic acid concentration in rabbit tears = 7.4200 ± 0.6602 mg dl^{-1} (N = 36). The observation that lactic acid increases with anoxia confirms the suggestions of RUBEN and CARRUTHERS (1972). In this experiment no contact lenses are worn therefore the effect of contact lenses in increasing tear lactic acid concentration can only be inferred from these results. Contact lenses have been shown to create a reduced oxygen environment at the lens, tear interface (FATT and ST. HELEN, 1971; TAKAHASHI et al., 1967) therefore such an inference is valid.

There is much conjecture on the origin of tear fluid lactic acid and this will be discussed more fully in context with other tissues of the cornea.

Aqueous Humour

Preparation of Aqueous Humour samples for lactic acid assay is the most simple and reliable of all the corneal tissues. Few operations are required and there are few possible sources of error.

Table 2 and Figure 9 present the data for Aqueous Humour lactic acid concentration and the effect of corneal anoxia on Aqueous Humour lactic acid concentration.

A student t-test comparison of lactic acid concentration in the right and left eyes of the (C) animals shows an insignificantly higher lactic acid concentration in the left eye. In sample preparation the right

eye is usually the first to be operated on, therefore the slightly higher concentration in the left eye could reflect an increase in lactic acid concentration during the time taken to prepare the right eye sample. However this is unlikely as the time taken to remove the corneal tissues is always less than two minutes. Furthermore, an additional check is made by reversing the order of operation and it is found that even when the left eye sample is taken first there is no difference between the lactic acid concentration in the right and left eye Aqueous Humour.

A comparison of the (C) eye with the (E.C.) eye after 3, 5 and 8 hours anoxia shows no significant difference at 3 and 5 hours, however a significant difference is obtained at 8 hours ($p < 0.02$).

Lactic acid in the Aqueous Humour is derived from two sources. The first is the result of glycolytic activity in the intra-ocular tissues of the eye, cornea, retina, lens and ciliary body. The second component originates from the ocular tissues and ocular blood supply. Whole body injection of ^{3}H- lactate shows that the blood lactate pool is capable of exchange with the Aqueous and Vitreous Humours. The concentration of ^{3}H-lactate between the blood and intraocular fluid reaching equilibrium within 40 minutes (RILEY, 1972). Other workers have demonstrated that the concentration of lactic acid in the Aqueous Humour is in excess of the plasma concentration (RILEY, 1972; SCHÜTTE et al., 1972 (a); REIM et al., 1969; BRUUN-LAURSEN and LORENTZEN, 1974). Radioactive tracer work of RILEY (1972) shows that ^{14}C - glucose derived lactic acid can enter and leave the cornea therefore plasma and intraocular lactic acid must be interchangeable. The excess of lactic acid

in the Aqueous Humour indicates that the intraocular fluid-plasma equilibrium is not balanced and that there is either a resistance to outward flow of lactic acid or intraocular lactic acid production is faster than the loss of lactic acid into the blood via the vasculature of the iris and corneal angle into the Aqueous vein.

Cyclodiathermy or coagulation of the long ciliary vessels has been shown to increase Aqueous Humour lactic acid concentration and reduce Aqueous Humour glucose levels (SCHÜTTE et al., 1972 (a), (b); PFISTER et al., 1971). An explanation for the increased Aqueous Humour lactic acid concentration in these eyes is that a reduction in the glucose concentration of the Aqueous Humour results in increased glycolysis, and utilisation of glycogen reserves, to produce lactic acid coupled with the reduced removal of lactic acid to the plasma. The mechanism by which reduced Aqueous Humour glucose triggers an increase in corneal lactic acid production is unknown.

An explanation for the increase in Aqueous Humour lactic acid concentration in the (E.C.) at 8 hours is that anaesthesia may, over a prolonged period, increase the plasma lactic acid concentration. This is not unreasonable, RILEY (1972) has shown that plasma lactic acid concentration can increase four fold under the stress of wrapping the rabbit in a towel in preparation for experimentation. The increased plasma lactic acid concentration over the longer period, i.e. above 5 hours, could lead to an increase in Aqueous Humour lactic acid which is in equilibrium with the plasma. Thus if the plasma/Aqueous Humour lactic acid equilibrium level rises and the lactic acid production from the intraocular tissues is maintained at a constant rate, then

the sum of the two components will increase in comparison to the normal (C) Aqueous Humour lactic acid concentration. A second but less likely factor could be a reduction in plasma glucose concentration as a consequence of anaesthesia. This could increase lactic acid production by the intraocular tissues (SCHÜTTE et al., 1972 (a)). Figure 12 shows a schematic interpretation of intraocular lactic acid dynamics in the normal rabbit eye.

After 3, 5 and 8 hours anoxia there is an increase in Aqueous Humour lactic acid concentration. At 3, 5 and 8 hours there is a significant difference ($P < 0.02$) from the (C). Comparison of the (E.C.) with the treated eyes shows a significant difference at 3 hours ($P < 0.05$), 5 hours ($P < 0.02$) and 8 hours ($P < 0.1$). The low degree of significance observed in the 8 hour (E) is due to the increased level of lactic acid produced in the (EC) after 8 hours. Furthermore the higher (EC) background concentrations result in increased variation of the test samples, which again reduces the significance level.

If the increase in Aqueous Humour lactic acid concentration between (E) and (EC) is represented as a percentage of the (C) the increase in lactic acid due to anoxia =

3 hours = 14.9930

5 hours = 13.4261

8 hours = 15.4609 Figure 13.

If the increase in lactic acid concentration in the 8 hour (EC) relative to the (C) lactic acid concentration is considered to be due to the effects of anaesthesia, and subtracted from the lactic acid

Figure 12 A schematic of Aqueous Humour lactic acid dynamics in the rabbit.

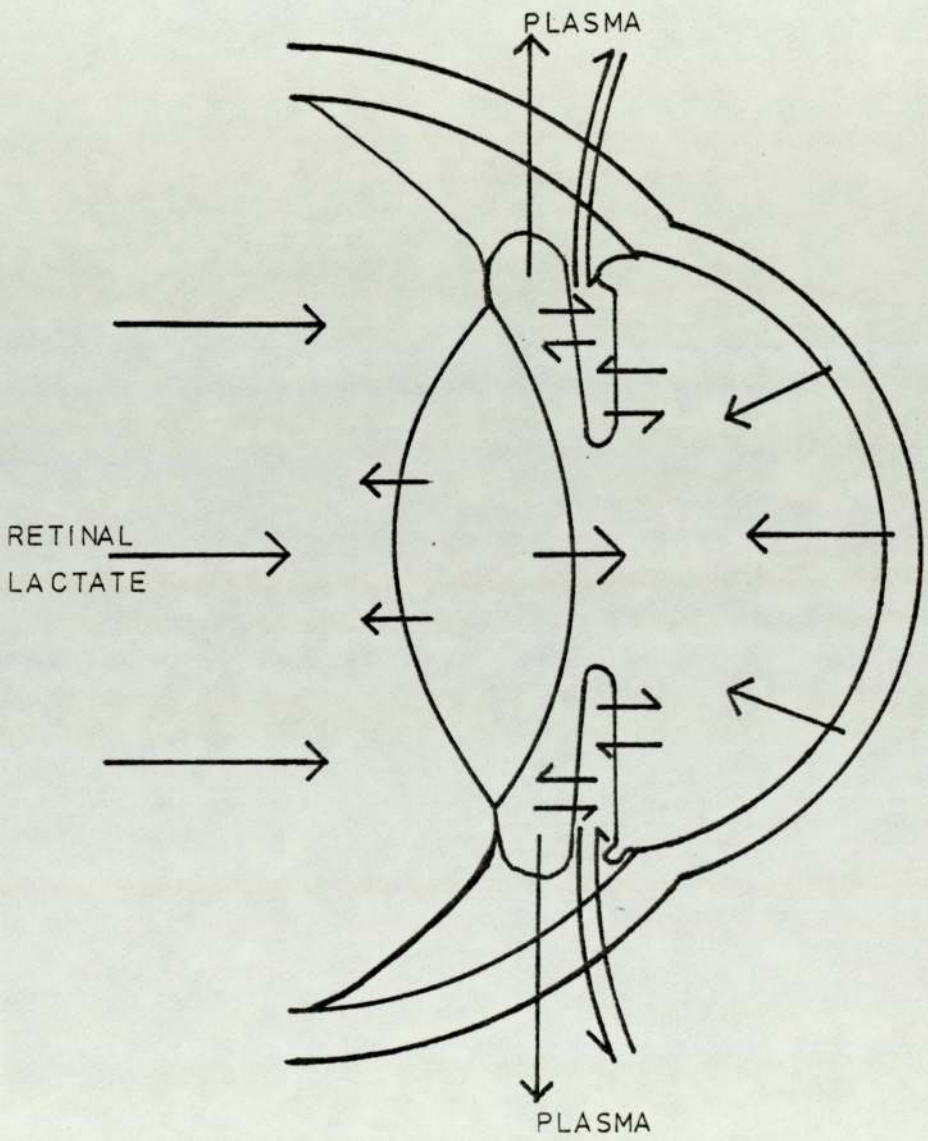
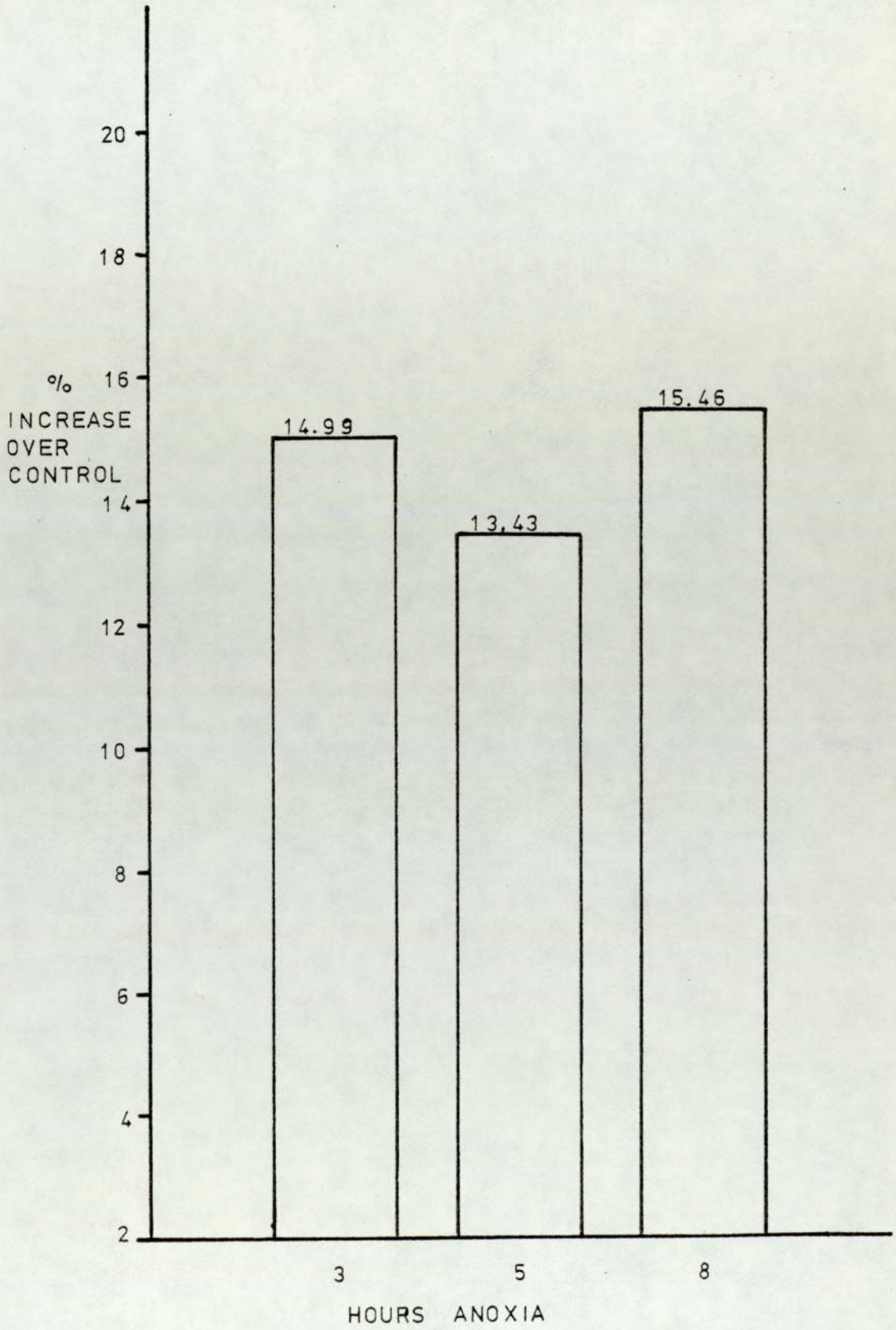


Figure 13 The effect of anoxia on Aqueous Humour lactic acid concentration.



concentration measured in the (E) at 8 hours, a figure of 123.74 $mgdl^{-1}$ is obtained for the concentration of lactic acid in the (E) after 8 hours anoxia. This concentration of lactic acid is similar to that measured after 3 and 5 hours anoxia and explains the similarity in percentage increase in Aqueous Humour lactic acid concentration due to anoxia.

Values for normal Aqueous Humour lactic acid concentration in the rabbit are shown in Table 7. It is evident from Table 7 that there is a large variation in the Aqueous Humour lactic acid concentration reported by various authors, these differences can be attributed to:-

(a) Species and age variation - Different sub-species of rabbit have been used and sub-species variation has been shown to exist in many biological systems, section 2.7, however BRUUN-LAURSON and LORENTZEN (1975) demonstrated that age has no effect on Aqueous Humour lactic acid concentration.

(b) Sample preparation - Methods of sample preparation vary from that described earlier in section 4.8, to heat fixation (BRUUN-LAURSON and LORENTZEN, 1975), sodium citrate (sodium iodoacetate) fixation (PRAUS et al., 1969) and other authors who do not specify sample treatment (RILEY, 1972). Speed and efficiency of inactivation of the glycolytic enzymes shown to be present in the Aqueous Humour can affect the Aqueous Humour lactic acid concentration (BITO and SALVADOR, 1970). If not fully inactivated enzymatic activity can take place leading to the formation or removal of lactic acid depending on the lactate/ pyruvate equilibrium.

Table 7

Lactic acid concentration in the Aqueous Humour of the rabbit.

AUTHOR	CONCENTRATION	UNITS	CONCENTRATION mg dl^{-1}
THIS WORK			98.5
Ruben and Carruthers 1972	4.3	$\mu M\ ml^{-1}$	38.74
Auricchio 1957	4.7	$mEq\ l^{-1}$	42.35
Kinsey 1953	12.1	$\mu M\ ml^{-1}$	109.02
Reddy and Kinsey 1960	9.3	$\mu M\ ml^{-1}$	83.79
Ridge 1956	9.68	$\mu M\ ml^{-1}$	87.22
Bito and Salvador 1970	2.93	$mM\ l^{-1}$	26.40
Reim et al. 1969	7.45	$mM\ l^{-1}$	67.12
" 1972	8.5	$mM\ l^{-1}$	76.59
Riley 1972	9.9	$mM\ l^{-1}$	89.20
Bruun-Laursen and Lorentzen 1975	5.2	$mM\ l^{-1}$	46.85
Praus et al. 1969	50.0	$\mu g100\ \mu l^{-1}$	50.0
Dreifus and Praus 1970	50	$\mu g100\ \mu l^{-1}$	50.0
Schütte et al. 1972 (a)	9.3	$\mu M\ gH_2O^{-1}$	83.79

Considering the preceding points the lactic acid concentrations between authors can be considered as being in agreement.

PRAUS et al., (1969) studied the effect of contact lens wear on Aqueous Humour lactic acid concentration over an eight hour period. Using 40% water content Geltact contact lenses covering 60 and 100% of the cornea they observed an increase of 104.4 and 111.2% respectively when compared to the control, which is taken as 100%. The percentage increase is therefore 4.4 and 11.2% of the control when these lenses are worn for eight hours. Further work by DREIFUS and PRAUS (1970) however only showed a 10% increase over the control over an eighteen hour test period with 100% of the cornea covered. RUBEN and CARRUTHERS (1972) used various types of contact lens and found percentage increases in the Aqueous Humour lactic acid over the control of:-

HeMa 40%: Worn for 1-4 weeks, constant wear = 74.42%

Small hard lens, 2-12 weeks wear = 13.95%

Large hard lens, 17 to 33 hours wear = 86.05%.

The percentage increases in Aqueous Humour lactic acid found by DREIFUS and PRAUS (1970), PRAUS et al., (1969) and RUBEN and CARRUTHERS (1972), (small hard lens) all fall within the range found in this system for 3, 5 and 8 hours anoxia.

The large increase in Aqueous Humour lactic acid concentration found in the gel lens and large hard lens system of RUBEN and CARRUTHERS (1972) cannot be demonstrated in this study. If the effects of anaesthesia on the (EC) level of lactic acid at eight hours is ignored and the (E) at eight hours compared to the (C) then the percentage increase

in lactic acid concentration over the (C) after eight hours anoxia = 51.72%. This figure compares more favourably with the figure of RUBEN and CARRUTHERS (1972), however it is difficult to make a true comparison on account of the observed increase in lactic acid concentration in the Aqueous Humour of the (EC) at eight hours.

It is possible that when soft and hard lenses are worn for longer periods of time, other factors are affecting lactic acid concentrations, for example a mechanical effect. However comparison of the work of DREIFUS and PRAUS (1970) with that of RUBEN and CARRUTHERS (1972) suggests that this is unlikely. Both authors used 40% water content lenses however RUBEN and CARRUTHERS (1972) show an increase 7 to 8 times that of DREIFUS and PRAUS (1970) with only six hours longer wear.

It is also possible that over longer periods, in excess of four days, lactic acid may concentrate in the Aqueous Humour. The results of this study show that after periods of anoxia up to eight hours the Aqueous Humour is capable of maintaining a high concentration of lactic acid, and that a similar high concentration of lactic acid is observed in the (EC) eye. Lactic acid excess = Aqueous Humour concentration minus the plasma concentration. In the normal rabbit Aqueous Humour = 98.5 - plasma concentration. RILEY (1972) gives a value for plasma lactic acid concentration of 50.46 $mgdl^{-1}$. Corrected for system differences plasma lactic acid concentration = 55.72 $mgdl^{-1}$.

Therefore in the normal rabbit Aqueous Humour lactic acid excess = 98.5 - 55.72 = 42.78 $mgdl^{-1}$.

After eight hours anoxia the excess = 149.56 - 55.72 = 93.84 $mgdl^{-1}$ or 123.74 - 55.72 (for the corrected concentration) = 68.02 $mgdl^{-1}$.

The lactic acid increase in the Aqueous Humour over the eight hour experimental period

= 149.56 - 98.5 = 51.0583 $mgdl^{-1}$

= 123.74 - 98.5 = 25.24 "

(or 5.6 mMl^{-1} and 2.80 mMl^{-1}).

RILEY (1972) has shown that the normal corneal lactic acid flux into the Aqueous Humour = 0.02 $\mu M min^{-1}$. If this is helping to create a lactic acid excess of 48.78 $mgdl^{-1}$, then 93.84 $mgdl^{-1}$ excess must be produced by a flux rate of:-

$$\frac{93.84}{42.78} \times 0.02 = \underline{0.044 \ \mu Mol \ min^{-1}}.$$

Epithelium

Figures 5 and 6 show that removal of the epithelium from the corneal stroma is complete. With practice the epithelium can be removed in a single piece, however there is usually some contamination by surface layers of the stroma. Avoiding such contamination is an extremely tedious procedure and largely unavoidable. It is appreciated that any stromal contamination will affect the final result, however the contamination is usually slight and since the concentration of lactic acid per unit weight of stroma is small it is unlikely to affect the concentration of lactic acid in the epithelial layer.

Sample preparation is relatively simple however great care must be taken to maintain the sample at $0^{o}C$.

Table 3 and Figure 10 represent the results obtained for epithelial lactic acid concentration and the effect of anoxia on epithelial lactic acid concentration.

A student t-test comparison of the (C) values for epithelial lactic acid concentration at 0, 3, 5 and 8 hours anaesthesia show no significant difference ($p < 0.1$).

There is however considerable variation in the results which can be explained by:-

(a) Depth of anaesthesia - this can affect lactic acid concentration, however various authors have suggested that this is unlikely (HERRMANN et al., 1942; AUGSBURGER and HILL, 1972, 1973).

(b) Varying amounts of stromal contamination will affect the concentration per unit weight relationship. As stated earlier this factor must be small since the cellular component of corneal stroma is approximately 1% (MAURICE and RILEY, 1970).

(c) It is suggested earlier that if the plasma glucose concentration falls as a consequence of anaesthesia then corneal glucose concentration will fall. A number of authors have shown that lactic acid concentration is increased when glucose concentration falls (REIM et al., 1972; THOFT and FRIEND, 1972; SCHÜTTE et al., 1972 (a)). If plasma glucose falls and Aqueous Humour glucose falls then corneal glucose may fall by a sufficient degree to affect metabolism in the epithelium. However it is highly unlikely that the plasma glucose would fall sufficiently to alter epithelial glucose concentration.

A student t-test performed on the experimental group shows no significant difference between the 3 and 5 hours anoxia treated epithelial lactic acid concentrations and the (C) concentration. There is a significant difference at eight hours ($p < 0.02$). A comparison of the (E) and (EC) at each time shows no significant difference at 0, 3 and 5 hours but a significant difference at eight hours ($p < 0.02$).

Table 8 shows the values for epithelial lactic acid concentration compared to those measured by other authors. Again there is a large variation, the range extending from 0.6206 (LANGHAM, 1952) to 2.712 (KILP, 1974) $mg.g^{-1}$ wet weight. Different methods of sample preparation and analysis could account for these differences (REIM et al., 1967 (b)).

Figure 10 shows that the concentration of lactic acid increases from 1.0381 $mg.g^{-1}$ wet weight to 1.3573 $mg.g^{-1}$ wet weight, this represents an increase of 30.7485% over the control in the eight hours of anoxia.

There have been a number of reports describing the effect of contact lens wear on epithelial lactic acid concentration. Since contact lenses reduce the oxygen tension at the epithelial surface it is assumed that the effect of contact lenses is to induce a state of anoxia at the corneal epithelial surface.

Table 8

Lactic acid concentration in normal rabbit corneal epithelium.

AUTHOR	CONCENTRATION	UNITS	CONCENTRATION mgg^{-1} wet wt.
THIS WORK			1.04
Morley and Toth 1961	996	μgg^{-1} wet weight	0.996
Kilp 1974	30.1	μMg^{-1} wet weight	2.71
Turss and Schebitz 1972	18.83	"	1.70
Reim et al 1967 (b)	13.58	"	1.22
Langham 1952	60.87	$mg\ 100g^{-1}$ wet weight	0.62

Author and treatment		% increase over the control
(a) MORLEY and McCULLOCH (1961) - Hard methyl methacrylate lenses worn on a rabbit eye.		
Period of wear in days =	1	56.52
	2	82.61
	3	102.17
(b) BURNS et al., (1971) - Various types of contact lens, each worn for 16 hours.		
Lens type = Silicon rubber		No effect
Carefully fitted methyl methacrylate		30.69
Randomly " " "		57.43
Geltact soft contact lens		57.43
(c) TURSS and SCHEBITZ (1972) - Hard contact lens worn for 24 hours.		38.24
(d) KILP (1974) - Hard and soft lens worn for 4 hours	Hard	59.80
	Soft	No effect

Nitrogen induced anoxia as seen in this experiment does not produce the severity of anoxia, as measured by increased epithelial lactic acid concentration, that might be expected. One possible explanation is that the eyelids are not restrained and the animal is free to blink, (ordinarily the animal does not blink under anaesthesia and the corneal epithelial surface must be kept moist by mechanically closing the eyelids at approximately half hourly intervals). The eyelids usually lie in close proximity to the cornea, and it is possible that some oxygen is diffusing from the blood vessels of the palpebral conjunctiva retarding both the onset of respiratory distress and the increase in epithelial lactic acid concentration.

Stroma Plus Endothelium

Small sample size and difficulty in isolating the endothelium makes the

preparation of separate endothelial and stromal samples impractical, and they are thus sampled together.

The collagenous fibres of the stroma make homogenation particularly tedious. Therefore a particular effort must be made to keep the tissue at $0^{\circ}C$ whilst homogenising.

Table 4 and Figure 11 present the data for the effect of anoxia on lactic acid concentration in the stroma plus endothelium. A student t-test comparison of the (C) with the (EC) shows no significant difference at 3 and 8 hours but a fall in lactic acid concentration at 5 hours ($p < 0.1$).

The observed fall in lactic acid control level at five hours reflects a trend seen in the other tissues. The only possible explanation is that anaesthesia is responsible for a decrease in corneal lactic acid. The mechanism by which anaesthesia exerts its effect is not known. Variation in the (C) eye is greater than that found in (EC) eyes. It is possible that not only does anaesthesia act to diminish lactic acid concentration in the stroma plus endothelium at five hours, but also to stabilise the lactic acid concentration.

A statistical comparison of the lactic acid concentration in the anoxic stroma plus endothelium at 3, 5 and 8 hours with the (C) using a student t-test shows no significant difference at 3 and 8 hours but a fall at 5 hours ($p < 0.02$). A comparison of the (E) with the (EC) at each time also shows no significant difference at 3 and 8 hours but a fall in lactic acid at 5 hours ($p < 0.05$).

Table 9 shows published data on the concentration of lactic acid in the stroma plus endothelium. Results obtained in this study are seen to compare favourably with those of the authors shown on Table 9. Slight differences can be attributed to different methods of sample preparation.

The results of a number of studies on the effect of contact lenses and anoxia on stroma plus endothelial lactic acid concentration are summarised below:-

Author and treatment		% increase over the control
(a) TURSS and SCHEBITZ (1972) - 24 hours hard contact lens wear		43%
(b) KILP (1974) - 4 hours of hard and hydrophilic (HEMA) contact lenses	Hard	11.80
	Hydrophilic	No effect
(c) MORLEY and McCULLOCH (1961) - hard contact lenses worn for 1 to 3 days continuously. Days wear =	1	46.15
"	2	59.62
"	3	75.0
(d) PRAUS et al., (1969) - 18 hours hard lens wear		50%
(e) DREIFUS and PRAUS (1970) - 18 hours hard lens wear		50%
(f) SMELSER and CHEN (1955)) (g) LANGHAM (1952)) (h) LANGHAM and TAYLOR (1956))	3 to 4 hours nitrogen induced anoxia	13.0
(i) THOFT and FRIEND (1972) - 30 minutes nitrogen induced anoxia		40.0

Values given by authors (d) to (i) for whole cornea lactic acid concentration increase. However epithelial and stroma plus endothelial

Table 9

Lactic acid concentration in the normal stroma + endothelium of rabbit cornea.

AUTHOR	CONCENTRATION		CONCENTRATION mgg^{-1} wet wt.
THIS WORK			0.54
Turss and Schebitz 1972	7.81	μMg^{-1} wet wt.	0.70
Kilp 1974	16.1	"	1.45
Morley and Toth 1961	771	$\mu g.g^{-1}$ wet wt.	0.77
Schütte et al 1972 (a)	13.0	μMg^{-1} H_2O	0.80
Reim et al 1972 (Endothelium removed)	12.1	μMg^{-1} H_2O	0.74

Other authors have measured whole cornea lactic acid concentration. Using Langham's (1952) estimate that lactic acid is distributed in the cornea of the normal eye in a 2:1 ratio, epithelium to stroma + endothelium, the following values are calculated.

Langham 1952			0.31
Langham 1954			0.29
Thoft and Friend 1972	4.15	μMg^{-1} wet wt.	0.37
Dreifus and Praus 1970	1434.5	$\mu g.g^{-1}$ wet wt.	1.44
Praus et al. 1969	1434.5	"	1.44

lactic acid concentration data show that the lactic acid concentration increases in all layers of the cornea.

The data obtained in these studies is unusual in that a decrease in lactic acid concentration in the stroma plus endothelium is observed after five hours. This decrease is indicative of:-

(a) An increase in lactic acid utilisation

(b) A mobilisation to another corneal component

(c) A decrease in lactic acid production.

The latter explanation is unlikely as any form of anoxia has been shown to increase lactic acid production in whole cornea (LANGHAM, 1952; SMELSER and CHEN, 1955). Further discussion of these possibilities is reserved for section 4.12.

There is surprisingly no increase in stroma plus endothelium lactic acid concentration after eight hours anoxia, compared to (C). There is however a significant increase over the five hours anoxic sample. It is possible that the stroma plus endothelial lactic acid concentration is increasing in this latter stage. Whether or not the stroma plus endothelial lactic acid concentration will increase over the (C) can only be determined by subjecting the cornea to longer periods of anoxia.

An explanation for the less severe effects of anoxia may be that oxygen is moving to the corneal tissues from the nearby palpebral conjunctiva. This will provide a higher oxygen tension at the corneal epithelial surface than expected by forced passage of nitrogen, or

Aqueous Humour substitution by a nitrogen bubble (LANGHAM, 1952; SMELSER and CHEN, 1955).

4.12 DISCUSSION

Section 1 (8) and (9) describe the changes that occur in the production and distribution of lactic acid in the cornea when subjected to anoxic stress either by reducing the oxygen tension at the epithelial surface or by wearing contact lenses.

The concentrations of lactic acid in the corneal tissues with exception of the tears and stroma plus endothelium compare favourably with data from other sources. Thus it can be assumed that despite species differences, such as that shown by KINOSHITA et al., (1955) and RILEY (1969 (a)) for the ratio of aerobic to anaerobic glycolysis in Ox and Rabbit cornea, similar respiratory pathways exist in the corneae of all animals, therefore a comparison between this work and other published data is quite valid.

There is little data regarding the concentration of lactic acid in tears either in the normal animal or one with an anoxic cornea. RUBEN and CARRUTHERS (1972), REIM et al., (1972) and KILP and HEISIG (1975) demonstrate the presence of lactic acid in the tears of a normal animal. However they differ with respect to the effect of contact lens wear. RUBEN and CARRUTHERS (1972) and KILP and HEISIG (1975) show that tear lactic acid concentration increases in contact lens wear, while REIM et al., (1972) find that the concentration of lactic acid in the tears remains constant regardless of treatment.

Unfortunately the technique used in this study like that of RUBEN and CARRUTHERS (1972) is only capable of giving qualitative data. The difficulty is collecting tear samples free from contamination by tears produced by reflex action.

A possible source of the measured increase in tear lactic acid is the corneal epithelium, since all the other tissues of the palpebral aperture are richly supplied with blood from a dense vascularisation thus maintaining an adequate oxygen supply. Although the epithelium is highly impermeable to organic ions, a slight flux occurs. Thus if the concentration in the epithelium becomes sufficiently high an outward flux to the tears of lactic acid will occur which can be measured. This increase in tear lactic acid concentration which can be simply measured by tear collection provides a method of measuring the metabolic status of the cornea without the use of surgical techniques.

However as discussed in section 4.11 before quantitative information can be obtained a more accurate method of sample collection must be conceived.

In the stroma plus endothelium, no anoxic increase in lactic acid is seen even after eight hours of anoxia. In fact at five hours a significant fall in stroma plus endothelial lactic acid concentration occurs.

KINOSHITA (1962), KINOSHITA and MASURAT (1954) and KUHLMAN and RESNIK (1959), have shown that in the normal cornea glycolysis takes place in the epithelium and stroma plus endothelium. These authors have also shown that in the epithelium two pathways of glycolysis are present,

the Embden-Myerhoff Pathway (E.M.P.) and an aerobic direct oxidative pathway, the Pentose Phosphate or Hexose Monophosphate Shunt (HMS). In the stroma the concentrations of the enzymes involved in the HMS are low and the activity of this pathway is assumed to be small. Oxidation of the products of glycolysis in the cornea is by the Krebs Citric Acid Cycle (TCA). The rate of oxidation by the TCA cycle in the corneal epithelium is insufficient to match the rate of lactic acid production by the glycolytic pathway, as a result lactic acid is always present in the epithelium. The rate of oxidation by the TCA cycle in the epithelium is much greater than in the stroma.

If the cornea is anoxic then the aerobic pathway will not operate and lactic acid concentration will increase. Normally the activity of the HMS pathway is high in the epithelium, and the inhibition of this pathway under anoxic conditions will lead to a transient fall in overall lactic acid production by glycolysis. The concentration of lactic acid in the corneal epithelium will be dependent on the anaerobic EMP pathway and will be constant while the TCA cycle and HMS pathways are inactive. Thus the flux of lactic acid into the Aqueous Humour will be reduced since the epithelium is temporarily producing less lactic acid. This transient reduction in flux is seen in the stroma plus endothelium sample as a fall in lactic acid concentration since lactic acid produced in the stroma is maintained at a constant rate, and Aqueous Humour oxygen supplies the respiratory needs of the endothelium thus lactic acid produced in the endothelium is constant. Therefore only the epithelium to Aqueous Humour flux affects the concentration of lactic acid in the stroma plus endothelium. A fall in stroma plus endothelial lactic acid concentration is seen for a short period; it

is assumed that this fall is represented by that seen at five hours in this study. Over the longer term lactic acid production in the epithelium by the EMP pathway increases due to the Pasteur effect, therefore lactic acid concentration in the epithelium will increase. The flux to the stroma plus endothelium and ultimately the Aqueous Humour will increase, which over a longer period will be reflected by an increase in the stroma plus endothelium concentration of lactic acid as seen in the work of other authors, and which would presumably be seen in this experiment had the anoxic period been longer.

Figure 14 shows a model of lactic acid production and movement in the normal and anoxic cornea.

4.13 CONCLUSION

Normal lactic acid concentrations in the constituent tissues of the cornea measured:-

Tears = 7.42 $mgdl^{-1}$

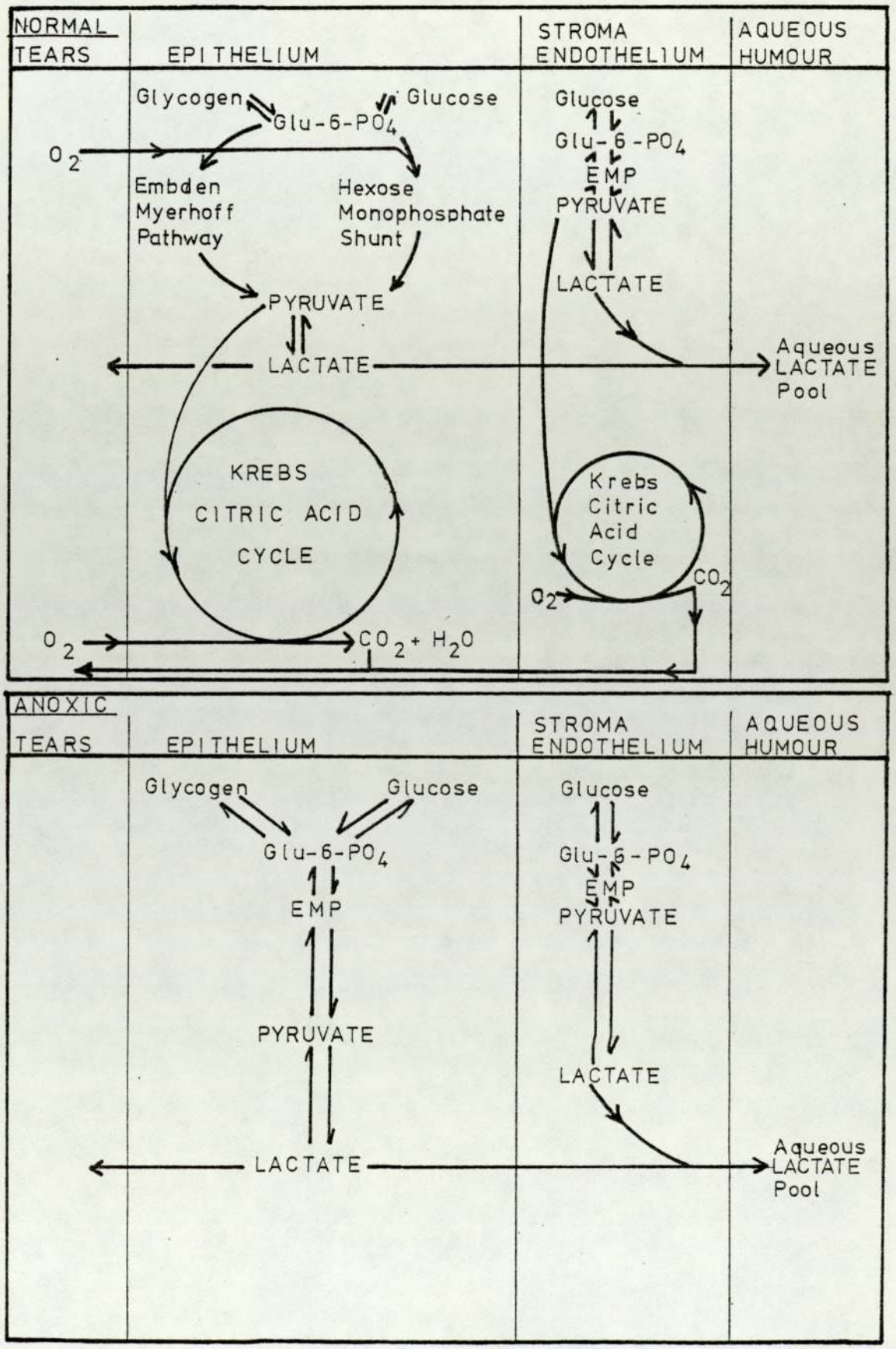
Aqueous Humour = 98.58 $mgdl^{-1}$

Epithelium = 1.04 mg g^{-1} wet weight

Stroma plus endothelium = 0.54 mg g^{-1} wet weight.

The levels of lactic acid in the corneal tissues are measured after exposing the epithelial surface to nitrogen gas for 3, 5 and 8 hours. The concentration of lactic acid increases progressively in the tears and Aqueous Humour. In the epithelium no significant increase is observed until eight hours. Stroma plus endothelial lactic acid shows no change at 3 hours, a significant fall at five hours and a return

Figure 14 A model of lactic acid production and dynamics in the normal and anoxic rabbit cornea.



to control levels at eight hours. Mechanisms are suggested for the various changes in lactic acid concentration in the corneal tissues.

Results describing the effects of anoxia on the cornea show similarities to the effects of contact lenses on corneal lactic acid concentration. It is suggested that contact lenses affect corneal lactic acid concentration by inducing anoxia at the corneal epithelial surface, and that results from anoxia and contact lens wear can be compared.

Contact lens wear results in an increase in corneal lactic acid concentration, therefore monitoring of corneal lactic acid can be used to determine one effect of contact lenses on the cornea, that is the interference with oxygen supply and corneal metabolism.

The only tissue which can be investigated for contact lens effects via the measurement of lactic acid concentration without intruding on the integrity of the cornea is the tears. Tear collection is inaccurate and only qualitative data can be obtained. If however tear collection can be established on a quantitative basis it would be possible to monitor <u>in vivo</u> effects of contact lens wear on the cornea which would be suitable for use in a clinical situation.

SECTION 5

Lactic acid in the tears using soft contact lenses as a collecting medium.

5.1 INTRODUCTION

Experiment 2, section 5, shows that the concentration of lactic acid in the component tissues of the cornea changes when the oxygen tension at the epithelial surface is reduced. Contact lenses are known to affect corneal lactic acid concentration and it is possible to gain insight as to the effects of contact lenses on corneal metabolism by observing the lactic acid concentrations of the cornea.

The only corneal tissue that can be collected, without interfering with the integrity of the cornea, is the tears. All attempts to obtain quantitative information regarding tear lactic acid concentration have been unsuccessful. One reason for the inability to derive quantitative information from the tears is the large variation in measured tear lactic acid concentrations in similarly treated animals. One reason for the large variation in the sampling procedure is the use of microcapillaries for collecting tear samples. This has a number of drawbacks:-

(a) The sample taken is only representative of the lactic acid concentration in the tears at the time of collection.

(b) Collection without inducing reflex tears is difficult.

(c) Sample sizes are small.

(d) There is a high risk of a capillary tube breaking in the eye.

RUBEN and CARRUTHERS (1972) used soft contact lenses as "sponges" to soak up tears. Tears absorbed by the lenses are subsequently elluted and subjected to enzymatic assay for lactic acid. The aim of these experiments is to use the basic method of RUBEN and CARRUTHERS (1972) as a model and to develop a method of quantifying the lactic acid concontration of tears produced under normal and anoxic conditions.

EXPERIMENT 1

The dynamics of lactic acid in Sauflon 85.

5.2 INTRODUCTION

RUBEN and CARRUTHERS (1972) and RUBEN (1976), report that hydrophilic contact lenses can absorb lactic acid from a tear solution. However they accept that the levels they measured may not be representative of the *in vivo* tear lactic acid concentration because the dynamics of solute movement in and out of soft lens polymers are not known.

Hydrophilic polymers of the type used in the manufacture of soft contact lenses have high water contents and as a result large pore sizes, (NG 1974; YASUDA and LAMAZE, 1971), this allows solutes with molecular radii of less than the pore size to enter the polymer and create an equilibrium between the solute in the bathing solution and the polymer. Thus the physical characteristics of the polymer have allowed the determination of lactic acid concentrations in the tear fluid.

If a quantitative study of solute movement in and out of the polymer is to be undertaken a number of parameters must be determined:-

(a) Pore size of the gel.

(b) Permeability of the gel to the solute in question.

(c) Rate of equilibrium of solute between the polymer and soaking solution.

(d) Binding of solute to polymer.

(e) Size of the polymer water reservoir.

In hydrophilic polymers pore size is proportional to water content. Thus if the water content is known pore size can be calculated (NG 1974; YASUDA and LAMAZE, 1971). The other parameters listed must be determined for each solute.

A number of papers have been published concerning the movement of solutes through hydrophilic polymer membranes (REFOJO, 1965; TAKAHASHI et al., 1966 (b); GUMPELMEYER and SCHWACH, 1972; FATT and ST. HELEN, 1971; FATT et al., 1977; YASUDA and LAMAZE, 1971). The method considered most suitable is described by GUMPELMEYER and SCHWACH (1972) who used radioactive tracers to observe the flow of solutes through hydrophilic polymer membranes.

This experiment combines the work of RUBEN and CARRUTHERS (1972) with that of GUMPELMEYER and SCHWACH (1972). The aim is to use radioactively labelled lactic acid and sensitive liquid scintillation counting techniques to determine:-

(a) Permeability
(b) Binding capacity
(c) Water content of the gel
(d) Estimation of gel pore size and size of the water reservoir
(e) Rate of equilibrium of lactic acid into a hydrophilic polymer.

5.3 APPARATUS AND METHODOLOGY

The aim of this experiment has been outlined in section 5.2. It is hoped to measure the rate of equilibrium, bonding and permeability of

lactic acid between two aqueous phases, one of which is a free solution and the other the water of hydration contained within the polymer mesh of a hydrophilic polymer gel.

Figure 1 shows a schema of the method to be used. Before the data can be obtained certain features of the system require analysis.

Choice of Polymer

The polymer if suitable will eventually be used in lens form in vivo, the gel must be suitable for patient use. Since the rate of movement of lactic acid molecules into the gel is being measured, a gel with a large pore radius will have a lower mechanical resistance to the movement of lactic acid molecules into and within the gel (YASUDA and LAMAZE, 1971). Polymer requirements for this study are:-

(a) Biocompatibility

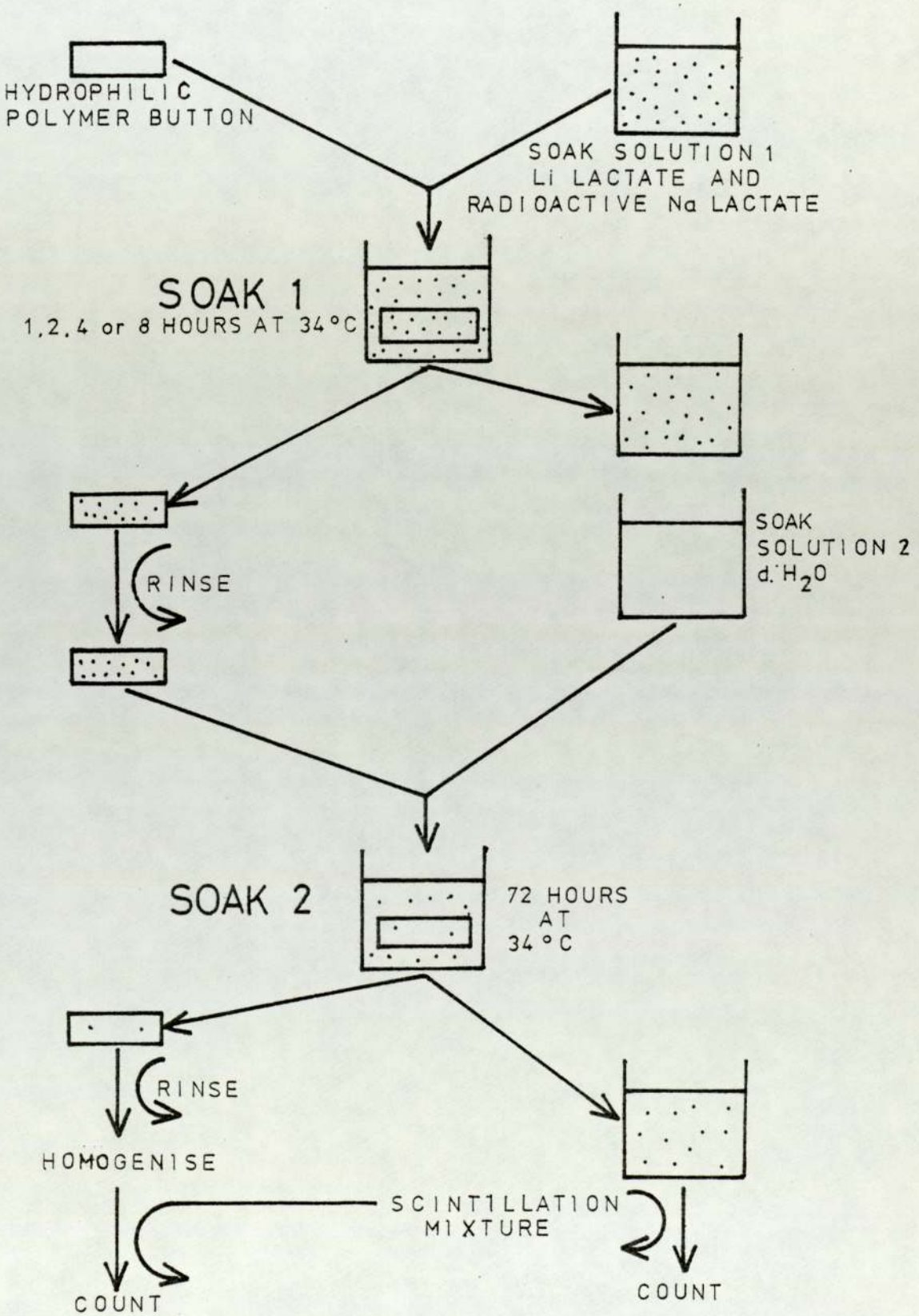
(b) High water content

(c) Large pore radius

(d) Acceptability for manufacture and use as contact lenses.

Sauflon 85 (British patent number 10186/71, 20.4.71, "Hydrophilic copolymers and articles formed therefrom") is widely used as a material for the manufacture of soft contact lenses, it has a high water content approximately 79% at 34°C in water, with a large pore radius = 20 Angstroms (NG, 1974; TIGHE, 1977) and a high patient acceptability (BURNETT-HODD, 1976, 1977).

Figure 1 Procedure for the determination of lactic acid dynamics in Sauflon 85.



Dehydrated rods of Sauflon 85 were the generous gift of Contact Lens Manufacturing Ltd.

Choice of Radioactive Tracer

The type of radioactive material and quantity required must be determined before any experiment using radioactive materials can be carried out.

A large number of radiochemicals are commercially available and it is frequently the case that not only is there a choice of radioisotope but also a choice of radioactive site in the molecule. Whenever possible a long lived radioisotope is chosen, one which is chemically stable and can be measured with a high efficiency.

The most common choice is between molecules having ^{14}C or ^{3}H as the isotope. In this experiment ^{14}C is chosen for a number of reasons:-

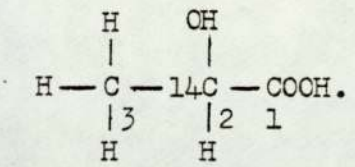
(a) The rate of decay of ^{14}C is slow, half-life = 5,760 years (NEAME and HOMEWOOD, 1974), therefore over a period of weeks no significant fall in activity will take place.

(b) Beta particles (β) emitted from ^{14}C have a higher energy and thus a higher counting efficiency than alpha particles (α) emitted from ^{3}H.

(c) The isotopic effect of ^{14}C is much less than that of ^{3}H.

(d) Molecular exchange of ^{14}C atoms is a less probably event than the exchange of ^{3}H atoms when in aqueous solution.

The ideal tracer is a lactic acid molecule labelled in the 2C position



This gives a molecule with all the features required and a labelled C atom in the centre of the molecule, making molecular exchange between the C atom of the lactic acid and the C atoms of the polymer chain unlikely. Any lactic acid left in the polymer other than that equilibrium fraction can be assumed to be bound to the polymer. Unfortunately this molecule is no longer available. The two isotopes currently available are L - (U14C) lactic acid and D.L. (1-14C) lactic acid. In either case any molecular exchange is difficult to determine as bonding to the polymer backbone is possible. The D-L (1-14C) form is employed to measure the counting efficiency while the L (U14C) form is used to determine the permeability and equilibration rate.

The problem of molecular exchange can only be answered empirically from the results. It can be assumed that any radioactivity remaining within the gel in excess of the expected concentration in the gel solution is either bound - lactic acid or due to molecular exchange.

In any investigation in which radioactive material is used it is necessary to know the amount of tracer and carrier required. The carrier concentration is set by the experimental requirements. Since

the aim of this experiment is to measure the uptake of lactic acid contained in the tears, a concentration representative of that present in the tears is used. When calculating the mass of carrier lactic acid plus tracer lactic acid the mass of tracer is so small that it can be considered negligible. The concentration of tracer depends on a number of factors:-

(a) The dilution expected

(b) The efficiency of counting

(c) The time available for counting

(d) The specific activity of the tracer.

The specific activity is obtained from specifications produced by the manufacturers. Time available for counting is set by the machine operators to allow the machine to be used at optimum capacity. The efficiency of counting must be determined experimentally for each system. An approximate value for the dilution factor can be calculated from the experimental details.

Preparation of Sample for Counting

Beta particles emitted by ^{14}C are measured by liquid scintillation counting techniques. Liquid scintillation counting is based on the transfer of energy from the β particles to a suitable scintillant which emits the energy as photons of light. Beta particles of sufficient energy excite solvent molecules which in turn excite others producing a cascade effect. This results in the transfer of approximately 5% of the energy from the original β particle (PRICE, 1973).

The photons of light are then detected by a photomultiplier tube and converted into electrical impluses.

The sample to be counted must be mixed with suitable solvents and scintillators in a transparent counting vial and lowered into the light-tight compartment of a liquid scintillation counter. The whole region of the counter must be shielded from extraneous radiation.

Unfortunately the number of counts recorded by a liquid scintillation counter is always less than the number of β particles emitted by the sample. This discrepancy is usually expressed in terms of counting efficiency, which is equal to the percentage of decaying muclei actually counted. Counting efficiency depends on a number of factors, some associated with the instrument, which in this case can be considered constant, and those associated with the sample.

The most important effect of the sample is quenching. This is the interference of β particle energy conversion to scintillations, or interference with light transmission within the solution. Both reduce the amount of light reaching the photomultiplier, as a result fewer scintillations are detected and the pulses generated are of lower voltage, and consequently efficiency is reduced. Unless quenching is known to be constant, or efficiency estimated, the comparison of count rates from different samples is meaningless.

To prepare the sample for counting it must first be mixed with primary solvents and scintillators. Unfortunately aqueous samples, as used in these experiments, are not soluble in these primary solvents used in liquid scintillation counting. Further materials, solubilisers, must

be added to the sample/solvent mixture to allow the incorporation of water. All such additions cause quenching as does water itself. Where aqueous samples are added, scintillation mixtures must be chosen carefully to enable the lower energy emissions to be detected and counted. The production of a scintillation mixture capable of solubilising aqueous samples is relatively simple. In this experiment a second problem arises in that the quantity of radioactivity contained in the water of hydration of the gel is required. This involves introducing the gel into the scintillation mixture. Hydrophilic gels are insoluble, therefore a method producing a suspension of the material must be used. Gels can be homogenised to a fine suspension in aqueous solution, however when placed in a standard scintillation mixture the count rate will fall progressively as the particles settle. An alternative method is to suspend the particles in a scintillation mixture that has a rigid or semi-rigid gel matrix. Solid materials are also self-quenching, this reduces the overall efficiency of counting, and as a result counting efficiency must be determined for each system.

Choice of Hydrating and Soak Solutions

A further consideration must be taken in the choice of solution to hydrate the gel. The choice is between water and 0.9% sodium chloride solution. One consideration is that if a button of polymer, hydrated in water, is placed in a solution of lactic acid in 0.9% sodium chloride solution, then the sodium chloride would diffuse down a concentration gradient from solution to gel. This would thus act as a carrier or diffusion pump and assist the movement of lactic acid

into the gel, and thereby accelerate the equilibrium process. This is an advantage in an experiment where rapid uptake of lactic acid into the polymer is desired. Conversely, if water is employed as the second soaking solution (Figure 1), it is hoped that the diffusion gradient of sodium chloride from gel to water will effect a similar outward movement of lactic acid.

Determination of Water Content of Sauflon 85

Rods of Sauflon 85 are cut into small buttons, 12 mm. in diameter and approximately 0.25 mm. thick. The discs are weighed and hydrated in either distilled water or 0.9% sodium chloride solution for three days at 34°C. When fully hydrated the gel discs are quickly dabbed dry and reweighed.

The gels are dehydrated in a vacuum oven, (Edwards High-Vac), at 60°C until repeated weighings show no change in weight and this final weight is noted. The ratio of dehydrated to hydrated weight expresses the water content of the gel.

Gelling of Scintillant

A scintillation "cocktail" suitable for the preparation of aqueous gel samples is the generous gift of Beckman Ltd. Ready-Solv E.P. scintillation cocktail is a mixture of: toluene, the primary solvent; Triton X-100, a solubiliser, and a special fluor system for producing photons of optimum wavelength.

The optimum ratio of scintillant and water (sample) for gel formation is determined by mixing varying amounts of scintillant and water, and observing the viscosity of the resulting gel. A ratio of 2:1 scintillant to water (sample) produced the most acceptable mixture.

Determination of Counting Efficiency, Polymer Quenching and Sedimentation with Sauflon 85

A solution of 10 $mgdl^{-1}$ derived from section 4.11 is taken as representative of lactic acid concentration in the tears. It is prepared by dissolving 0.010g. of L-Lithium lactate in 100ml. of 0.9% sodium chloride solution.

A measured volume of Sauflon 85 is homogenised to a fine suspension in 1ml. of 10 $mgdl^{-1}$ lactic acid solution in a 1ml. glass homogeniser (Jencons Scientific Ltd.). When the fine suspension is prepared the volume is made up to 3 ml. using the 10 $mgdl^{-1}$ lithium lactate solution. Varying quantities of gel suspension are made up to 4.8ml. with the lithium lactate standard solution and placed in scintillation vials (Beckman value vial TMII Catalogue Number 566408).

Fifty microcuries (μCi) of DL (1-14C) lactate, sodium salt, (Radiochemical Centre, Amersham), with a specific activity of 324 μCi mg^{-1}, is dissolved in 2 ml. of distilled water. Further dilutions are with standard 10 $mgdl^{-1}$ lactic acid in 0.9% sodium chloride solution. The radioactive stock solution is diluted in order to obtain a radioactive count of 2220 disintigrations per minute (D.P.M.) per 0.2 ml. aliquot. This concentration of radioactivity is used

since only ten minutes is available for counting each vial. For a standard deviation of 1% a minimum figure of 10,000 counts is required (NEAME and HOMEWOOD, 1974). Thus 2220 DPM allows sufficient radioactivity despite the possibility of low counting efficiencies. Diluted stock (0.2 ml. of 1.11×10^4 DPM ml^{-1}, 2220 DPM) is added to each vial and the solutions thoroughly mixed using a vortex mixer.

Ten ml. of Beckman Ready-Solv E.P. scintillation fluid is added to each vial. Each vial is capped and the contents mixed thoroughly.

The vials are appropriately labelled and placed in a Beckman LS 230 liquid scintillation counter. The counter is set to detect photons obtained from ^{14}C and ^{3}H energy β emissions. Each vial is counted for ten minutes. In order that no settling should occur, before the initial count, each vial is shaken immediately prior to placing into the counting chamber. When the rate of sedimentation is being measured each vial is shaken before the initial count and then left untouched for subsequent counts that are taken every hour for six hours.

One vial contains only 15 ml. of scintillant and is used to determine the background count rate. Another vial contains no polymer and acts as a control to determine the efficiency of counting and to allow the determination of quenching by the polymer.

Lactic Acid Uptake by Sauflon 85

A standard lactic acid solution is prepared by dissolving 0.010g. of Lithium lactate in 100 ml. of 0.9% sodium chloride solution. Forty ml. of this solution are placed in a 100 ml. volumetric flask, and 0.2 ml. (200 µl) removed using an automatic pipette.

A 5.55×10^7 D.P.M. ml^{-1} stock solution of L-(U14C) lactic acid sodium salt is prepared by dissolving 50 µCi of L-(U14C) lactic acid sodium salt, of specific activity 870 µCi mg^{-1}, in 2 ml. of distilled water. Out of this solution are taken 0.2 ml., which is then added to the volumetric flask containing 39.8 ml. of 10 $mgdl^{-1}$ Lithium lactate solution. This gives a stock radioactive lactate solution containing:-

(i) 10 $mgdl^{-1}$ L-Lithium lactate carrier

(ii) 2.775×10^5 DPM ml^{-1}.

(The error in lactic acid concentration caused by adding 0.2 ml. of distilled water is less than 0.5% and is ignored).

Since 1 ml. of this stock solution contains 0.1 mg. of lactate the activity in terms of mass of lactate = 2.775×10^6 DPM mg^{-1} of lactate.

Eight buttons of Sauflon 85 are weighed and hydrated, four in 0.9% sodium chloride solution and four in distilled water. Each button is dabbed dry and placed in a 10 ml. glass bottle. To this bottle is added 5 ml. of radioactive standard lactate solution. The button is allowed to soak for 1, 2, 4 or 8 hours at 34°C. After the appropriate time interval the buttons are removed and rinsed with distilled water, dabbed dry, and placed in another bottle containing 5 ml. of distilled

water at 34°C. The buttons are left to soak for 72 hours and the bottles are shaken occasionally to allow complete exposure of the buttons to the solution.

After 72 hours each button is removed, rinsed, dabbed dry and homogenised to a fine suspension in a 1.0 ml. homogeniser containing 2.0 ml. of water. The homogeniser is rinsed with a further 3 ml. of water and the total volume of 5 ml. transferred to a scintillation vial. The second soaking solution is also placed in a scintillation vial.

Two sets of samples are obtained, a gel suspension and the second soak solution (see Figure 1). To each vial is added 10 ml. of scintillation fluid. The vials are capped and thoroughly mixed. Vials are placed in the scintillation counter and each vial counted for ten minutes. Vials are left for one hour in the counter before counting.

5.4 RESULTS

Determination of Water Content

Buttons of Sauflon 85 are cut from rods and hydrated in 0.9% sodium chloride solution or water. All are hydrated at 34°C:-

Water hydrated buttons, mean weight = 0.1493 g.

After dehydration to constant weight the mean weight = 0.0310 g.

The percentage water content in water at 34°C = 0.1493 - 0.0310 = 0.1183 = water portion.

Therefore percentage water content = $\frac{0.1183}{0.1493} \times 100$

$$= 79.2364\%$$

0.9% sodium chloride solution hydrated buttons:-

Mean weight = 0.1520 g.

Mean dehydrated weight = 0.0331 g.

Percentage water content = 0.1520 - 0.0331 = 0.1189

$$= \frac{0.1189}{0.1520} \times 100$$

$$= 78.2237\%$$

Determination of Gel Quenching, Sedimentation and Counting Efficiency

Six sample vials are prepared:-

Vials 1 to 4 - Lactate solution, a spike of radioactive lactate solution containing 2220 D.P.M., homogenised Sauflon 85 and 10 ml. of scintillation cocktail.

Vial 5 - As for vials 1 to 4 without Sauflon 85.

Vial 6 - 15 ml. of scintillation cocktail.

Each vial is shaken and counted immediately, and then untouched for six hours, the activity being measured every hour.

Raw data for each vial is presented in Table 1. Vial 6 represents background activity. The measured activity of vials 1 to 5 must be corrected for background by subtracting the activity of vial 6. Mean background count = 101.9571 counts per minute (C.P.M.).

Table 1

Raw data for the determination of quenching and sedimentation effect of Sauflon 85. Values are counts per minute (C.P.M.) minus background count.

VIAL NO.	Hydrated weight of gel.g.	TIME OF COUNT HOURS 0	1	2	3	4	5	6	MEAN	SEM
1	0.0094	1404.04	1435.34	1436.34	1453.24	1449.64	1428.14	1430.04	1433.8	6.1135
2	0.0234	1426.14	1426.04	1413.14	1417.34	1414.34	1391.64	1367.94	1408.86	7.9892
3	0.0469	1386.44	1429.94	1407.44	1418.84	1388.54	1364.14	1387.74	1397.59	8.4773
4	0.0937	1378.44	1421.44	1450.54	1431.04	1376.44	1388.54	1397.24	1406.24	10.7611
MEAN		1398.77	1426.89	1426.89	1430.12	1407.24	1393.12	1395.74		
SEM		9.1591	2.5285	8.7178	7.1848	14.0234	11.4279	11.2248		
5		1406.84	1457.24	1458.24	1424.64	1404.24	1404.24	1426.64	1426.01	8.9069

Vials 1 to 4 contain varying quantities of Sauflon 85, with the aim of determining the quenching effect of the gel. Figure 2 shows the mean plus or minus the standard error of the mean ($\pm$ SEM) of counts taken at 0 to 6 hours. Vial 5 acts as a control since no gel is present. Comparison of vials 1 to 4 against control vial 5 using a student t-test is shown on Table 2. Vial 3 is significantly different from the control ($p < 0.05$), vials 1, 2 and 4 show no significant difference. Comparisons between vials 2, 3 and 4, show that the effect of polymer quenching can be ignored if the button weights are of the same order of magnitude.

Figure 3 shows a plot of count rate minus background for vials 1 to 5 at hourly intervals up to six hours. From a visual analysis there is little settling of the scintillation cocktail mixture as shown by vial 5. Vials 1 to 5 all exhibit an increase in activity after the first hour which is maintained up to three hours, followed by a gradual fall. A possible explanation for the increased activity after the first hour is that at zero hours each vial is shaken, due to the viscous nature of the scintillation cocktail/aqueous sample mixture some of the mixture adheres to the vial walls higher than the photo-multiplier tube can "see". As a result some activity may escape uncounted. After standing this "unseen" portion may fall back into the main volume and become effective. This could produce the increased count rate observed after the first hour. This count is maintained for the next two hours after which a decay in activity takes place.

Comparing vial 1 with vials 2, 3 and 4 shows that the gel has some settling effect. The weight of the gel in vial 1 is an order of

Figure 2 Determination of the quenching effect of Sauflon 85. Values are the mean ± SEM of counts taken every hour for 6 hours.

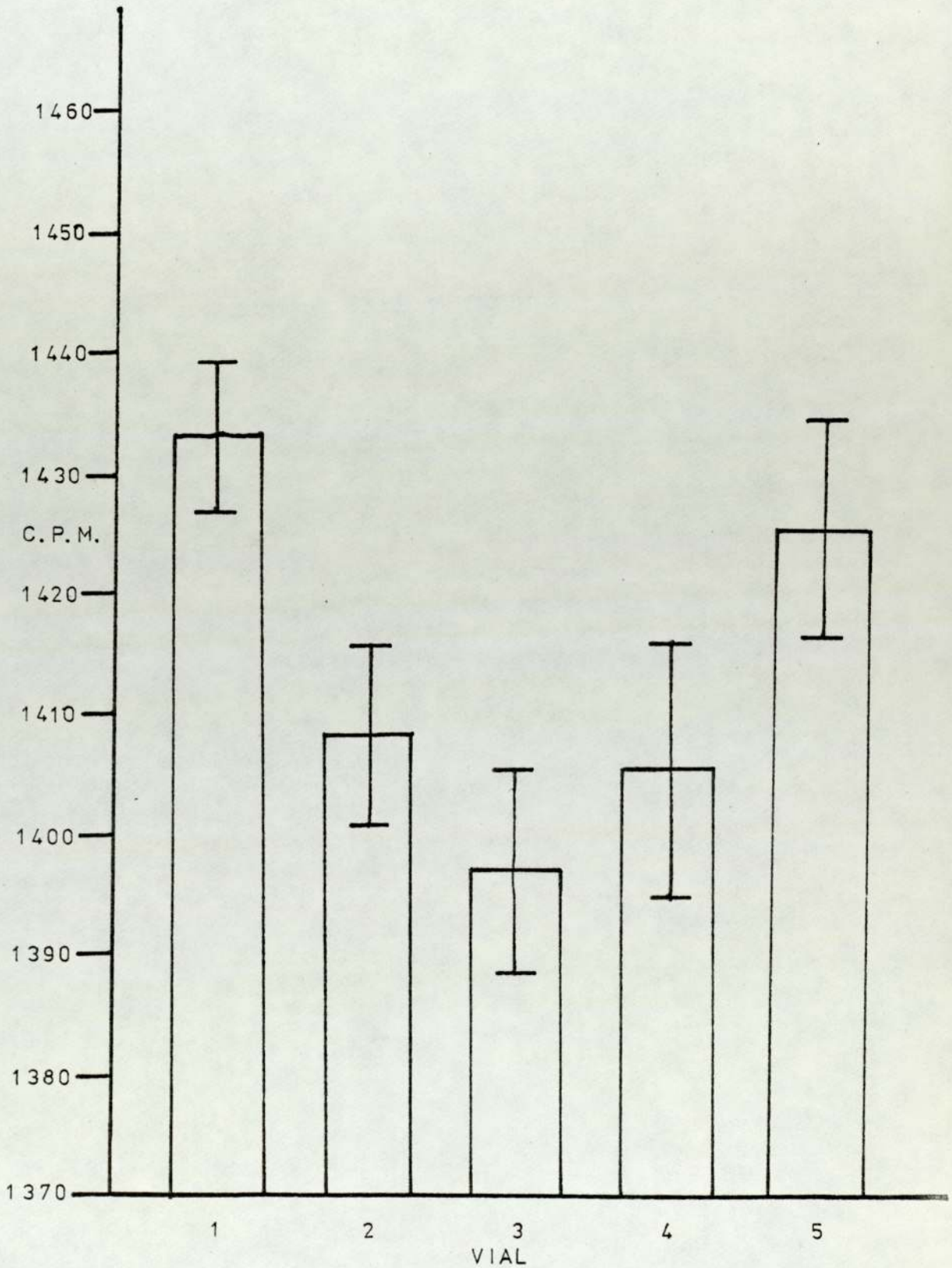
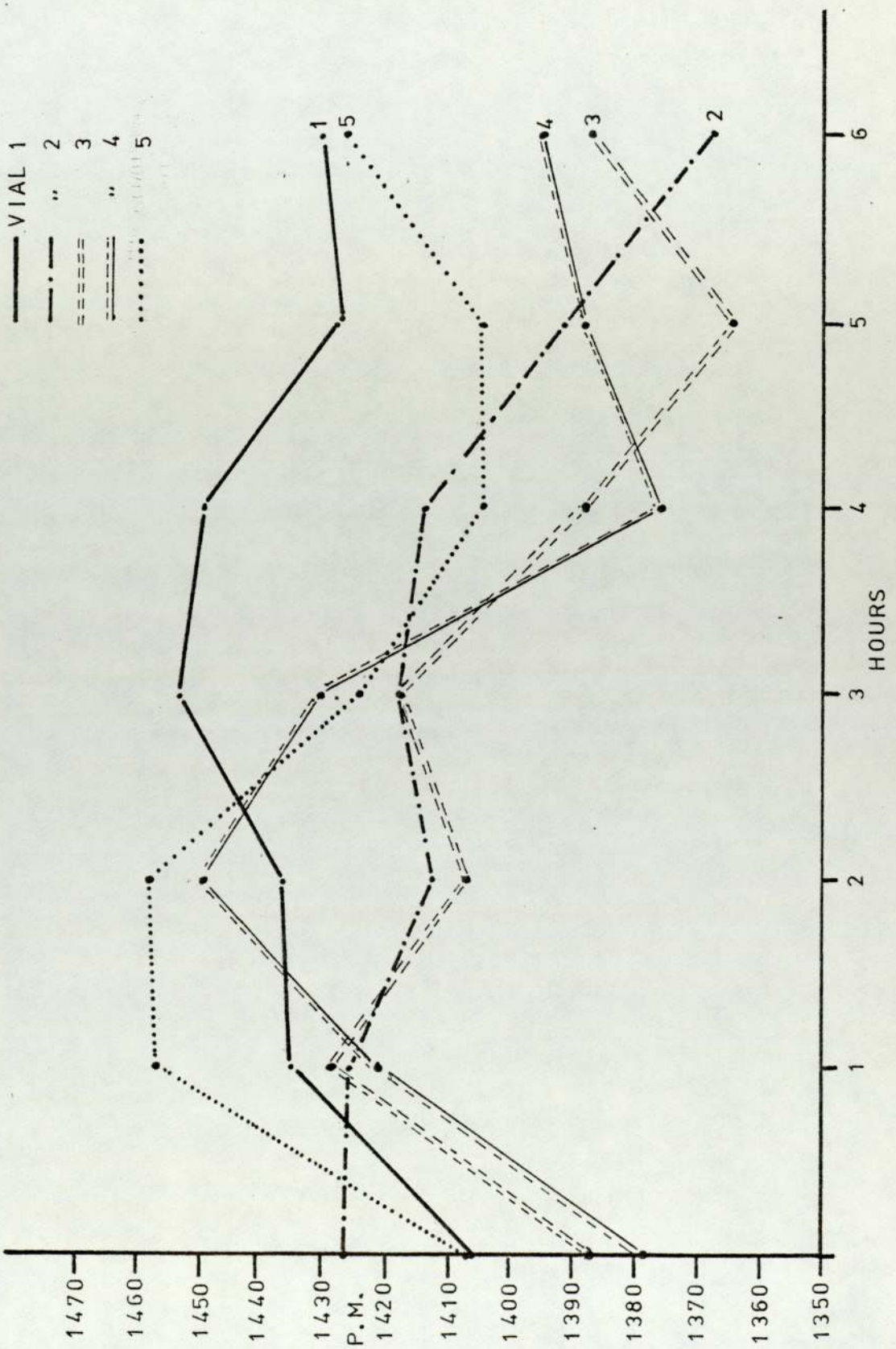


Table 2

Statistical data for a comparison of the control with varying quantities of polymer.

VIAL NO.	Weight of gel. g. hydrated	MEAN COUNT C.P.M.	S.E.M.	COMPARISON	σ_F	t	SIGNIFICANCE LEVEL		
							p=0.1	p=0.05	p=0.02
1	0.0094	1433.80	6.1135	1 v 5	12	0.7207	NO	NO	NO
2	0.0023	1408.86	7.9892	2 v 5	12	1.4984	NO	NO	NO
3	0.0469	1397.59	8.4773	3 v 5	12	2.3120	YES	YES	NO
4	0.0937	1406.24	10.7611	4 v 5	12	1.4154	NO	NO	NO
5	-	1426.01	8.9069	-	-	-	-	-	-

Figure 3 Concentration and sedimentation effects of Sauflon 85 on count rate.



magnitude different from vials 2, 3 and 4. Vial 1 shows no visible decay in activity due to settling over the six hour period. Vials 2 to 4 show a slight fall in activity. Compared to the zero hours control, there is no visible difference in vials 2 to 4 at six hours, however compared to the increased activities obtained at two hours there is a slight fall in activity. It can be concluded that if the vials are counted between 1 and 3 hours after shaking the effects of settling can be ignored. Figure 3 also shows that between 1 and 3 hours the quenching effect of the gel is small and can be ignored if the relative activities are high.

Having shown that settling can be ignored, and that no quench correction due to the gel is necessary if the vials are counted between 1 and 3 hours of shaking, the counting efficiency of the system can be calculated.

Since 2220 D.P.M. are added to each vial, the ratio of observed counts to actual counts expressed as a percentage yields a value for counting efficiency of the system.

Actual counts = 2220 D.P.M.

Observed counts = Mean value of the activities in vials 1 to 5 at 0 to 6 hours = 1414.2029

Therefore the counting efficiency = $\frac{1414.2029}{2220} \times 100$

$= \underline{63.7028\% \text{ efficiency}}$.

Lactic Acid Uptake by Sauflon 85

Having calculated the water content and counting efficiency it is possible to relate the activities observed in the aqueous and button portions described in section 5.2 to actual quantities of lactic acid (lactate) taken up and released by the gel.

Figure 4 shows a modified scheme of that given in section 5.3. Table 3 shows the raw data for the determination of lactate absorbed by Sauflon 85.

Background count = 93.1 C.P.M.

Counting efficiency = 63.7028%

Water content of Sauflon 85 in water = 79.2364%

" " " " " in 0.9% sodium chloride solution = 78.2237%.

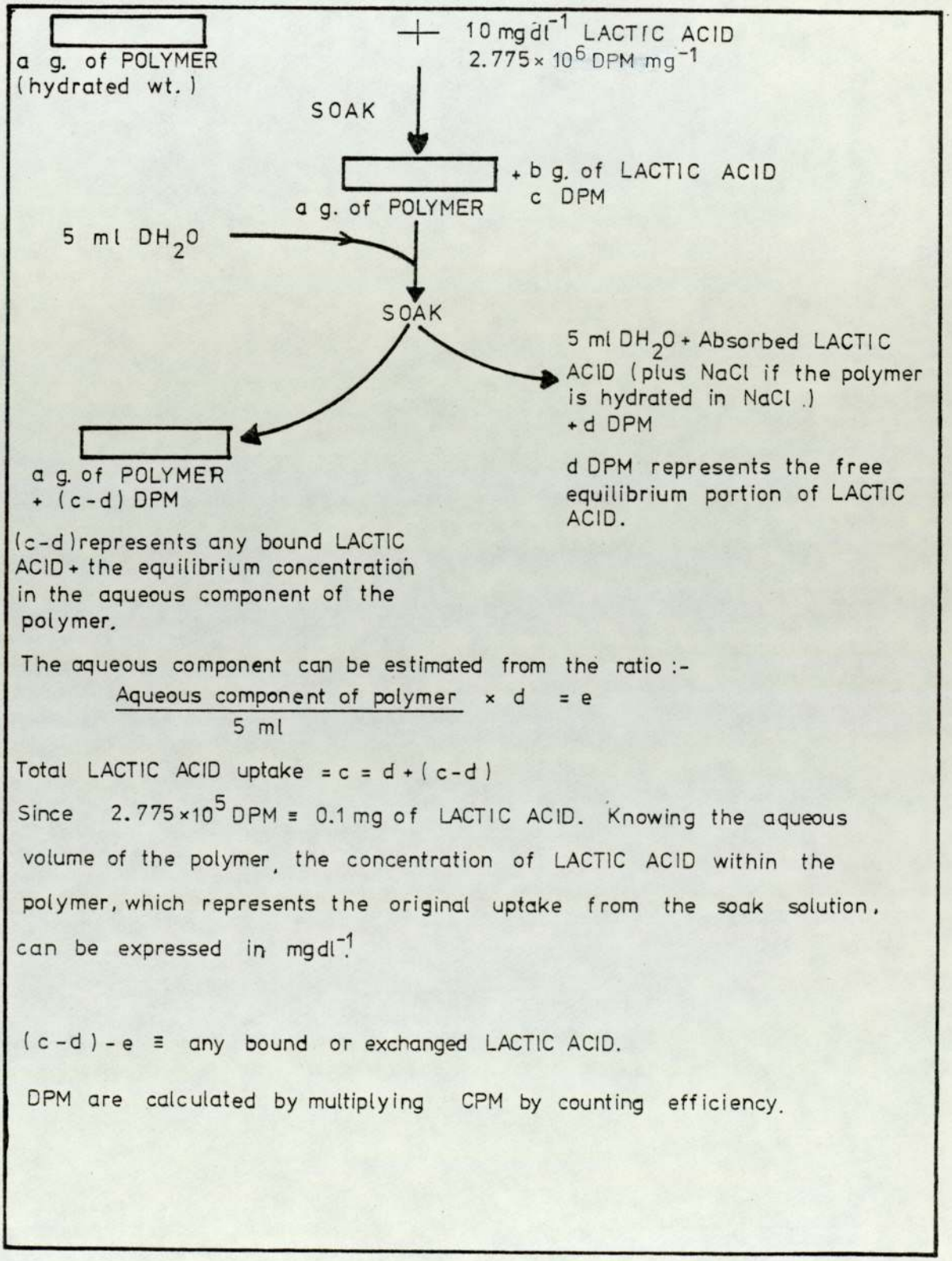
In order to make a comparison between the various treatments and to calculate the rate of lactate uptake, the raw data must be adjusted:-

(a) Each vial contains varying weights of polymer, therefore actual D.P.M.'s must be converted to counts on a unit weight basis. Actual D.P.M.'s are converted to D.P.M.'s per 0.1g. of polymer.

(b) The sum of D.P.M.'s for gel and solution represent the value C on Figure 4, i.e. the total radioactivity taken up by the gel button.

(c) C divided by the mg. equivalent for radioactivity and lactate, 2.7750×10^{6} D.P.M. mg^{-1} lactate, gives a value for the actual quantity of lactate taken up by the gel in mg.

Figure 4 A model of lactic acid dynamics in Sauflon 85.



The aqueous component can be estimated from the ratio :-

$$\frac{\text{Aqueous component of polymer}}{5\ \text{ml}} \times d = e$$

Total LACTIC ACID uptake = c = d + (c-d)

Since 2.775×10^5 DPM ≡ 0.1 mg of LACTIC ACID. Knowing the aqueous volume of the polymer, the concentration of LACTIC ACID within the polymer, which represents the original uptake from the soak solution, can be expressed in mg dl^{-1}.

(c-d) - e ≡ any bound or exchanged LACTIC ACID.

DPM are calculated by multiplying CPM by counting efficiency.

Table 3

Raw data for the determination of lactic acid absorbtion and release by Sauflon 85.

TREATMENT	CPM	CPM-Background	CPM x $\frac{\text{D.P.M.}}{\text{C.Efficiency}}$	Hydrated weight g.
Polymer				
H_2O 1hrs.	154.10	61.0	95.76	0.0910
H_2O 2hrs.	114.85	27.75	34.14	0.0715
H_2O 4hrs.	103.70	10.60	16.64	0.0826
H_2O 8hrs.	159.60	66.5	104.39	0.10
NaCl 1hrs.	123.60	30.50	47.88	0.1069
NaCl 2hrs.	142.90	49.80	78.18	0.1088
NaCl 4hrs.	128.50	35.40	55.57	0.0941
NaCl 8hrs.	133.90	40.80	64.05	0.0875
Solution				
H_2O 1hrs.	2005.40	1912.3	3001.91	0.0910
H_2O 2hrs.	338.0	244.5	383.81	0.0715
H_2O 4hrs.	642.30	549.2	862.13	0.0826
H_2O 8hrs.	1473.90	1380.8	2167.57	0.10
NaCl 1hrs.	1316.80	1223.7	1920.95	0.1069
NaCl 2hrs.	2522.40	2429.3	3813.49	0.1088
NaCl 4hrs.	1658.30	1565.2	2457.03	0.0941
NaCl 8hrs.	2038.80	1945.7	3054.34	0.0875

(d) Conversion of the value of lactate taken up by the polymer button in mg. to $mgdl^{-1}$ of polymer solution can be calculated by multiplying C by $\frac{1}{0.07924} \times 100$. This converts mg. of lactate in 0.07924 ml. of polymer solution into $mgdl^{-1}$ of polymer solution.

(e) To determine that quantity of lactic acid not in equilibrium with the soak solution, the expected value for count rate in the polymer must be calculated. This is done as follows:-

There are n D.P.M. in 0.1g polymer.

Therefore 78.2237% of 0.1g = 0.078224 g.

Assuming 1g of 0.9% sodium chloride solution is equivalent to 1 ml. of 0.9% sodium chloride solution.

The 0.07822 g of sodium chloride solution ≡ 0.07822 ml. of sodium chloride solution.

Therefore $\frac{5.0}{0.07822}$ = (the volume of soak solution in equilibrium with the polymer solution).

= Ratio of solution in the final soak solution to the solution in the polymer = 63.7841.

Therefore if the activity in the 5.0 ml. of soak solution is in equilibrium with the polymer solution then the expected activity

= $\frac{d}{63.784}$ D.P.M. in the aqueous portion of the polymer (d is obtained from Figure 4).

For water hydrated buttons = $\frac{5.00}{0.07924}$

$= 63.0040$

$= \frac{d}{63.0040}$

This calculated value subtracted from the actual activity in the

polymer button, n, gives that portion of radioactivity, and by inference lactate, either bound to the polymer or due to exchange of carbon atoms between the labelled lactic acid and the polymer backbone.

Table 4 shows the treated data.

Figure 5 shows the activity in D.P.M.'s per 0.1g of polymer for each treatment.

5.5 DISCUSSION

Homogenised hydrated Sauflon 85 is a fine suspension of polymer particles. The high efficiency of counting shown in this system suggests that the fine particles of polymer are transparent to the β particles emitted by the radioactive tracer, and also to the photons of light emitted by the scintillant.

The particles of gel created by the homogenising procedure are small enough to give a slow sedimentation rate, and therefore decay in activity due to settling is small. Some decrease in activity with time does occur, this is due to the increased self-absorbtion of the gel as it concentrates at the bottom of the vial.

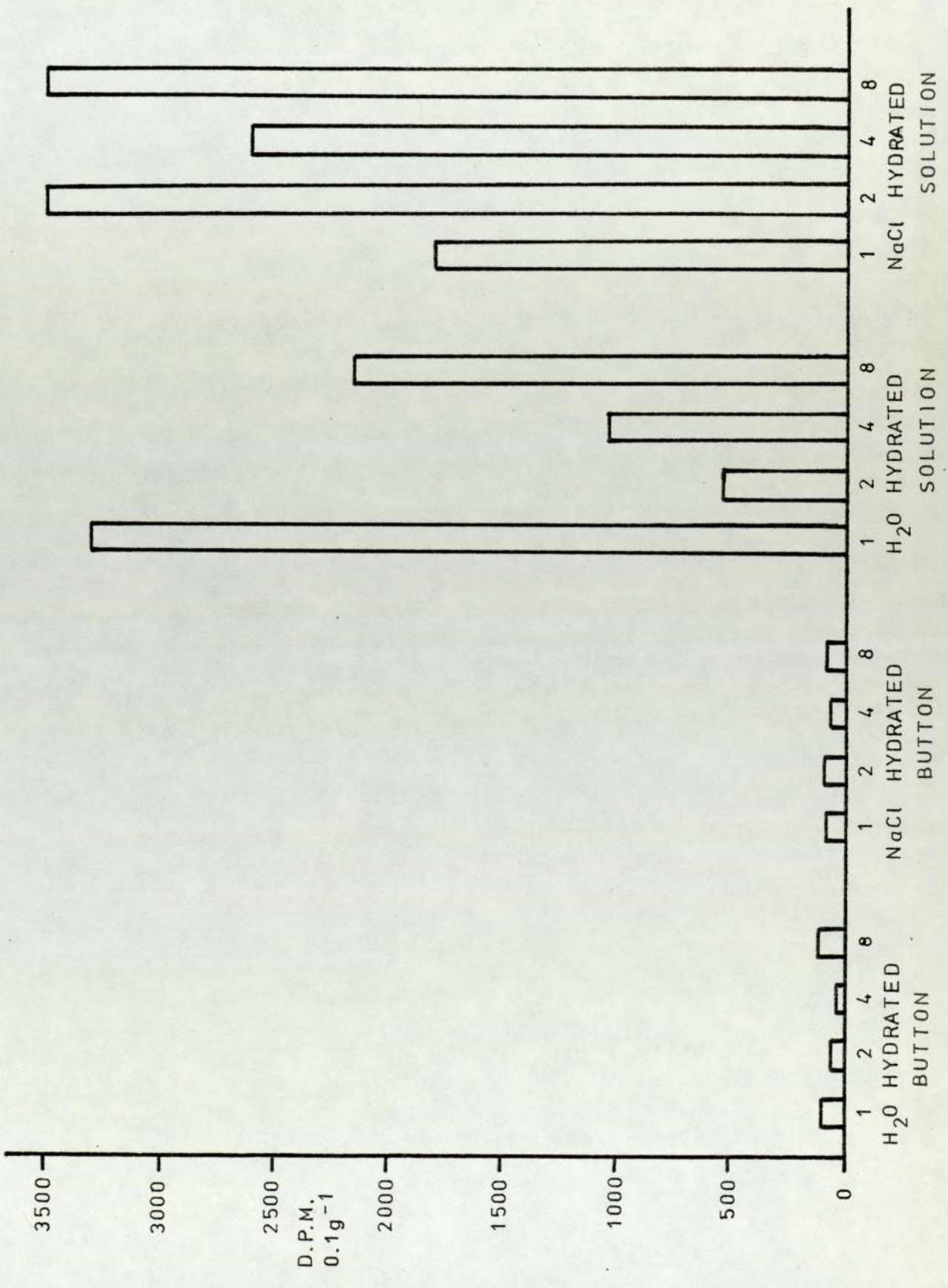
The low sedimentation rate, quenching and high efficiency of counting in this system suggest that this method may be suitable for use in a millipore filter system.

Table 4

Data for the determination of lactic acid absorbtion and release by Sauflon 85.

TREATMENT	(C-d) DPM per 0.1g polymer	(d) DPM in 0.1g soaking solution	(C-d)+d=C Total Transferred Radioactivity	$\frac{C}{2.775 \times 10^{-6}}$ =(f) mg. lactic acid	$\frac{(f) \times 100}{\text{vol of polymer } H_2O}$ mg dl-1	Estimated free lactic acid in solution DPM (g)	Bound Lactic acid DPM (C-d)-(g)
H_2O 1hr.	105.23	3298.80	3404.03	1.23×10^{-3}	1.55	52.36	52.87
H_2O 2hrs.	47.75	536.80	584.55	2.11×10^{-4}	0.27	8.52	39.23
H_2O 4hrs.	20.15	1043.74	1063.88	3.83×10^{-4}	0.48	16.57	3.58
H_2O 8hrs.	104.39	2167.57	2271.96	8.19×10^{-4}	1.03	34.40	69.99
NaCl 1hrs.	44.79	1796.96	1841.78	6.64×10^{-4}	0.84	28.17	16.62
NaCl 2hrs.	71.85	3505.05	3576.90	1.29×10^{-3}	1.63	54.95	16.90
NaCl 4hrs.	59.05	2611.09	2670.14	9.62×10^{-4}	1.21	40.94	18.12
NaCl 8hrs.	73.20	3490.67	3563.87	1.28×10^{-3}	1.62	54.73	18.47

Figure 5 The effect of soak time and hydrating solution on lactic acid absorbtion and release by Sauflon 85.



During rinsing care must be taken to prevent any contamination. The concentration of radioactivity in the original soak solution is much greater than the concentration in the button after elution, suggesting that the slightest contamination will render the information invalid.

Data shown in Table 3 show that lactate can enter the polymer mesh of Sauflon 85. This is as expected since the pore size of Sauflon 85 = 20 Å and the radius of a lactate molecule = 5 Å (TIGHE, 1977).

The data shown in Table 3 also show that absorbed lactate can be eluted. After 72 hours elution, the quantity of lactate remaining in the button is small in comparison to the total activity. Calculation of the expected lactate remaining in the button after elution shows that there is a very small bound portion. The bound portion is so small, that it lies within the limits of experimental error, therefore any conclusions regarding molecular exchange and chemical binding of lactic acid to the polymer backbone must be purely speculative.

A comparison of the residual lactate concentration in the buttons of water and sodium chloride solution hydrated polymer, shows no difference. The elution procedure must be similar for both types of polymer. Observation of the lactate obtained from the free aqueous compartment of the button shows that the sodium chloride solution hydrated button is capable of absorbing lactate faster than the water hydrated button. A comparison of the sequential uptake of lactate into the buttons shows that the concentration of lactate within the button is increasing with time, and that the lactate concentrations in the sodium chloride solution hydrated buttons are consistantly

higher than the water hydrated buttons. Values for the water hydrated button at one hour and the sodium chloride solution hydrated button at two hours may be in error due to contamination.

The levels of lactate in the buttons are increasing with time, it can be assumed that if left longer the buttons could absorb more lactate. The values given for actual lactate concentration in the aqueous compartment of the button, in terms of $mgdl^{-1}$, presented in Table 4, show that only 10% of the lactate concentration in the soaking solution is attained. This reinforces the statement that absorbtion is still taking place at eight hours and that the solution and aqueous compartment are not in equilibrium.

The time taken for complete equilibrium between the soaking solution and aqueous compartment of the button can only be measured by using longer soak times. GUMPELMEYER and SCHWACH (1972) have described the movement of water, glucose and amino-acids through a number of hydrophilic soft lens materials. Whereby a hydrated polymer membrane separates two components, one containing a radioactively labelled solute and the other sodium chloride solution, the time taken for the appearance of the radioactive solute on the opposite side of the membrane is determined. These authors have shown that water, glucose and amino-acids move freely through the materials tested. The rates of movement for each solute differ for each material, however a 5% increase over the control is found over a period of 40 to 60 hours for glucose and amino-acids.

Comparing the results of this study with those of GUMPELMEYER and SCHWACH (1972) it appears that the rate of movement of lactate into Sauflon 85 is of the same order as that found for glucose and amino-acids. The fact that those buttons hydrated in sodium chloride solution absorb lactate quicker than water hydrated buttons disproves the hypothesis that movement of lactate into the button may be assisted by a diffusion pump. Other methods of solute movement within the polymer must be occurring. REFOJO (1965) has shown that viscous flow can occur through hydrophilic polymers but that as water content increases diffusion plays an increasingly important role. The movements of solvents and solutes through hydrophilic polymers is extremely complex and beyond the scope of this study (YASUDA and LAMAZE, 1971; ROGERS and STERNBERG, 1971).

5.6 CONCLUSION

Absorbtion of lactic acid into Sauflon 85 does occur. The rate of absorbtion is higher in buttons hydrated in sodium chloride solution, than in water hydrated buttons.

Over an eight hour period the concentration of lactic acid in the aqueous component of the polymer has reached approximately 10% of that present in the soaking solution.

Lactate absorbed by Sauflon 85 can easily be eluted by soaking the button in water for 72 hours. After elution a concentration of lactate remains in the button, equivalent to that concentration expected from an equilibrium between button and eluting solution. Also present is a

portion of lactate that cannot be explained in terms of equilibrium values and is considered to be a bound portion, however the quantity is so small that it lies within the range of experimental error of the system.

Since Sauflon 85 is seen to absorb and release lactate, its further use as a "sponge" in an *in vivo* system is suggested.

The time taken for equilibrium remains to be measured, until the experiment can be repeated using longer soak times, the results from any system utilising the uptake and release of lactate from a hydrophilic polymer "sponge" will yield only qualitative information.

EXPERIMENT 2

Lactic acid concentration in the tears of normal and anoxic rabbit eyes.

5.7 INTRODUCTION

Experiment 2 section 4 shows that tear fluid lactic acid concentration increases when the eye is anoxic. Due to difficulties in sample collection large variations in the data occur and only qualitative information can be derived.

RUBEN and CARRUTHERS (1972) used soft contact lenses as "sponges" to determine the lactic acid concentration of the tears. Unfortunately due to a lack of information regarding the permeability characteristics of soft lens polymers to lactic acid, only qualitative information can be derived from these authors results. Experiment 1 section 5 has determined some of the permeability characteristics of one hydrophilic soft lens polymer, Sauflon 85.

The aim of this experiment is to use Sauflon 85 soft contact lenses, in the system of RUBEN and CARRUTHERS (1972), to measure the concentration of lactic acid in the tear fluid of normal and anoxic rabbit eyes. The ultimate aim is to produce a system to monitor the concentration of lactic acid in tear fluid under clinical conditions.

5.8 APPARATUS AND METHODOLOGY

Sauflon 85 soft contact lenses generously provided by Contact Lens Manufacturing Ltd. are used. All lenses are quality rejects and each

has the dimensions:-

Diameter = 12.0 mm.

Power = +0.75

Radius of curvature = 7.8.

Each lens is carefully dabbed dry using Kleenex Mediwipes and quickly weighed. The lenses are placed back in their storage bottles and sterilised before use. All lenses are soaked in 0.9% sodium chloride solution.

Male New Zealand white rabbits between 2 and 3 kg. body weight are used.

Control rabbits have a lens placed in each eye for eight hours, no anaesthesia is necessary.

The experimental animals are anaesthetised as described in section 4.8 and a lens placed in each eye. A goggle is placed over the experimental eye and warm humidified nitrogen gas blown through the goggle for eight hours.

After eight hours each lens is removed and immediately rinsed in 5ml. of ice cold 0.6 M Perchloric acid, to remove any tears present on the lens surface. The lenses are then placed in polycarbonate tubes containing 0.2 ml of 0.6 M Perchloric acid and left to soak for six hours at $0^{\circ}C$. Each tube is then placed in a water bath at $34^{\circ}C$ for a further four hours, before returning to the refrigerator for a further 48 hours.

After soaking each tube is shaken vigorously using a vortex mixer. The soaking solution is then placed in a haematocrit tube, sealed and centrifuged for six minutes as described in section 4.8. The tubes are then stored at $0^{\circ}C$ until the assays are performed. Samples are always assayed within four days of spinning. Each sample is assayed according to the method set out in section 4.8.

5.9 RESULTS AND INTERPRETATION OF DATA

Lactic acid concentrations in the samples are calculated as for a tissue sample by the method described in section 4.9. Raw data for lactic acid concentration in the 0.2 ml. of 0.6 M Perchloric acid soak solution is given below.

R.Eye. Control = 1.48, 1.72. Mean = 1.60 $mgdl^{-1}$

L.Eye. Control = 1.31, 1.45. Mean = 1.53 $mgdl^{-1}$.

L.Eye. Experimental control = 1.48, 1.72. Mean = 1.60 $mgdl^{-1}$

R.Eye. 8 hours anoxia = 4.93, 5.17. Mean = 5.05 $mgdl^{-1}$.

The actual mass of lactic acid remaining in the lens has been shown to be insignificant, section 5 Experiment 1, therefore lactic acid in the 0.2 ml of soak solution is representative of the fraction absorbed by the lens from the tears.

The concentration of lactic acid is given in $mgdl^{-1}$, therefore 0.2 ml. of solution will contain.a $mgdl^{-1}$. $\frac{0.2}{100}$ mg of lactic acid = b.

The lenses all have different weights therefore the mass of lactic acid in mg. per g. of lens is calculated as follows:-

$$\text{Lactic acid } g^{-1} \text{ of lens} = b \times \frac{1}{\text{weight of lens}} \text{ mg. } g^{-1}.$$

Converting all the previous values and taking a mean gives the following values for mass of lactic acid absorbed by the lens.

R.Eye. Control = 0.074 mg g^{-1} of hydrated polymer

L.Eye. Control = 0.076 mg g^{-1} " " " .

L.Eye. Experimental control = 0.068 mg g^{-1} of hydrated polymer

R.Eye. 8 hours anoxia = 0.231 " " " " .

In terms of those units given by RUBEN and CARRUTHERS (1972), μMg^{-1} lens:-

R.Eye. Control = 0.82 μMg^{-1}

L.Eye. Control = 0.85 " .

L. Eye. Experimental control = 0.71 "

R. Eye. 8 hours anoxia = 2.56 " .

The concentration of lactic acid in the aqueous component of the lens = Weight of lactic acid g^{-1}. $\frac{78.2237}{100}$ mg ml^{-1} of lens solution, (this assumes that 1 g. of 0.9% sodium chloride solution is equivalent to 1 ml of 0.9% sodium chloride solution).

The concentration of lactic acid in the free water of hydration in the lens = Concentration in mg g^{-1}. $\frac{100}{0.07822}$ $mgdl^{-1}$.

Converting the earlier values, the concentration of lactic acid in the free water of hydration within the polymer for the various treatment are given below:-

R.Eye. Control = 9.47 $mgdl^{-1}$

L.Eye. Control = 9.77 " .

L.Eye. Experimental control = 8.73 $mgdl^{-1}$

R.Eye. 8 hours anoxia = 29.48 " .

5.10 DISCUSSION

The results from this experiment confirm those obtained in section 4, Experiment 2, that lactic acid efflux from the corneal epithelium increases when the cornea is anoxic. In this experiment eight hours anoxia results in a three-fold increase in lactic acid concentration within a soft contact lens placed on the cornea.

A comparison of the data in this study with that of RUBEN and CARRUTHERS (1972) shows that the concentrations of lactic acid in the lens in units μMg^{-1} of lens are very similar. RUBEN and CARRUTHERS (1972) give a mean concentration of 1.2 μMg^{-1} in a lens worn continually on human eyes for one to six months. This compares well with the mean control value in this study of 0.79 μMg^{-1}. It seems peculiar that the concentrations present in lenses worn on rabbit eyes for only eight hours should be similar to those from lenses worn on human eyes continuously for one to six months. It is possible however that the concentration of lactic acid in rabbit tears is greater than that of humans.

After eight hours anoxia the concentration of lactic acid in the lens taken from the rabbit cornea is 2.56 μMg^{-1}. This compares favourably with the maximum lactic acid concentration of 2.36 μMg^{-1} measured in lenses worn by humans (RUBEN and CARRUTHERS, 1972).

Concentrations of lactic acid in the aqueous compartment of the lens also approximate to levels found in the tears measured by the direct sampling method in section 4, Experiment 2. The high concentration of 29.48 $mgdl^{-1}$ obtained in the aqueous of a contact lens worn on an anoxic cornea shows that the upward trend seen in section 4.10 for tear fluid lactic acid concentration is not an artifact. Experiment 1, section 5 shows that only 10% of the soaking solution lactic acid enters the lens in eight hours. If this is the case then the concentrations of lactic acid measured in the tears in this study are unusually high. A possible explanation is that the animals blink occasionally which can compress the lens with a resulting sponge action on the tears. Also the lenses used in this study are extremely thin, with a small mass to surface area ratio. It is possible that movement of lactic acid into the lenses is easier than into the buttons of polymer. This can be checked by repeating Experiment 1 with lenses of Sauflon 85.

However there is a conclusive increase in tear lactic acid when the cornea is subjected to oxygen deprivation. The work of RUBEN and CARRUTHERS,(1972) has shown elevated tear lactic acid levels after extended contact lens wear, therefore contact lens wear can be further linked to oxygen deprivation.

The lactic acid leaving the epithelial surface of the anoxic eye can be measured quantitatively. If the increase in lens lactic acid concentration is taken as representative of the lactic acid released from the corneal epithelium, then in eight hours 5.05 - 1.60 $mgdl^{-1}$ leave the cornea. This is equivalent to 6.88 µg produced in eight hours, 6.88 µg is equivalent to 0.076 µM of lactic acid per hour per cornea.

REIM et al. (1967 (b)) have shown that the normal rabbit cornea produces 0.9 µM of lactic acid $cm.^{-2}$ $hour^{-1}$, and DE ROETTH (1951) has reported a figure of 1.2 µM $cm.^{-2}$ hr^{-1}. In comparison, the quantity of lactic acid produced by the cornea over an eight hour period of anoxia in this study, 0.0763 µM can be considered to be small. It is suggested that the corneal epithelium is only slightly permeable to lactic acid and that most of the lactic acid produced in the corneal epithelium during anoxia passes into the Aqueous Humour.

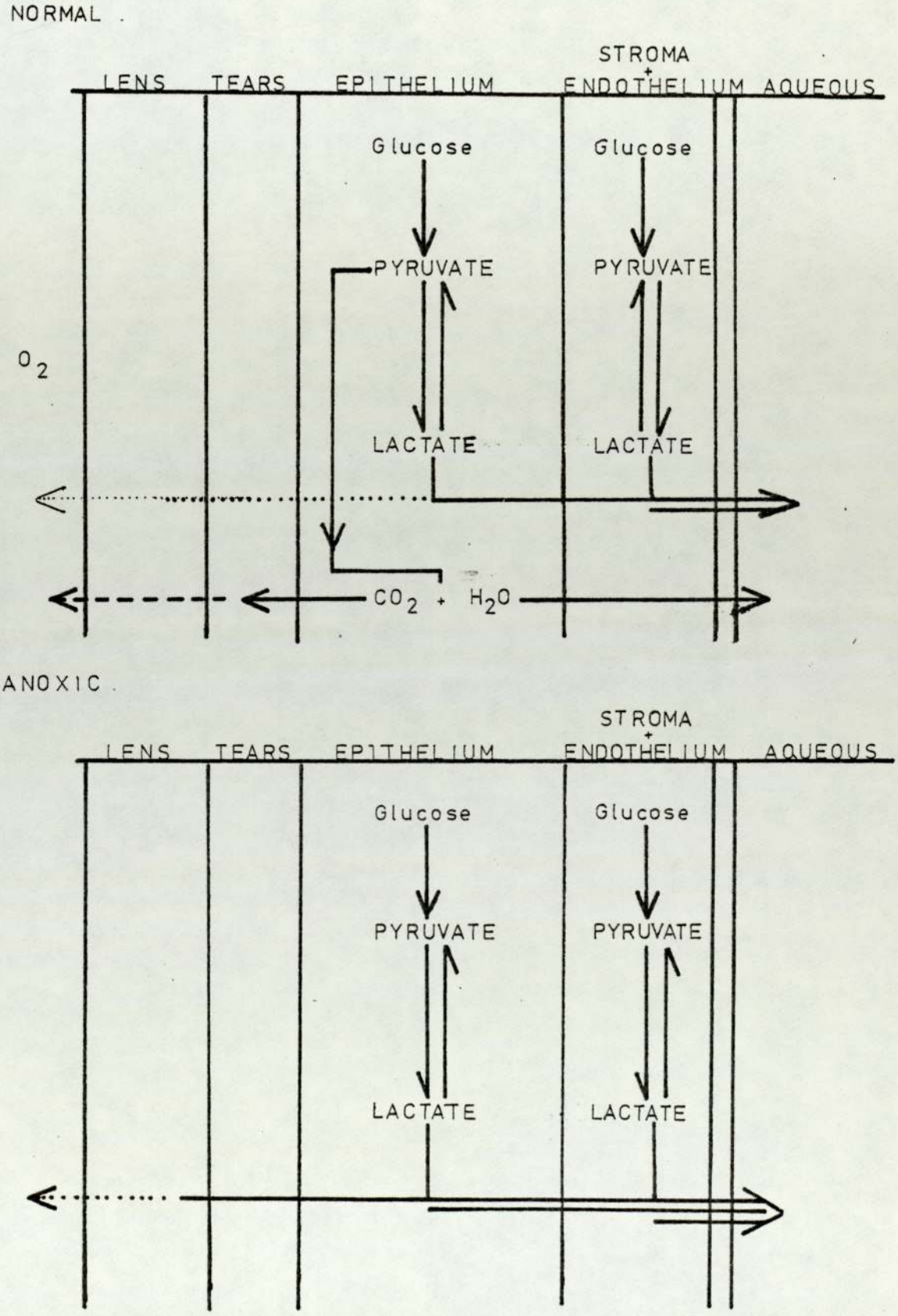
Thus one of the principal aims of this experiment, which is to develop a method of measuring the concentration of lactic acid in the tears that poses no danger to the patient and which is simple and relatively inexpensive, has been achieved.

Figure 6 is a schema of the production and movement of lactic acid in the normal and anoxic cornea with a soft contact lens in position.

5.11 CONCLUSION

The mean concentration of lactic acid in soft contact lenses worn on

Figure 6 A model of lactic acid production and flux in a normal and anoxic rabbit cornea with a soft contact lens in situ.



rabbit corneae for eight hours = 9.32 $mgdl^{-1}$. After eight hours anoxia this concentration increases to 29.48 $mgdl^{-1}$.

These results confirm that:-

(a) Lactic acid can move through the corneal epithelium and that the quantity moving is proportional to the concentration of lactic acid in the cornea.

(b) Soft lenses provide a reliable technique for tear collection.

(c) Because of the minimum requirement on equipment, simplicity of procedure and lack of stress to the patient, soft lenses can be used to collect and monitor tear lactic acid concentration.

The dynamics of lactic acid movement into the lens when on the eye compared to the static system described in Experiment 1, section 5, show that with the information currently available, measured tear lactic acid concentrations must only be used qualitatively.

However if a study of tear fluid lactic acid concentration is undertaken for a large group, using the soft lens collecting technique, then trends may occur that can be applied to the individual patient, allowing an approximation of the state of metabolism of the cornea in that patient.

SECTION 6

GENERAL DISCUSSION

6.1 COMMENT ON RESEARCH STRATEGY

The present study has investigated *in vivo* methods of observing corneal metabolism and function, in the normal eye and in the eye under conditions representative of contact lens wear.

In all the experiments attention has been paid to produce *in vivo* techniques that are suitable for use in the clinical environment. This is considered to be an original contribution to the field of corneal metabolism and function related to contact lens wear.

Reference to the previous literature reveals a dearth of information on questions concerned with the practical application of the techniques used to study corneal metabolism and function. Consequently information regarding the functional application of *in vivo* techniques has been purely speculative.

The results of the present study recommend certain techniques for clinical use whereas others are clearly unsuitable.

6.2 REVIEW OF EXPERIMENTAL RESULTS AND SUGGESTIONS FOR FURTHER WORK

The results of section 2, show that *in vivo* measurement of corneal oxygen consumption rate is possible. The experimental technique is simple, uses relatively inexpensive equipment, has a low risk factor and is therefore suitable for routine use in a clinical environment.

The apparatus is capable of producing accurate results. However as with most in vivo applications not all variables can be controlled and inaccuracies do occur. The main source of error in this system is uncontrolled movement of the patients eye, which produces fluctuations in the trace and introduces inaccuracies in trace analysis. The system is however sufficiently accurate to show individual variations and physiological trends.

Although the risk of damage to the patient is low, no patient having suffered any damage in this study, further modifications can be made to improve patient comfort. Cutting a concave curve into the sensor tip to match the corneal curvature will allow the sensor to rest more comfortably against the cornea, and also reduce the likelihood of oxygen penetrating the system by lateral movement. Using sleeves individually fitted to the eye will also increase patient comfort and reduce lateral movement of oxygen.

Other authors have shown in the laboratory, that corneal anoxia and contact lens wear affect normal oxygen consumption rate and that these changes give an indication of the degree of interference by the contact lenses on normal corneal metabolism. In this study a system has been described that is capable of use in the clinical environment. It should therefore be possible to use this system to study patient reaction and tolerance to contact lenses in the clinical environment. However before such a study can be undertaken, more information regarding the natural variations in oxygen consumption rate are required. More readings must be taken to determine diurnal variations

in both males and females, and to determine menstrual variations, since a large percentage of contact lens wearers are female.

Section 3 has shown that it is possible to measure corneal carbon dioxide efflux rate in vivo. The equipment used is relatively inexpensive and the procedure simple. However the system has a number of limitations, those of size and speed of electrode response, that make the system unsuitable for clinical use. The results also contain inherent inaccuracies, due to difficulties in calibration, trace collection and calculation, that emphasise its strictly limited use.

Even if it were possible to produce a system of sufficient accuracy and comfort, it is unlikely that it could find use in determining contact lens effects. The predominant effect of contact lens wear is reduction of oxygen tension at the epithelial surface, inducing anoxia within the cornea. As a result of anoxia, carbon dioxide production by the corneal tissues will fall, making what in the normal eye is a small flux even smaller and therefore more difficult to detect.

Section 4, Experiment 1 has shown that lactic acid concentration of the tears cannot be measured using thin-layer chromatography. The technique is insufficiently sensitive to detect the low concentrations of lactic acid present in the tears.

The use of enzymatic assay methods, in Experiment 2, section 4, confirms the presence of lactic acid in the tears. Measurement of lactic acid concentration in the component tissues of the cornea under anoxia,

in an attempt to emulate contact lens effect, shows that lactic acid concentrations increase throughout the cornea with the exception of the cornea plus endothelium. Under anoxic conditions, epithelial lactic acid concentration is sufficiently high to induce a corresponding outward flux of lactic acid into the tears. This suggests that monitoring of tear fluid lactic acid will provide a technique for determining corneal respiratory well-being during periods of contact lens wear. The simplicity, accuracy and low cost recommends the use of this technique by the clinician.

However collecting tears by capillary only provides information on the state of metabolism at the time of collection and not over the whole period of wear. The difficulties associated with tear collection and resulting variability in measured tear lactic acid concentration, reduce the usefulness of information derived from tear lactic acid data.

The results of section 5 show that lactic acid can enter and leave Sauflon 85 polymer. However comparison of the freedom of movement measured in buttons, Experiment 1, with that in lenses, Experiment 2, indicates a difference in mobility between button and lens. Before any quantitative information on tear fluid lactic acid concentration, using Sauflon 85 as the collecting medium, can be obtained, the permeability of Sauflon 85 lenses to radioactive lactic acid must be determined. Although the information at the moment is not quantitative, the simplicity of procedure, low cost, safety and the fact that the sample is representative of the whole wearing period, recommend the use of Sauflon 85 lenses as a collecting medium for measurement of tear lactic acid concentration.

In this study lenses removed from the eye are fixed in perchloric acid, this necessitates the use of a new lens for each experiment, prohibiting routine use. If however the rate of conversion or degradation of lactic acid in the lens is measured without the use of a fixative, and found to be acceptably low, then it will be possible to use and re-use the lenses for collecting tear lactic acid.

If a study of tear lactic acid concentration is undertaken for a large number of patients, in the normal and contact lens wear situations, then it may be possible to detect changes that can be used to determine the corneal respiratory and metabolic status.

6.3 COMMENT ON THE APPLICATION OF IN VIVO MONITORING TECHNIQUES TO THE CLINICAL ENVIRONMENT

At the present time monitoring of the cornea during periods of contact lens wear utilises mainly optical techniques to determine changes in corneal thickness caused by oedema and increased hydration.

The information obtained from such studies is limited, any changes can only be attributed to a general malaise. The results of this study show that it is possible to monitor certain aspects of corneal metabolism by observing substrate supply and the movements of metabolic end-products. By using these techniques coupled with those already in use, it will be possible, to monitor the effects of contact lenses on the cornea with more accuracy, and even to pinpoint the mechanism by which the contact lens is interfering with normal corneal function.

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APPENDIX 1

UNIVERSITY OF ASTON IN BIRMINGHAM

DEPARTMENT OF OPHTHALMIC OPTICS

SOFT LENS RESEARCH

DETERMINATION OF CORNEAL OXYGEN CONSUMPTION

The aim of this experiment is to measure the rate of oxygen consumption by the corneal epithelium, with a view to producing a clinical procedure for assessing corneal trauma during contact lens wear.

You will be asked to wear a specially designed soft contact lens containing a micro-oxygen probe.

Should you wish to have the lens removed at any time, please tell the practitioner and this will be done immediately.

Your eyes will be examined before and after the experiment for any side effects. In addition you may be asked to return for a short confirmatory examination within one or two days of wearing the lens.

Dr J R LARKE

NOTES FOR GUIDANCE OF EXPERIMENTAL VOLUNTEER SUBJECTS

In agreeing to take part in an experimental procedure, involving contact lenses and your eyes, you are necessarily subjecting yourself to a greater degree of risk than would be the case were you to be fitted with a conventional form of contact lens. Various measures have been taken to minimise this risk, principally by means of animal studies prior to contact lens wear, and also in designing the experiment in which you take part. However, it is a necessary feature of any experimental procedure that the final outcome is to an extent unknown.

During the course of an experiment there are various safeguards which may be adopted. Firstly, if at any point in the procedure you feel in any distress, you may ask for the experiment to be stopped, and all of the experimenters are under written instructions to observe your request. Secondly, at the completion of an experiment your eyes will be examined by a registered Ophthalmic Optician for signs of an adverse response, and you may be given some form of treatment. After you leave the laboratory should you feel that there is any reasonable doubt about the health of your eyes, please do not hesitate to ring. If it is a week-end, or at night, you may ring my home telephone number which is given below, if you are unable to contact me please go to the Casualty Department of the Birmingham and Midland Eye Hospital, in Church Street.

Dr J R Larke

Home telephone number: 021 449 0202

UNIVERSITY OF ASTON IN BIRMINGHAM

DEPARTMENT OF OPHTHALMIC OPTICS

SOFT LENS RESEARCH

DECLARATION to be signed by experimental patients on initial registration.

I have read the notes for guidance and information of patients attending the Soft Lens Research Clinic, and have received and read a written description of the experiment in which I am to take part.

I hereby agree to act as a volunteer experimental subject.

Signed .....

Date .....

NAME
(Block Capitals) .....

ADDRESS .....

.....

.....

.....

J.R.L. 14

THE UNIVERSITY OF ASTON IN BIRMINGHAM

SOFT LENS RESEARCH

CLINICAL EVALUATION OF CONTINUOUS WEAR

SOFT CONTACT LENSES

CONFIDENTIAL (To be completed by patient)

Mr. Miss Mrs. Official Patient No: _____

Surname _____ Other names _____

Home Address _____ Term Address _____

_____ _____

_____ _____

Tel: _____ Tel: _____

Occupation: _____

Name of regular Optician (if any) Family Doctor

_____ _____

Address _____ Address _____

_____ _____

_____ _____

Visual "correction" Wearing at present: Spectacles Contact Lenses Both None

_____

If spectacles worn:- Age when spectacles first prescribed _____

Full time Part time All distances Long distance Close Work

_____

If contact lenses worn:- Every day Intermittantly Infrequently Discontinued

_____

When was your last eye examination?

_____

Have you any complaints on the performance of your present lenses?

_____

Family History: Are there any instances of eye diseases* in a

	e.g. *Glaucoma
Grandparent	Retinal Detachment
Parent	Cataracts
Brother/sister	Blindness or very poor sight of unknown origin.
None known	

Personal History

General Health:	Any past:	Any eye treatment other than glasses
Good	Eye disease	Yes
Indifferent	Eye injury	No
Poor	Neither	

Do you suffer from any of the following conditions:-

Frequent colds	Red eyes
Catarrh	Red eyelids
Sinus trouble	Scaly eyelashes
Hay fever	Styes
Asthma	Sore or gritty eyes
Food allergies	Itching eyes
Drug allergies	Watering eye(s)
Boils, abscesses	Sticky eyes
Pimples, Acne	Discharging eyes
Lip cold sores	Intolerance to light
Headaches/Migraine	Double vision
Dandruff	Intermittent "steamy" vision

Are you at present taking any regular pills, tablets or medicines prescribed by your doctor? (please state what, if known) Yes / No

---

Other relevant comments:

SEEN BY ..... DATE .....

EXPERIMENT ..... LENSES .....

EYES PRIOR TO EXPERIMENT

SYMPTOMS

COMMENTS

EYES FOLLOWING EXPERIMENT

SYMPTOMS

COMMENTS

TREATMENT GIVEN

NEXT APPOINTMENT