CLINICAL AND EXPERIMENTAL STUDIES ON NORMAL AND DRY EYES

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A thesis submitted to the University of Glasgow in candidature for the degree of MSc (Medi Sci) in the Faculty of Medicine.

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September 1992

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ACKNOWLEDGEMENT

I am thankful to Dr. G. N. Dutton for his help and support throughout the period of my research and during the writing of the thesis. Dr. Dutton was kind enough to read the whole thesis and corrected my spelling mistakes however the whole thesis was written and typed by myself. I am also grateful to Mrs Dorothy Aitken for her immense help throughout my research but more so with the Electron Microscopy Study. I am also grateful to Dr Anita Philips who guided me through the basic techniques of cell culture used in this research I am indebted to Dr. P-J Lamey for providing the patients with Sjogren Syndrome who in turn were very helpful and supportive during the Vitamin A clinical trials. I am thankful to Dr. Alistair Adams for providing practical information about the Impression Cytology technique and the Applicator he invented in his research. I am grateful to Dr Donald Allen for the statistical analysis in chapters five and six.

I would like to thank Dr. Jeffrey Jay for reading Chapters three and four about the conjunvtiva and Dry Eye and to Professor W. R. Lee for his full support throughout my laboratory work. Also my thanks to Mrs. S. Cameron for her help during the preparation and staining of the "Millipore filter papers" in the Impression Cytology technique.

I would like to thank Professor W. S. Foulds for giving me the opportunity to be part of the Tennent Institute during that short period of time and to enable me to do a wide range of research with a friendly and helpful staff in a scientific environment which will always gives me pleasant memories. Last but not least my thanks go to my family for their full support throughout the period of research.

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Abbreviation

Apo-R.B.P.	Apo-Retinol Binding Protein
B.U.T.	Break-Up Time
C.R.A.B.P.	Cellular Retinoic Acid Binding Protein
C.R.B.P.	Cellular Retinol Binding Protein
EGF	Epidermal Growth Factor
holo-R.B.P.	holo-Retinol Binding Protein
IgA	Immunoglobulin A
IgG	Immunoglobulin G
I.U.	International Unit
K.C.S.	Kerato-conjunctivitis sicca
P.A.S.	Periodic Acid Schiff's reagent
pre-R.B.P.	pre-Retinol Binding Protein
R.B.P.	Retinol Binding Protein
S.E.M.	Scanning Electron Microscopy
W.H.O.	World Health Organisation
T.E.M.	Transmission Electron Microscopy

Summary

The clinical manifestations of dry eye are a common complaint which accounts for about 10% of patients attending a general ophthalmic clinic. Dry eyes are commonly caused by a deficiency in one or more of the constituents of the tear film, an irregularity of the ocular surface or a defect in the lid margin. The treatment of dry eye has been directed generally to the relief of symptoms as there is no permanent cure in the majority of cases.

The tear film is a complex trilaminar structure. It plays an important function as an extra layer situated between the lid and the ocular surface. The formation of the tear film within a very short period of time, from three different types of glands situated at different parts of the eyelid and the ocular surface, is one of the most fascinating observations in ophthalmology. However the present knowledge of the different aspects that influence the tear film is incomplete.

This thesis includes clinical trials and laboratory research using "Millipore filter paper" which has the advantage of removing one or maybe two layers of the conjunctiva under local anaesthesia. The conjunctival cells were examined under light and electron microscopy.

Vitamin A as Retinol acetate eye ointment was used on patients with Sjögren's syndrome who have also dry eye symptoms. The aim of this trial was to assess the effect of retinol acetate on the conjunctival epithelium.

The thesis is divided into two parts. The first half includes a general reviews of the tear film and vitamin A. The first chapter is a brief historical account of our knowledge of the anatomy and physiology of the lacrimal glands and the tear film from ancient times to the present day. It also gives a brief historical account of the clinical manifestations of dry eyes and the different modalities of treatment. In the second chapter, a brief anatomical description of the conjuntiva, the goblet cells, the main and accessory lacrimal glands and the meibomian glands, which all contribute to the formation of the trilaminar structure of the tear film with an analysis of the normal composition of the tear film. There is a section on the clinical manifestations and diagnostic tests of dry eye with an emphasis on Sjögren Syndrome. The last part of this chapter deals with the present day management of dry eyes and the new drugs that hopefuly will give a longer relief of symptoms in the near future.

Vitamin A was used topically for the treatment of dry eyes with Sjögren syndrome patients. For this reason a brief history of Vitamin A was included in chapter

three. In chapter four a brief review of the structures and properties of vitamin A. This includes a very brief description of the types of vitamin A and its effects on the body. Vitamin A is an essential nutritional substance and therefore its deficiency or excess is harmful. These effects include night blindness and degenerative changes on the conjunctiva and the cornea.

Impression cytology is a new technique which assess the conjunctival epithelial cells under light microscopy. It is a quick non-invasive test with wide applications. "Millipore filter paper" is used to remove one or two layers of the conjunctiva at one time. Chapter five is a description of a clinical and a laboratory trial to choose the better filter paper surface with the maximum number of cells and the minimum amount of mucus. It showed that the dull surface of the filter paper gives a better yield of cells than the smooth surface.

Chapter six is a double blind clinical trial of the effect of topical retinol acetate on patients with Sjögren syndrome. The results of the trial showed a reduction of foreign body sensation and photophobia in two third of cases but it has no significant improvement on the burning and itchy sensation. There was no significant improvement on the tear meniscus or Break-up time.

Chapter seven includes an attempt to culture a first line cell culture using "Millipore filter paper". Then the cell cultures were tested with therapeutic and toxic doses of vitamin A. The result showed that the cell culture continue to multiply with the therapeutic doses but showed minimal growth with the toxic doses.

Scanning Electron microscopy was used to examine the deeper layers of the conjunctiva by using "Millipore filter paper". This is a new technique which has not been used before and has wide clinical applications. Also the superficial conjunctival epithelial layer was examined from patients with Sjögren syndrome. It showed cuboidal and columnar shaped epithelial cells.

This thesis confirmed the following, firstly that the treatment of topical vitamin A to dry eye patients has no significant effect. Secondly the establishment of a first line cell culture of epithelial cells will give a wide range of tests eg immunological studies and the testing of new drugs on the epithelial cells. Thirdly the Scanning Electron microscopy of the epithelial cells is a unique and hopefully interesting test that may answer the morphological changes of the epithelial cells from longitudinal to cuboidal shape then flat cells at the superficial layer.

The clinical diagnostic tests and treatments for dry eyes have changed little compared to the progress

achieved in the mangement of other eye diseases however in order to achieve any progress, a coherent effort is required between scientists of different disciplines eg Biochemists, Biologists, Pharmacologists and Clinicians to undertake a unified meaningful research for the benefit of the large numbers of dry eye sufferers.

CHAPTER ONE

A Brief History of The Tear Film and the Dry Eye

1-1 Introduction

The role of tears as an expression of happiness and sadness has been recognised throughout human history. This chapter details in brief, the progress of our knowledge in the anatomy and physiology of tears and dry eye disorders.

1-2 The Anatomy of the Lacrimal System

The theory and practice of modern medicine took its roots from ancient Greece. Galen (131-201 AD), the great Greek physician, described two tear glands in the medial canthus where tears were conducted through the puncta into the palpepral fissure and then were drained back through the puncta to the nose and the mouth. He also described the orbital lacrimal gland but since he did not see any ducts he did not recognise its function. In 1662, Steno of Padua, discovered the ducts of the lacrimal gland by using the hair of a wild boar as a probe. Rosenmuller in 1797, described the main lacrimal gland. The tarsal glands were described by Meibomius in 1666 (Del Castillo, 1986).

In the nineteenth century, different glands were discovered in the eye lids. These were the glands of Zeis in 1835, the accessory glands of W. Kraus in 1854, the glands of Wolfring in 1872 and the glands of Moll in 1857 (Del Castillo, 1986).

Zinn in 1780, described the vascular supply of the lacrimal glands while the *lacrimal lymphatics* were first reported by Mascagni in 1787 (Del Castillo, 1986).

Several experimental trials were carried out during the last century to discover *the peripheral lacrimal secretory nerves*. Early experiments were unsuccessful in producing tears by stimulating the trigeminal nerve. Later in 1876 and 1893, Goldzieher concluded that the lacrimal secretory pathway runs first with the facial nerve and then it joins the second branch of the trigeminal nerve via the sphenopalatine ganglion and subsequently reaches the lacrimal gland (Del Castillo, 1986).

1-3 The History of Goblet Cells

Goblet cells were first identified in the small intestine by Henle in 1837. Schulze in 1866, called them the goblet cells due to the similarity in shape with a drinking goblet. Green in 1894 and Parsons in 1904, claimed that goblet cells often have a foot process which reaches the basement membrane (Kessing, 1986).

The goblet cells in the conjunctiva were first described in 1867 by Stieda although Green in 1894, was the first to establish that goblet cells were present normally as independent anatomical structure at the basal layer of the epithelium. However in most subsequent publications it is assumed that the conjunctival goblet cells form from intermediate or superficial cells. The question of the origin of the goblet cells has not been answered to this day (Kessing, 1986).

1-4 Development of the Lacrimal Glands

Speciale-Cirincione in 1909, was the first to discover that the main lacrimal gland emerges in the embryo at 22 -25mm stage, from the basal cells lining the temporal portion of the upper fornix of the conjunctiva. This was confirmed by Kiebel & Mall in 1912 (Duke-Elder, 1963).

The accessory lacrimal glands were discovered by Falchi in 1905, and confirmed by Ask in 1910. They found an ectodermal invagination from the conjunctiva at the 170mm stage of fetal life. However these glands, even at birth, are not fully developed (Duke-Elder, 1963). The development of the sebaceous glands was reported by Contino in 1907, who also reported on the development of the conjunctival mucous glands (Duke -Elder, 1963).

Apt and Cullen (1964) established that the lacrimal glands begin their basal secretion some months before birth and that reflex secretion begins during a period between a few weeks before to a few weeks after birth.

1-5 Physiology of the Lacrimal Glands

Hippocrates' (460-375 BC) concept that the tears secreted directly from the brain had dominated the teaching in medical school in Arabia and Europe for more than twenty centuries. Hunain Ibn Ishaq (809-877 AD) agreed with the theory of Hippocrates with one exception, that the tears were secreted by the temporal and frontal veins when these veins were dilated. Al-Razi (840-926 AD) thought that the lacrimal pathway connected the brain with the lacrimal puncta. In 1609, Casserius of Padua argued that the tear pathway passed through the

lamina cribrosa whilst Wathonin in 1656, claimed that the tears were conducted along the nerve pathway. The concept of the tear secretion by the brain was changed by Steno (1638-1686) when he discovered tiny ducts joining the lacrimal gland to the upper fornix. Martini in 1884, proposed the existence of another secretory site since tears were present after the removal of the main lacrimal gland (Del Castillo, 1986).

1-5-1 Types of Lacrimation

The three fundamental types of secretory tears are based on their mode of action. The types of lacrimation are psychic, basal and reflex. Other reflexes that may induce lacrimation are the visual stimulation reflex, irritation of the facial nerve and the gustatory lacrimal reflex.

Psychic Lacrimation is normally associated with emotional states. Of all the vertebrates, only humans possess this type of lacrimation (Jones, 1973). Voluntary lacrimation is exceptional but it has been observed by Gualdi in 1930 & 1931. Hysterical lacrimation has been documented by Berger in 1895 and tends to appear in violent paroxysms (Del Castillo, 1986).

Janin in 1772, suspected the presence of the *basal Secretion* (Del Castillo, 1986). Later, several researchers including Schirmer (1903) and Jones (1966) studied its characters and varieties. They claimed that the basal tear secretion is produced by the accessory lacrimal glands only. This idea has been rejected lately since the parasympathetic nervous system supplies both types of lacrimal glands and in turn they respond as one unit to varying

stimuli (Wright, 1985a).

Ocular *Reflex Secretion* has been recognised for a long time. It is produced by an irritation of the trigeminal nerve. It was recognised by Badal in 1885 and Schirmer in 1903 (Del Castillo, 1986).

1-5-2 The Mechanism of Lacrimal Secretion

Both Czermak in 1860 and Goldzieher in 1893, found, by stimulating the nerve electrically, reached the conclusion that the main secretory pathway runs with the facial nerve first then joins the trigeminal nerve. Demtschenko in 1872, concluded that the sympathetic nerves control the basal lacrimal secretion and the cranial nerves control the reflex and the psychic secretion. Crespijaumi in 1942, confirmed these findings and added that the basal tear secretion is controlled by sympathetic regulation of the blood flow while the parasympathetic nerves control the lacrimal glands directly. Ruskel in 1975 and Pita, Salori and Berges in 1982, have demonstrated the existence of sympathetic nerve endings in the lacrimal glands (Del Castillo, 1986).

1-5-3 The Quantity of Lacrimal Secretion

Early efforts to measure the tear secretion were started in the eighteenth century by obtaining tears from an eye with ectropion and in another instance from an inflammatory fistula of the lacrimal gland (Del Castillo, 1986). Schirmer (1903) used strips of absorbing paper to measure the amount of tear secreted. DeRotth in 1941, standarised the test by using Whatman 41 filter paper. Accurate measurements of the basal tear secretion using coloured markers were

applied by Zintz and Schilling in 1964. Later, several workers estimated it to be on average about 1µl /min (Del Castillo, 1986).

1-5-4 The Physico-chemical Character of Tears

Magaard in 1882 described tears as a clear, salty, slightly alkaline fluid which varies in appearance and composition on whether it is collected from the ducts of the lacrimal glands or from the conjunctival sac (Duke- Elder,1968).

The osmotic pressure of tears was estimated by Massart in 1889, to be above that of plasma while Ridley in 1930, maintained that it was below that of plasma. More recently, Schaeffer in 1950 and Betelho in 1964, have suggested that the tear film is approximately isotonic with plasma (Duke-Elder, 1968).

The refractive index of the lacrimal fluid has been estimated by von Röth in 1922, to be 1.3369 and confirmed by Fischer in 1928 (Duke-Elder, 1968).

The pH was determined by Arlt in 1855, and by Ahlstrom in 1895, to be slightly alkaline. Early this century, a number of researchers estimated the pH to be around 7.4 - 7.5 . Charlton in 1921 and Oguchi and Nakashima in 1928, (Duke-Elder, 1968).

The surface tension of the tear film was recorded by Cerrano (1910) at 38°c, using lacrimal fluid from a calf, to be 72.3 dynes/cm (Del Castillo, 1986).

1-6 Tear Composition

Fourcroy and Vauquelin in 1791, were the first to determine the components of tears. Frerich's analysis in 1846, showed that tears

are composed of water 98.7-99.06% , albumin 0.08-0.1% and the rest is mucus, lipids, sodium chloride and phosphate. Glucose was first detected by Wada in 1920 (Del Castillo, 1986). Vitamin A was identified in tears by Ubels and MacRae (1984).

The antibacterial properties of lacrimal secretion were considered by Schirmer in 1904 to be insignificant. Helleberg in 1901, found that this property was abolished by heat (Del Castillo, 1986).

The main types of protein discovered in tears are seven. These are IgA, IgG, prealbumin, serum albumin, ceruloplasmin, transferrin, and lysozyme (Spase et al, 1968). The secretory IgA was noted as a major separate component in tears (Chodirker & Tomasi, 1963). IgE was detected by radioimmunodiffusion in tears. It was found to increase in cases of allergic conditions (Brauninger & Centifano, 1971). The prealbumin component in tears was not identified in serum, spinal fluid or urine (Josephson & Weiner, 1968). Lysozyme was discovered in tears, saliva and egg yolk (Fleming & Allison, 1922). Ridley studied its effects on several bacterial inflammatory diseases of the eye (Ridley, 1930).

1-7 Preocular Tear Film

Terson in 1892, recognised the three components of the tear film (Del Castillo, 1986). Wolff (1946), postulated the stratification of the three layers. Several workers have studied the tear film in relation to the wetting time and specifically in relation to contact lens wear.

1-8 History of the Dry Eye

Diseases of the lacrimal system have been recorded as far back as the second millennium B.C., in Hammurabi's Code of Law during his rule as king of Sumaria in Mesopotamia (Del Castillo, 1986). The conditions causing dry eyes were either due to malnutrition or to infection like trachoma which was described in the Papyrus Ebers. Paul of Aegina (625-696 AD) described the dry eye as "pruriginous affection of the eye". It was cured by baths and a wholesome diet. Applications of an acrid nature were utilized to promote flow of tears and to soften the hardness" (Paulus Aeginata, 1884).

Bitot in 1859, was the first to describe the symptoms of xerophthalmia (Del Castillo, 1986). The link between the reduced secretion of the salivary and lacrimal glands was first reported by Hadden (1888). In Japan at the time of famine, Mori noticed in 1904 that children between 2 - 5 years old, developed xerosis of the conjunctiva and scleromalacia which led to blindness. It was also noted at that time that this condition did not occur in children from fishing communities. Mori (1921) and Yudkin (1922) demonstrated the histological features of the lacrimal glands in rats deprived of vitamin A. Kreiker (1930) observed that the goblet cells were decreased in number, early in the degenerative process.

The association of xerophthalmia and systemic diseases of the connective tissue was suspected by Paul of Aegina in the eighth century. Fischer in 1883, recognised the association between xerophthalmia and arthritis deformans (Del- Castillo, 1986).

In the last century, Leber (1882) reported a case of xerophthalmia which he called " Filamentous Keratitis ". Gougerot

(1926) reported the association of dry mouth, dry eye and dry vagina. Sjögren (1933) described " Kerato - conjunctivitis Sicca " as a syndrome with the association of dry mouth, dry eye and arthritis.

Other well known conditions associated with xerophthalmia have been described. These include dermato-stomatitis by Stevens and Johnson in 1922, familial dysautonomia by Riley and Day and ocular pemphigoid by Lortat Jacob in 1956 (Del Castillo, 1986).

1-9 History of Therapeutic Modalities

A wide range of substances has been used throughout history for the treatment of dry eyes but with no definite cure. Saliva and egg white have each been tried as tear substitutes since ancient times. Berger in 1894 proposed the use of saline. Cantonnet in 1908, introduced the term "artificial tears" (Del Castillo, 1986). More than 40 years ago, methyl cellulose, an inert substance of high viscosity was introduced into artificial solutions (Swan, 1945). More than a decade later, Barrie Jones tried a number of preparations like B.J.6 and hydroxymethyl cellose (Jones and Coop, 1965). Polyvinyl alcohol 1.4% was introduced as a non-viscous dilute polymer solution (Krishna & Brown, 1964). Mucolytic agents have been tried to counteract the highly viscous tears in KCS. In 1928, fibrinolysin was suggested as a useful agent in lysing mucin molecules (Weve, 1928) . 10% N-Acetylcysteine topical preparations have been used successfuly in breaking down viscous tears in dry eye diseases (Absolon and Brown, 1968). Chondroitin sulphate A was introduced by Deschamps in 1981 whilst Holly in 1982 tried lecithin (Del Castillo, 1986). Slow releasing artificial tear inserts have been tried recently.

Gel tears of acrylic acid polymers have also been used (Leibowitz, Chang and Mandell, 1984).

Certain drugs have been used to stimulate tear secretion. Pilocarpine eye drops were used early this century with little effect. Eledoisin, is a drug belonging to a group of tachykinins which has been used mainly in Germany with good effect in the management of KCS (Bietti, 1973). Other drugs have been proposed which include fluid secretion stimulators such as bromhexine (Frost -Larsen, Isager and Manthorpe, 1978; Prause et al, 1984).

Simple surgical procedures such as punctal occlusion by electrocautery was first described in 1935 (Beetham, 1935) with good results. A drastic procedure like transplanting a Stensen duct into the conjunctival sac, to supplement tears with saliva has been tried.

Flynn (1967), a priest and ophthalmologist living in Australia, who suffered from a dry eye condition, designed a reservoir attached to the frame of his glasses with a connecting tube passes from the reservoir to each eye to provide a continous supply of fluid.

CHAPTER TWO

The Conjuctiva, The Tear Film and The Dry Eye - A Brief Review

2-1 The Conjunctiva

The conjunctiva is a thin transparent multi-layered epithelium. It covers the tarsus of both eye lids. It reflects at the fornix onto the anterior surface of the eye globe where it merges with the corneal epithelium. Although the conjunctiva unites the eyelids and the eye globe, their movements are independent from each other. The conjunctiva is formed of two layers, a superficial layer called the epithelium and a basal layer called the substantia propria (Last, 1968).

The conjunctiva contributes to the maintenance of the preocular tear film by the secretions of its specialised glands as well as acting as a partial reservoir of the tear film. Also the conjunctival epithelial cells can transdifferentiate into corneal epithelial cells and cover the corneal surface in case of total loss of corneal epithelium (Aitken et al, 1988).

The conjunctival epithelium is a non-keratinized squamous epithelial tissue. It is divided for the purpose of description into **palpebral, fornix** and **bulbar** parts.

The palpebral conjunctiva is subdivided into *marginal, tarsal* and *orbital* zones. The palpebral conjunctiva is closely adherent to the whole tarsus of the upper lid but only to half the width of the

tarsus of the lower lid. The orbital conjunctiva lies between the upper border of the tarsal plate and the fornix. It lies loosely in horizontal folds on the underlying muscles of Müller. These folds appear first after birth. They almost disappear when the eyes are shut (Last, 1968).

The fornical conjuctiva is adherent to loose fibrous tissue which is derived from fascial expansions of the sheaths of the levator and recti muscles. The contraction of these muscles deepens the fornical conjunctiva. Glands of Krause are present in this part of the conjunctiva. The fornical conjunctiva reaches superiorly to 10mm from the limbus, inferiorly to 8mm from the limbus, laterally 14mm from the limbus and medially 7mm from the limbus. It forms a recess at all directions except at the medial side (Last, 1968).

The bulbar conjunctiva lies loosely on the underlying Tenon's capsule. About 3mm from the cornea, the conjunctiva, Tenon's capsule and sclera become closely united (Last, 1968) .

The epithelium, at the tarsal zone of the upper lid, is formed of two layers, a superficial layer of cylindrical cells with basal nuclei and a deep layer of flat cells with oval nuclei. On the other hand, the epithelium at the tarsal zone of the lower lid is formed of three to four layers. There are cone shaped cells at the superficial layer, wedge shaped cells and polygonal cells on the intermediate layer and cubical cells at the basal layer.

The bulbar conjunctiva has a similar structure to that of the lower tarsal conjunctiva except that the polygonal cells in the middle layer are without prickle cell formation and contain round central nuclei. Pigment granules appeared at the deeper cells of the

bulbar conjunctiva.

The Substantia Propria is formed of two layers, an adenoid layer and a fibrous layer. The adenoid layer is thin. It is formed first in the fornix region at 3- 4 months. It is well developed in the fornix, being here 50-70μ in thickness, which contributes to the formation of folds in the fornix. The adenoid layer ceases at the subtarsal fold at the lid margin. The adenoid layer consists of a fine connective tissue reticulum which contains lymphocytes. Nodules of lymphocytes are found towards the angles but usually fade off at the periphery. The fibrous layer is generally thicker than the adenoid layer but is almost non- existent over the tarsus. The conjunctival vessels and nerves, Müller's unstriped muscle and Krause's glands are present in the fibrous layer (Last, 1968).

The epithelium contains goblet cells, melanocytes and Langerhans cells (Bron, Mengher and Davey, 1985). The inflammatory cells normally present in the epithelium, are the neutrophils (mean of 6000 cells/cubic mm) and lymphocytes (mean 14 000 cells/cubic mm) (Allansmith, Greiner and Baird, 1978). Langerhans cells average 200 - 400 cells / square mm. (Gillette, Chandler and Greiner, 1982). The substantia propria contains neutrophils (mean 100 000 cells / cubic mm), lymphocytes (mean 18 000 cells/ cubic mm), plasma cells (mean 7 000 cells/ cubic mm) and mast cells (mean 7 000 cells/ cubic mm). No eosinophils or basophils are found in the normal conjunctiva (Allansmith, Greiner and Baird, 1978) (Table 1).

Goblet cells whose name implies their shape, are found in all portions of the epithelial conjunctiva. A flat nucleus is situated near the base. Kessing (1986) classified the mucous gland system of

	Epithelial cells/mm3	Substantia Propria -cells/mm3
Neutrophils	4 000 - 6 000 (26 000)	2 000 (10 000)
Lymphocytes	5 000 - 14 000 (46 000)	100 000 (337 000)
Plasma Cells	_____	18000-46000 (100 000)
Mast Cells	_____	5 000- 7 000 (11 000)
Langerhans Cells	200 - 400 cell/mm2	

Table 1. The average number of inflammatory cells in normal conjunctiva. The values in parenthesis are of the upper normal limits.

the conjunctiva into two main types. In the first type the goblet cells open independently onto the surface whilst in the second type the goblet cells open into crypts that open to the surface. In the bulbar area, the width of a mature goblet cell is 15-20μ and its height 10-15μ. Goblet cells are apocrine glands. The density of the goblet cells is highest nasally. They are absent in the 4- 5mm area around the limbus (Kessing, 1966). The goblet cell free area is absent in the foetus and in infants up to one year of age while it is seen in 73% of whole mount conjunctivae in the age group 1-25 years and 100% in the age group 26-75 years of age (Kessing, 1986).

A reduction in the number of goblet cells was found in cases of KCS, Stevens-Johnson syndrome, ocular pemphigoid and acute alkali burns (Ralph,1975). It also occurs as an early stage of xerosis due to vitamin A deficiency which indicates that vitamin A is an important factor in goblet cell formation. The number of goblet cells may increase in inflammatory conditions (Kessing, 1986).

Under electron microscpy, the epithelial cells are divided either according to their morphology and electron intensity as in scanning electron microscopy (SEM) or according to their ultrastructural contents as in transmission electron microscopy (TEM). In SEM, the epithelial cells have been divided into light, dark and medium cells. The light cells which are the predominant type, are smaller in size with longitudinal shape and smooth surface while the dark cells are large with irregular surface (Greiner, Covington and Allansmith, 1977). Rohan and Steuhl (1982) divided the superficial layer of the conjunctival epithelium by TEM into five types. Type I comprise the goblet cells. Type II are characterised by

their contents of small electron dense granules while type III are rich in golgi material. Type IV contain a large amount of endoplasmic reticulum. Type V show an exceptionally large number of mitochondria.

The accessory lacrimal glands of Krause are placed deep in the subconjunctival connective tissue. There are some 42 glands in the upper lid and 6-8 glands in the lower lid. The glands of Wolfring which are larger than the glands of Krause, are situated above the tarsus. There are about 2-5 glands in the upper lid and 2 glands in the lower lid (Last, 1968).

The conjunctiva is innervated by the sensory nerves of the trigeminal nerve and by sympathetic nerves which accompany the blood vessels. The bulbar conjunctiva is supplied by the ciliary nerves. The superior palpebral conjunctiva is supplied by the frontal nerve medially and lacrimal nerve laterally. Sensory innervation of the inferior palpebral conjunctiva is by the lateral palpebral branch of the lacrimal branch of the ophthalmic nerve and the infraorbital branch of the maxillary nerve (Newell, 1978).

The sensory nerve fibres terminate in the conjunctiva in one of two ways; either as free nerve endings which comprise the majority or as compact encapsulated nerve endings which include "Krause end bulbs" in variable number (Oppenheimer, Palmer & Weddell, 1958). The highest sensitivity of the ocular surface is at the cornea whilst the lowest sensitivity is at the bulbar conjunctiva. The degree of sensitivity of the upper and lower lid margins and the caruncle is between that of the cornea and bulbar conjunctiva (Norn, 1973)

2-2 The Tear Film

The tear film is important in maintaining a healthy ocular surface by containing the necessary nutritional materials for the cornea, including oxygen and carbon dioxide exchange. Continued tear flow across the ocular surface washes any materials such as micro-organisms, debris or foreign particles (Smolin, 1987) and reduces the friction between the ocular surface and the eyelids (Kessing, 1986). The tear film also contains bactericidal elements but its main action is that of lubricant (Holly & Lemp, 1977).

The basal tear secrection is defined in this thesis as the physiological non-reflex lacrimation. This lacrimation is formed from the main and accessory lacrimal glands, mucous glands, meibomian glands and the glands of Zeiss and Moll (Del Castillo, 1987).

The average basal tear volume in the conjunctival sac is 5-9 µl with no significant difference between age groups, sexes and fellow eyes. The average estimation of flow rate of tears is 1.2µl /min with a range of 0.5-2.2 µl/min. The tear volume increases proportionally with the tear flow (Mishima et al, 1966). There is a circadian rhythm of tear turnover with an increase of tears in the morning than in the afternoon. Also there is no difference in the tear turnover between the sexes or in the dark or light colored irises (Webber, Jones & Wright, 1984). The average normal wetting time is 30 seconds. The wetting time is shorter in females and in people over 70 years of age (Norn, 1969).

The normal osmolarity of the tear film ranges between 295 and 309. These values increase in patients with KCS to an average of 312-424 (Gilbard, Farris and Santamaria, 1978). The mean pH of the

tears, 7.11, is slightly alkaline. In older women, the alkaline shift is more than in any other age group. The tear pH, after awakening, is slightly acidic but with the loss of CO2 in the open eye, the pH return to normal within an hour (Coles & Jarvis, 1984). Although any variation in the pH will increase lacrimation (Conrad, Reay, Polcyn and Robinson, 1978), tests have shown that diurnal pattern changes of pH amplitudes and periods are distinct to each normal individual (Carney & Hill, 1976).

The precorneal tear film has a uniform thickness over both the conjunctiva and the cornea which measures 6.5μ (Mishima, 1965). The vertical position of the tear film is maintained by the existence of the epithelial microvilli, the hydrophilic material on the epithelial surface and the stabilising factor of the oily layer of the tear film (Brown, 1973).

Bron (1985) claimed that the major preportion of tears is drained through the canaliculi (about 95%) whilst the remaining 5% is lost through evaporation (about 0.085 μ/min) and through absorption by the conjunctiva leading to inward pumping of sodium across the epithelium.

2-2-1 The Tear Film Layers

The unique trilaminar structure of the tear film reflects the complexity of the formation and stability of the tear film. Since Wolff's (1946) description of the three layered tear film, studies have been directed on the anatomical structure, the physiological and biological inter- relationship of the tear film layer, and to the ocular surface, the lids and the atmosphere outside the tear film.

The mucin layer is the inner layer of the tear film. Mucus is secreted by the goblet cells and the subepithelial vesicles of the conjunctival epithelium (Wanko, Lloyd and Mathews, 1964). The mucous layer, 0.2- 0.4 µm thick, is adsorbed onto the superficial epithelial cell layer (Norn, 1969b). Blinking causes mucus to move to the medial canthus where it compacts and is pushed onto the skin surface (Adams, 1979). The mean flow rate of mucus is 1.1 mm/min. This value is independent of age, sex, environmental temperature and moisture. The mucus flow is reduced in facial paralysis, entropion and KCS. The mucus flow is arrested by sleep and anaesthesia (Norn, 1969b).

The two types of glycoproteins in the body are the plasma and mucin glycoproteins. The mucin glycoproteins have a high carbohydrate content (more than 50%) compared to less than 25% in the plasma glycoproteins (Wright & Mackie, 1977). Mucin is formed mainly from high molecular weight glycoproteins which are formed of a protein core with carbohydrate chains attached to their sides (Holly, 1973). Glycoproteins are negatively charged due to the presence of a high amount of sialic acid and a small amount of weakly sulphated mucopolysaccharides (Moore & Tiffany, 1979; Jensen & Falbe-Hansen, 1969).

The corneal epithelium has a lipophilic surface (Lemp et al, 1970). The mucus changes the hydrophobic character of the ocular surface to a hydrophilic type by reducing the surface tension of the aqueous part of the tear film and helps it to adsorb to the ocular surface. Subepithelial vesicles which contain long chain mucin glycoprotein molecules, bind to the overlying mucus of the goblet

cells (Dilly, 1985a). The mucin as a surfactant has the ability to form hydrogen bonds with water. It holds water to the surface lipid layer as well as to the lipoidal corneal epithelium (Havener, 1983).

The Aqueous Layer is the middle layer of the tear film. It is about 6-10 µm thick. The aqueous is produced by the palpebral and orbital parts of the main lacrimal gland and the accessory glands of Krause and Wolfring (Jones, 1973). About 95% of the aqueous layer is produced by the main lacrimal gland while about 5% is formed by the accessory lacrimal glands. Lacrimal secretion is controlled by parasympathetic nerves to the main lacrimal glands and probably to the accessory lacrimal glands. Prolactin and adreno -corticotrophic hormones have been found in the lacrimal gland and may be involved in stimulating tear production and secretion (Frey et al, 1986).

Lysozyme, lactoferrin and secretory IgA are produced by the acini of the lacrimal glands (Bron, 1985). Secretory IgA is also produced by the conjunctival epithelial cells but not the corneal epithelial cells (Liu et al, 1981; Janssen & van Bijsterveld, 1983). There is small amount of sialomucin dissolved in the aqueous tear film which originates in the tear glands and lowers the surface tension (Holly & Lemp, 1977).

The Lipid Layer is the superficial anterior layer of the tear film with a minimum thickness of 0.2µ (Brown & Dervichian, 1969a). It covers the ocular surface between the lid margins. The lipids are secreted from the meibomian glands and probably the glands of Zeis. The positions of the meibomian duct orifices are ideal in spreading their lipid contents over the aqueous layer of the tear film. The lipids are composed of non-polar sterols (cholesterol esters) and wax

esters. There are small amounts of free fatty acids and triglycerides (Tiffany, 1985). There are also phospholipids which act as polar fractions (Andrews, 1970). The meibomian secretion is solid below 35°C and fluid above that temperature. The lipid layer is formed mainly of non - polar oils which are slow to diffuse and a polar component (about 4% of the total secretion) which spreads easily above 35°C (Brown & Dervichian, 1969b).

The lipid layer retards the evaporation of the tear film and lowers the surface tension of the tear film (Bron, 1985) making it thicker and more stable tear film (Brown & Dervichian, 1969a). Closure of meibomian gland orifices in rabbit eyes, increases the tear film osmolarity in the presence of normal lacrimal gland function and caused ocular surface abnormalities similar to KCS (Gilbard, Rossi and Heyda, 1989). It also prevents the contamination of the tear film by the polar lipids of the skin (McDonald & Brubaker, 1971).

2-3 Composition of the Tear Film

The tear film is composed mainly of water, about 98.2 % (Kikkawa, 1969). The concentration of sodium and urea in tears is similar to that in plasma whilst the concentrations of potassium (6- 42 mmol /L) and chloride (106-138 mmol/ L) are higher in tears than in plasma (Thaysen and Thorn, 1954). The calcium level in tears (0.3- 2.0 mmol/ L) is higher than in plasma (Uotila, Soble and Savory, 1972). The copper value is 1.5 mg / L. Most of the copper is free, and part of it is combined with caeruloplasmin (del Castillo, 1986). The glucose level in tears (0.2 mmol / L) is very low compared

to its level in plasma (Giardini & Roberts, 1950).

The average protein content in tears is 8 gm/ L (Allansmith, 1973) with a higher protein content in females than in males (Tapasztó, 1973). The protein content is divided between 60% albumin, 20% globulin and 20% lysozyme (Milder, 1981).

The tear film plays a vital role in combating infection. The main protein components are **IgA, IgG, lactoferrin, caeruloplasmin** and **lysozyme**.

Secretory IgA is a major component of the immunoglobulins in tears yet it is present in small amount in serum (Chodirker & Tomasi, 1963). It is composed of two molecules of IgA, similar to serum IgA molecules, but they are linked together by a cysteine - rich polypeptide J-chain which prevents the IgA from being broken down by tear enzymes (McGill, 1985). The average concentration of IgA in tears ranges between 170 mg / L (Sen et al, 1978) and 400 mg/ L (McGill et al, 1984) which is a higher concentration than that of plasma (McClellan et al, 1973). The IgA level in tears is higher in females than in males. No relationship with age or sex has been established with any of the other immunoglobulins (Sen et al, 1978). IgA levels in tears can vary in any one subject over a period of time. IgA acts to protect mucosal surfaces by preventing bacteria from adhering to the surface and then dispose of these bacteria (McGill, 1985).

The average value for **IgG** is 140 mg / L. It is formed either by transudate from serum or by active transport from blood (McGill, 1985). Therefore any increase in IgG value may be due to an increase in permeability of the blood vessels in case of acute inflammation

particularly when there is tissue damage (McClellan et al, 1973). IgG neutralises viruses and toxins, lyses bacteria, enhances opsonisation and forms immune complexes. IgG and IgA tear levels are less stable than those in serum (Allansmith, 1973).

Lysozymes in tears are formed in the main and accessory lacrimal glands (Gillette, Greiner and Allansmith, 1981). The average concentration of **lysozymes** in tears varies between 1gm /L and 2mg/ L (McGill, 1985) with higher values in males than in females (Tapaszò, 1973). Its level decreases linearly after the age of 40 years by 10 mg / L per year. Its value also decreases in irritable eyes (Spase, Bonavida and Stone, 1968). Lysozymes enhance the action of bacteriolysis by the secretory IgA (Smolin, 1987).

Lactoferrin forms 25% of tear protein. The level of lactoferrin in tears ranges between 1.4 gm/ L (McGill, 1985) and 2.2 gm/ L. This value is not influenced by age or sex (Kijlstra, Jeurissen and Koning, 1983). Lactoferrin is mainly produced (90%) by the acinar cells of the lacrimal gland (Gillette et al, 1980). Its antibacterial action is due to its chelating property which denies the invading organisms their nutrient iron (McGill, 1985).

Tear Caeruloplasmin is a copper -containing protein. Its exact function has yet to be determined. Tear caeruloplasmin value is 160mg /L (McGill, 1985).

In an established sicca condition, the levels of lysozyme and lactoferrin are reduced while the IgG and caeruloplasmin are increased (Mackie and Seal, 1984).

The **histamine** level in tears of normal subjects is 10.3ng/ml with a range of 2.2- 36 ng/ml. This level increases to 38.2 ng/ml

in patients with vernal conjunctivitis (Abelson et al, 1977).

The **lipid** content in normal tears, averages 2.2 gm / L. Its level in tears is higher in females than in males (Tapaszto, 1973).

The main components of tears are listed in table 2.

2-4 Dynamics of the Tear Film Formation

Although the eyelids play an important role in the formation and distribution of the tear film, they have no effect on tear production (Wright, 1985). The rate of blinking depends on the individual and on the environment. The time factor in each blink averages 0.32 sec. (Beard, 1981). The upper eyelid descends with a speed ranging between 17 and 20 cm/sec but it may reach 40 cm/sec while the lower lid moves horizontally and nasally at a speed of 0.2 -0.5cm/sec. (Doane, 1980). The lower eyelid movement precedes that of the upper lid by about 0.02 sec. The closure of the lid fissure starts at the lateral canthus and progresses medially (Beard, 1981). The eye globe retracts by 1-6 mm due to the pressure of the descending upper lid on the eye globe (Doane, 1980). Kessing (1967), using a tomographic x-ray technique with barium sulphate as a contrast, showed that only the lid margin of the upper lid and the lid margin and the tarsal area of the lower lid are in contact with the eye globe.

The tear flow which appears to be intermittent in most normal people, streams along the lid margin of the upper eyelid nasally and temporally and along the lower lid nasally to reach the canaliculi (Maurice, 1973). When the upper lid descends, the shear force of the lid removes the old tear film and redistributes the mucous layer

Inorganic substances		
Water	98.2%	
Oxygen (pO2 at sea level)		
Eyes are open	159	mmHg
Eyes are closed	35-55	mmHg
Carbon dioxide		
pCO2	0.2	mmHg
Sodium	80-170	mmol/L
Pottasium	6-42	mmol/L
Chloride	106-138	mmol/L
Calcium	0.3-2.0	mmol/L
Magnesium	0.3-1.1	mmol/L
Bicarbonate	25	mmol/L
Carbohydrates		
Glucose	0.2	mmol/ L
Lactate	1-5	mmol/ L
Pyruvate	0.05-0.35	mmol/ L
Lipids		
Lipid- Total	2200	mg/ L
Cholesterol	200	mg/ L
Nonproteins		
Urea	0.4	mg/ L
Histamine	0.01	mg/ L
Proteins		
Protein- Total	8	gm/ L
Caeruloplasmin	150	mg/ L
Lactoferrin	1.4- 2.2	gm/ L
lysozyme	1 - 2	gm/ L
IgA	170-400	mg/ L
IgG	140	mg/ L
IgM	<5	mg/100ml
IgD	<1	mg/100ml
IgE	250	ng/ml

Table 2. Composition of the Tear Film

over the ocular surface (Norn, 1969a). As the upper eyelid elevates, a thin aqueous layer is formed by the lid margin. The lipid layer formation follows shortly with an added thin layer of aqueous (Brown & Dervichian, 1969a).

The tear film remains stable for less than a minute when part of the superficial lipid layer migrates to the tear - epithelium interface creating small areas of high interfacial tension leading to the break-up of the tear film (Holly & Lemp, 1977).

2-5 Wettability and Stability of the Tear Film

The wettability of the ocular surface is important first, in forming a uniform adsorptive tear film and second in maintaining its stability. This is achieved by reducing the surface tension of tears to equal the adhesive forces of the mucus layer formed by the goblet and subepithelial cells of the conjuctiva.

The wettability of the ocular surface depends on a negative surface tension between the tear film and the epithelial surface layer. The aqueous fluid on an epithelial layer surface, free of mucus, has a surface tension of 70 dynes/cm (Holly, 1973). Applying mucin to the epithelial surface reduce the surface tension to about 46 dynes/ cm (Miller, 1969).

The lipid layer, which contains mucin, forms a mucin - lipid interface that reduces further the surface tension of the tear film (Holly, 1973). On the other hand at the ocular surface, the mucoproteins, produced by the subepithelial surface, and the mucus layer form an adhesive force that increase the surface tension from 28 dynes/cm to 38 dynes/cm (Dilly, 1985).

With regard to the break down of the tear film, there are several hypotheses. The observations of Holly (1973) indicate that the formation of dry spots is due to contamination of the adsorbed mucin layer at the ocular epithelial surface by a migrating lipid layer which renders the ocular surface hydrophobic.

2-6 Dry eye conditions

The incidence of KCS in systemic diseases is difficult to assess, but the incidence of KCS in rheumatoid arthritis is about 14% (Wright, 1987).

The diagnosis of dry eye is based on a number of vague symptoms which can develop in many local and systemic conditions. In order to consider the causes of dry eye, it is practical to apply the anatomical components involved as the basis for classification of tear film disorders (Table 3). This classification is derived from several sources (Holly & Lemp, 1977; Wright, 1986; Roy, 1984; Duke- Elder, 1965).

The eyelid is vital for the distribution and formation of the tear film. It expels any superfluous fluid (Jones, 1973). Any weakness or irregularity in the congruity of the lids may affect tear film formation. Patients with KCS have a statistically significant increase in the evaporation of tears compared to normal subjects (Rolando, Refojo & Kenyon, 1983).

The changes in the mucus layer may be due to an insult to the ocular surface including the goblet cells, either directly as in a chemical burns or as a general reaction of the mucous membranes of the body. Other causes such as vitamin A deficiency, may lead to

I Lid	Paralysis
	Proptosis
	Levator spasm
	Ectropion
	Infrequent blinking eg progressive supranuclear palsy
II Mucus	Vitamin A deficiency
	Ocular cicatrical pemphigoid
	Erythema multiforme
	Drug induced (systemic and topical)
	Chemical burns
	Trachoma
III Aqueous	Keratoconjunctivitis sicca
	Riley - Day syndrome
	Congenital alacrima
	Idiopathic
	Neurogenic- Central-Pontine lesion
	Basal fracture
	Otitis media.
	Peripheral-Skull fracture
	Associate neoplasia.
	Lesion of fifth cranial nerve
	Parasympathetic blocking scopolamin.
	Ramsey Hunt syndrome.
IV Lipid	Chronic blepharo-conjunctivitis
V Ocular surface	Pterygium
	Dellen
	Symblepharon
	Scars of the ocular surface.
VI Associated systemic diseases	Rheumatoid arthritis
	Sjögren syndrome
	Sarcoidosis
	Rosacea keratitis
	Inflamatory bowel disease
	Immune complex disease
	Systemic lupus erythrematosis
	Polyarteritis nodosa
	Scleroderma

Table 3. Classification of dry eye disorders

metaplasia of the epithelial surface.

Aqueous secretion may be reduced in the absence of the lacrimal glands, in peripheral or central neurogenic causes or as part of a systemic conditions.

The lipid layer changes may be due to infection or blockage of the meibomian glands. Lipids from the surrounding skin destabilise the tear film.

Any irregularities in the ocular surface such as a pterygium or a scar will disturb the uniformity of the tear film and reduce the break -up time of the tear film.

2-7 Sjögren's Syndrome

Sjögren's syndrome is a chronic inflammatory disease characterised by remissions and exacerbations (Williamson et al, 1974). The aetiology is unknown although auto - immunity, vitamin deficiency or disturbance of endocrine function may be involved (Sjögren & Bloch, 1971). The presence of at least two of the following three criteria must be included. These are KCS, xerostomia and one of the following connective tissue diseases which include rheumatoid arthritis, systemic lupus erythematosus, progressive systemic sclerosis, polyarteritis nodosa and polymyositis . Primary Sjögren's syndrome comprises KCS and xerostomia, whilst secondary Sjögren's syndrome includes an additional connective tissue disease. Sjögren's syndrome is predominant in females with a ratio of 9 /1 (Frost-Larsen, et al, 1980). The age peak is between 40-60 years of age but it may occur in patients as young as 10 years of age. There is no

predominance of race in this condition (Baum, 1973).

In Sjögren's syndrome, a definite sicca syndrome has been found in 3.5% of females and in 2.8% of males (Isenberg & Crisp, 1985). The values of lactoferrin in tears, break-up time of the tear film and Schirmer's test are lower in Sjögren's syndrome patients than of those with KCS and no systemic disease (Mackor and van Bijsterveld, 1988). There is early damage to the acinar cells of the main and accessory lacrimal glands (Chomette, Auriol and Liotet, 1986) which leads to the reduction of lactoferrin in tears. The reduction of aqueous in tears leads to an epithelial degeneration with inter- and intracellular oedema of the superficial epithelial layers. Goblet cells population may be reduced but the decrease in mucus is proportionately less than that of aqueous production (Frost-Larsen et al, 1980). The bulbar conjunctiva rarely looks dry and it is uncommon to find infection of the lid margins or conjunctiva or both. Lacrimal glands are rarely enlarged in this condition (Baum, 1973).

Diminished salivary secretion may precipitate dental caries and increases thirst. There is also a burning sensation in the mouth with difficulty in swallowing and speech. Swelling of the salivary glands may occur. Labial biopsy has been used to confirm the diagnosis. A positive biopsy shows acinar atrophy and lymphocytic infiltration .

Systemically, arthritis with bone erosion occur in a high proportion of cases. There is a reduction of secretion in the bronchi, the oesophagus, the vagina and the skin. The gastric secretion may also be reduced (Frost-Larsen et al, 1980).

Immunoglobulin abnormalities in Sjögren's syndrome predominately reflect the marked hyperactivity of β lymphocytes with hypergamma globulinaemia and a variety of circulating autoantibodies (Maddison, 1985). Several investigators have shown an increase of HLA-A1, HLA-B8, DW2 and or DW3. In primary Sjögren's syndrome, the frequencies of HLA-A1, B8, and DW3 are 88%, 94% and 74% respectively where as in Sjögren's syndrome with rheumatoid arthritis, the frequencies of the above antigens are 38%, 24% and 14% respectively (Isenberg & Crisp, 1985). The risk of developing lymphoid neoplasia may be up to 44% (Frost -Larsen et al, 1980). Of all the patients who have Sjögren syndrome, some 10% develop eye complications which include corneal erosion , corneal perforation and symblepharon (Williamson, 1987).

Many conditions are frequently associated with Sjögren's syndrome. They include Waldenström purpura, Raynaud's phenomena, Hashimoto disease, pulmonary fibrosis; chronic hepato- biliary disease and thrombocytopenic purpura (Frost- Larsen et al, 1980).

2-8 Signs of dry eye

The most practical means of diagnosing these conditions is by careful history taking and clinical examination with the slit lamp (Doughman, 1973). The signs of a dry eye vary from one patient to another and from time to time in the same patient. They include grittiness, foreign body sensation, an itchy sensation and a burning sensation. There may be heaviness or tiredness of the lids giving a "pseudoptosis" which occur in 20% of patients with aqueous deficiency (Williamson, 1982). Dry eye sensation is rare. Photosensitivity

may be felt by some patients. There may be slight redness of the eye. Stringy mucus discharge, debris of the epithelial cells and flakes of mucus are seen in the conjunctival sac. There is also dullness of the conjunctiva and the corneal surfaces (Sjögren and Bloch, 1971).

2-9 Diagnosis of dry eye

Several clinical tests have been employed to diagnose this condition yet no one simple reliable test exists to confirm the diagnosis (Wright, 1987). Tests for the assessment of tear quantity include the tear meniscus, the fluorescein staining test, the Schirmer tests and rose bengal test while tests for assessing the quality of the tear film comprise the break-up time and impression cytology (Doughman, 1973).

2-9-1 Tear Meniscus

The tear meniscus height ranges between 0.1- 0.6mm (Lamberts, Foster and Perry, 1979). Any irregularity in the width of the meniscus strip or frank areas of discontinuity strongly suggests the presence of aqueous tear deficiency or possibly a lipid abnormality (Holly & Lemp, 1977).

2-9-2 Fluorescein Staining

Fluorescein stains the de-epithelialised ocular surface. The area mostly affected is the palpebral fissure. Fluorescein staining viewed with a cobalt blue light at the slit lamp may show fine

multiple puncti or dense conglomerate staining areas of the conjunctiva alone or the conjuctiva and the cornea.

2-9-3 Tear Film Break-up time (BUT)

This test examines directly with the slit lamp the functional ability of the mucus layer of the tear film to maintain its integrity and indicate the presence of any lipid abnormalities (Holly and Lemp, 1977).

The test is performed by instilling one drop of 1% fluorescein solution into the conjunctival sac. The patient is asked to blink several times. With the aid of a slit lamp and a cobalt-filtered light and narrow beam, the tear film is scanned for any breakdown in the tear film. The time is measured from the last blink to the first break in the tear film. The test is repeated three times and a mean is taken. The breakdown in the tear film should occur at different part of the cornea. If the breakdown occured in the same site, then a local abnormality of the ocular surface is the cause. The normal range of BUT is between 15 -34 seconds. Local anaesthesia reduces the BUT due to its affect on microvilli (Lemp, 1973b). Fluorescein dye affects the stability of the tear film and BUT (Mengher et al, 1985). Furthermore benzalkonium as a preservative commonly used in different eye drop preparations reduces the BUT through its detergent effect on the oily layer of the tear film leading to increased evaporation of water from the tear film (Wilson, Duncan and Jay, 1975). No correlation was found between BUT, corneal sensation, age and sex (Lemp, 1973b). The validity of this test has been questioned as the results are

not reproducible (Vanley, Leopold and Gregg, 1977). Yet there is an agreement that any result below 10 seconds is considered to be abnormal (Lemp, 1973b; Bron, 1985).

2-9-4 Rose Bengal Test

Rose bengal is an iodine derivative of fluorescein, supplied at 1% concentration (Baum, 1985). It stains dead and degenerating epithelial cells of the ocular surface (Woldoff & Haddad, 1973). In dry eye conditions, it usually stains the epithelial cells at the palpebral fissure as fine spots or coarse conglomerate areas. In few normal cases, an unusual corneal stain may occur. Therefore the conjunctiva alone or the conjunctiva and the cornea together must show the staining as a positive sign (Doughman, 1973). 1% Rose bengal is irritant and uncomfortable whilst 0.5% rose bengal is less irritant but equally effective. (Woldoff & Haddad, 1973).

2-9-5 Schirmer tests

These tests are simple and easy to use. For more than 50 years, very little change has been introduced into these tests. Whatman 41 filter paper is accepted as the standard filter paper. It is capable of absorbing about 0.5µl of water /mm strip of 5mm in width (Holly, Lamberts & Esquivel, 1983). Jones (1966) has described these tests in detail. The tests comprise the *Schirmer test 1, the Schirmer test 2* and the *Basic Secretion test*. These tests should be performed in a quiet room with a dim light and away from draught.

Schirmer test value is normally reduced in old age. There is no difference between the two sexes. The majority of normal people

have a difference of 3 mm or less between the two eyes (Henderson and Prough, 1950). Local anaesthesia reduces the test value by about 40% (Lambert, Foster and Perry, 1979). In an anaesthetized eye, the rate of wetting a Schirmer paper has a rapid phase of 50% in the first two minutes. This clearly is not a basal tear secretion but has a slight reflex component (Clinch, 1983) however the use of local anaesthetic should abolish most of the reflex component giving a basal tear secretion value.

As far as the validity of Schirmer's tests is concerned, the majority of researchers and clinicians believe that these tests are unreliable and unreproducible (Frost - Larsen et al, 1980 ; Van Bijsterveld, 1969). Furthermore the addition of local anaesthetic will further distort the result (Wright, 1987). Nevertheless there is a general agreement that a result below 5mm in 5 minutes indicate a reduction of the tear secretion (DeRoeth, 1952; Doughman, 1973; Holly & Lemp, 1977).

2-9-6 Impression cytology

This is a qualitative non - invasive technique that has been used to examine the epithelial and goblet cells of the conjunctiva in a wide range of diseases by taking an imprint using cellulose acetate filter paper. In dry eye conditions, the assessment of the nucleus / cytoplasm ratio (Tseng, 1985) and the density of the goblet cells (Ohji et al, 1987) provides a useful guide to histopathological changes of the conjunctiva. Impression cytology has been used to detect the typical fibrillo - granular inclusions present in the conjunctival epithelial cells of a baby wih muco - polysaccharidosis

(Maskin & Bodé, 1986). This technique has been used to detect chlamydia in neonatal conjunctivitis (Maskin, Heitman & Yee, 1986). Further discussion regarding the application of this technique will be described in normal and dry eyes with Sjögren's syndrome patients in chapters five and six.

2-10 Manaement of dry eyes

In most cases there is no cure for dry eye disorder. Therefore the aim of therapy should be directed toward symptomatic relief. This is achieved by keeping the ocular surface moist for longer periods of time by first instilling tear substitutes. If the symptoms still persist then punctal occlusion will preserve the remaining tears in the conjunctival sac. Hydrophilic contact lenses have been used in cases of filamentous keratitis (Lemp, 1987). Topical retinoid preparations have been used successfully in certain dry eye conditions (Tseng et al, 1985; Wright, 1985b). This mode of treatment is discussed in chapter 6. The treatment of any infection at the lid margin or of the ocular surface may improve the stability of the tear film.

Our limited knowledge in diagnosing specific tear film layer deficiency result in emperical and frequently unsatisfactory therapy (Gilbard, 1985). Furthemore, there are no artificial eye drops commercially, which give satisfactory results for most patients. Hypotonic solutions of 150 m Osm/ L have been more suitable than isotonic solutions in patients with KCS (Gilbard and Farris, 1979). Hypo-osmolarity alone does not guarantee the relief of symtoms in KCS, but other factors such as viscosity and colloid osmotic pressure

may be more significant (Wright, Cooper & Gilvarry, 1987).

The combination of artificial tears and mucus is more effective in reducing the surface tension and interfacial tension than artificial tears alone or a combination of artificial tears, mucus and protein (Lemp et al, 1970).

Methyl cellulose polymers have higher viscosity than other polymers and therefore maintain a longer retension time of the tear film (Gilbard, 1985). The mean retension time of hydroxypropyl methyl cellulose is 5.1 min. (Lemp, 1973a). Hydroxypropyl methylcellulose did not improve the tear film stability or have any effect on the conjunctival epithelium (Versura et al, 1989). High viscosity substitutes are associated with discomfort due to poor lubrication, crusting of the lids and blurred vision (Laflamme & Swieca, 1988). 1.4% Polyvinyl alcohol eye drops have favorable wetting abilities by the action of the macromolecules in adsorbing to the epithelial surface and interface of the tear film. The mean retension time of polyvinyl alcohol is 2.9 minutes (Lemp, 1973a).

Tear substitute therapy has given a subjective and objective improvement in 50% of patients with Sjögren's syndrome over 5 years period (Williamson et al, 1974).

Preservatives which are normally added to artificial tear substitutes, are used to retard the growth of microbial organisms but at the same time have a toxic effect on the ocular cells. The main preservatives used are benzalkonium, chlorobutanol, thiomersol and chlohexidine. Benzalkonium chloride is a cationic detergent which emulsifies the lipid cell wall (Lemp, 1987). A concentration of more than 0.01% can damage the corneal epithelium leading to superficial

punctate keratitis, corneal oedema, photobia and lacrimation (Lemp, 1973b). Preservative - free tear substitutes reduce dry eye induced keratitis, mucus strands, irritation and burning sensations (Laflamme & Swieca, 1988).

5% N-Acetylcysteine eye drops have been used successfully in patients with KCS who have mucus deposits. N-Acetylcysteine cleaves the high molecular weight glycoproteins in mucus leading to a redcuction in the stability of the tear film (Lemp, 1973a).

Other topical preparations which have recently been used in trials include Eledoisin and fibronectin. Eledoisin is a potent vasodilator which has been used as eye drops with good results in filamentous keratitis (Jaeger, Götz & Kaercher, 1985). Fibronectin is a high molecular weight plasma protein which has been used in Sjögren's syndrome patients with improvement in the clinical manifestations (Kono et al, 1985).

The main disadvantage of artificial tear preparations in use at present, are their short action, the presence of preservatives and the need for frequent application. Instilled solutions are diluted to half its strength by every 45 seconds and to 1/1000 of its original concentration within eight minutes (Lemp, 1973a).

Eye ointment gives long periods of hydration yet it interfere with vision however its use overnight is useful as an adjunct to artificial tears during the day.

Gel tears of acrylic acid polymers were used with prolonged subjective improvement in dry eye diseases (Leibowitz, Chang and Mandell, 1984). Unfortunately no such preparations are commercially available yet and therefore a definite and prolonged assessment is

required.

Ocular inserts placed in the conjunctival cul-de sac, for constant release of fixed amount of methyl cellulose have been favored by some patients who have mucus deficiency conditions (Pavan - Langston, 1973).

In cases of chronic blepharitis and secondary dry eyes, the lipases produced by the bacteria break the lipids into free fatty acids and destabilise the tear film. In meibomian gland dysfuction, obstruction is the primary cause rather than inflammation. Treatment therefore is directed toward eyelid hygiene with local and systemic antibiotics (Bowman, Dougherty & McCulley, 1987).

Bromhexine increases the bronchial secretion and decreases its viscosity. It has been found during the treatment of patients with cystic fibrosis, that the symptoms of dry eyes improved (Isenberg and Crisp, 1985). In Sjögren's syndrome patients, Bromhexine, taken orally, gave good improvement in the signs and symptoms of dry eyes (Frost - Larsen, Isager and Manthorpe, 1978).

Punctal occlusion is useful in patients for whom the artificial eye drops are not relieving the symptoms satisfactorly, the Schirmer test is less than 5mm and the rose bengal stains the ocular surface (Dohlman, 1978). Temporary occlusion with gelatin rod inserts into the four canaliculi has been advised as a temporary assessment before carrying out a permanant punctal occlusion (Foulds, 1961). Punctal occlusion under local anaesthesia, should include the canaliculi to obtain permanant occlusion (Knapp et al, 1989).

Surgical treatment for any abnormality in the eyelid may be helpful. Lateral tarsorraphy to a wide palberal fissure improves the

symtoms. Buccal mucous membrane grafts have been tried with very limited sucess (Lemp, 1987). Conjunctival transplantation in cases of thermal and chemical burns has been used by Thoft (1977) with encouraging results. Normal conjunctival graft provides a significant advantage over the use of buccal mucous membrane grafts (Vastine, Stewart and Schwab, 1982).

CHAPTER THREE

A BRIEF HISTORY OF VITAMIN A

3-1 Introduction

The history of vitamin A demonstrates the curious landmarks in the development of human knowledge and progress, with its golden as well as its dark ages. In order to recognise and appraise our present achievements, it is important to appreciate how and when our ancestors knew about vitamin A deficiency and how they dealt with it.

A brief history of vitamin A is included with a small section on the history of vitamin A and the eye.

3-2 History of Retinoids and Carotenoids

Night blindness was first recorded as an early manifestasion of nutritional deficiency in Eber's papyrus, an ancient Egyptian medical treatise of about 1500 B.C. which recommended an ox-liver, roasted and crushed, as a curative agent (Ebers, 1937). In ancient Greece, Hippocrates (460-377 B.C.) prescribed ox-liver eaten raw with honey. Galen (130-200 AD) recommended "continuous eating of roasted or boiled liver of goat" and "painting the eyes with the juice of the roasted liver", a residue of the old Egyptian influence (Wolf, 1978).

Surprisingly any serious attempt to identify vitamin A started only early this century. Stepp in 1909, discovered a fat - soluble extract in egg yolk whilst in Wisconsin University, McCollum and Davis (1915) discovered the existence of two factors, a fat-soluble substance A and water - soluble substance B. Furthermore, they

established that fat- soluble A resists the action of alkali. Hopkins (1920) showed that fat-soluble substance A in butter resisted heat up to 120°C but was readily destroyed by oxidation.

Steenbock (1919) recognised the relation between fat-soluble factor A and the yellow coloration of butter and cod liver oil. Also McCollum, Simmonds and Pitz (1917) and Osborne and Mendel (1919) concluded that certain green vegetables has high activity as sources of vitamins A.

Carotenoid has long been known to give a transient blue colour with concentrated sulphuric acid. This test gave the same colour reaction when tested with liver oils of fish, birds and mammals. The colour was lost when the oils were heated and aerated (Drummond and Watson, 1922). Carr and Price (1926) found that saturated antimony trichloride in chloroform gave a stable colour with cod liver oil and thus provided a better test for vitamin A than the concentrated sulphuric acid assay.

Euler, Euler and Hellstrom (1928) showed that carotene was active in curing vitamin A deficient rats. They also showed that carotenoids gave a blue colour similar to that of vitamin A with the antimony trichloride reaction. Moore in 1929, confirmed that carotene has vitamin A activity although it is different from the vitamin A found in cod liver oil. He also showed that carotene given to vitamin A deficient rats became stored in the liver in the form of vitamin A (Moore, 1957).

In 1931, Karrer, Morf and Schopp in Zurich, obtained highly purified vitamin A and proposed its structural formula. Moreover he and others were able to formulate the structure of ß carotene. Kuhn

and Brockmann (1931) showed that both α and ß forms of carotene were biologically active although the ß carotene is more active. Karrer and Eugster (1950) eventually succeeded in synthesising ß carotene and several other pigments.

Holmes and Corbett (1937) crystallised vitamin A. Arens and Van Dorp (1947) and Isler et al (1947) succeeded in producing vitamin A in pure form and in substantial quantities. Morton (1944) identified the structures of retinal and vitamin A aldehyde. Figure 1 gives a brief details of the history of vitamin A and carotene.

3-3 Recent History of vitamin A and the eye

The relationship between vitamin A and the retina began with Franz Boll who presentd a paper at the Berlin Academy in November 1876, describing a freshly excised pink retina of a frog which became "bleached " on exposure to light. Yudkin in 1931 discovered the presence of vitamin A in the retinae of normal animals. Wald in 1933, whilst working in Germany, was responsible for the recognition of the visual purple as a protein complex of vitamin A. He was awarded the Nobel prize for his work.

Wolbach and Howe (1925) were the first to report that the keratinisation of the cornea is due to vitamin A deficiency.

Therapeutically, the Russians in the nineteen thirties, were the first to use topical vitamin A with good results in phlyctenular keratoconjunctivitis, various types of keratitis, corneal erosions and trachoma (De Grosz, 1939). Balachovski of Rostov (1934) called attention to a possible local vitamin A deficiency. De Grosz (1939)

THE PAPYRUS EBERS (1500 B.C.)
(Recommended Ox-liver eaten)

↓ ↓

HIPPOCRATES (460-377)
(Recommended ox-liver
eaten raw with honey)

↓ ↓

GALEN (130-200 A.D.)
(Recommended roasted Ox-liver eaten and
the juice painted on the eyes)

↓ ↓

STEPP (1909)
(Discovered fat soluble substance
in egg yolk)

↓ ↓

McCOLLUM & DAVIS (1915)
(Dicovered a fat-soluble factor A and
water-soluble factor B)

↓ ↓

McCOLLUM, SIMMONDS & PITZ (1917) and
OSBORNE & MENDEL (1919)
(Concluded that certain vegetables have
high activity as source of vitamin A)

↓ ↓

MOORE (1929)
(Confirmed that Carotene has
vitamin A activity)

↓ ↓

KARRER, MORF & SCHOPP (1931)
(Obtained highly purified vitamin A)

↓ ↓

MORTON (1944)
(Identified the structure of retinal
and vitamin A aldehyde)

↓ ↓

KARRER & EUGSTER (1950)
(synthesized β carotene)

Figure 1. Landmarks in the history of
Vitamin A and carotene.

claimed good results with topical vitamin A in eye injuries including chemical burns, blepharitis and filamentous keratitis.

CHAPTER FOUR

VITAMIN A - A BRIEF REVIEW

4-1 Introduction

Since the discovery of vitamin A at the start of this century, there has been an explosive increase in research into basic, nutritional and biochemical aspects of vitamin A, including the introduction of different synthetic products.

Our knowledge of the metabolism of vitamin A in the body and particularly in the retina is not complete. A brief summary of the metabolism of vitamin A and the manifestations of its deficiency and excess, in general, and the eye in particular, will be discussed.

4-2 The Retinoids

Retinoids were defined as synthetic substances which at present exceed one thousand in number (Frickel, 1984). According to the old definition by the International Union of Pure and Applied Chemistry (IUPAC-IUB), retinoids are "Those diterpenoids derived from a monocyclic parent compound containing 5 carbon-carbon double bonds and a functional group at the terminus of the acyclic portion". New retinoid derivatives that are tricyclic or tetracyclic retinoidal benzoic acid derivative, are not included in this definition, yet these substances are more than a thousand times as potent as retinol or its esters (Sporn and Roberts, 1985). Some of these retinoids have been shown to cure vitamin A deficient

rats (Stephens- Jarnagin, Miller and De Luca, 1985). Although their chemical structure is different from that of vitamin A derivatives, they have similar action. It is not yet known how this mechanism is mediated at a cellular level but both types have the ability to stimulate the receptors concerned (Roth and Taylor, 1983). Therefore a new definition has been proposed for retinoids as "Substances that can elicit biological responses by binding to and activating a specific receptor or set of receptors. The programme for the biological response of the target cells resides in the retinoid receptors rather than the retinoid itself". Retinoids affect the differentiation and the proliferation of many types of cells whether ectodermal, endodermal or mesodermal in origin or whether neoplastic or non-neoplastic (Sporn & Roberts, 1985).

The term "retinoid" will be used in this thesis in its generic sense, while the names "retinol", "retinal" or "retinaldehyde" will be reserved for the specific chemical structure.

4-3 Vitamin A Properties, Nomenclature and Formulae

Vitamin A compounds and β carotene derivatives are necessary for the maintenance of vision, reproduction, epithelial cell growth and differentiation. Also they appear to be involved in bone development, the immune response, mucopolysaccharide synthesis and adrenocortical function (Chader, 1984).

Retinoids are derived from units of isoprene which are 5 carbon building units (Figure 2). Isoprenoids are units of isoprenes, which include retinoids, various oils as cholesterol, resins, steroids, steroid hormones and carotenes (Chader, 1984).

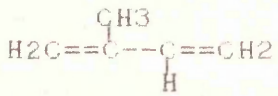


Figure 2. Isoprene unit structure.

β- ionone ring | Polyene chain | Polar end "R" ring

Retinol	R= CH2OH
Retinal	R= CHO
Retinoic acid	R= COOH

Figure 3. The chemical structure of natural retinoids.

The structure of natural retinoids consists of a β ionine ring, unsaturated side chain and the terminal polar end group which is important in conferring vitamin A activity on the molecule (Figure 3). The hydroxyl group of retinol maintains all the functions of vitamin A, while the aldehyde group of retinal is involved in vision. The acid group of retinoic acid has the same actions of retinol except in the maintenance of normal vision (Dowling & Wald, 1960) and in reproduction (Thompson, Howell and Pitt, 1961).

The all-trans configuration of the side chain is the most common type found in nature and it is the form that is transported in the blood. The 11-cis isomer is important in the visual process and is found mainly in the retina, while the 13-cis isomer is involved in cellular differentiation and function (Chader, 1984). The all-trans retinal is the principal and biologically the most active substance. It may be synthesised in a pure state where in its natural sources, it is accompanied by many esters (European Pharmacopeia, 1975). Esters of all -trans retinol are called retinyl esters which are stable and found commonly in nature.

The all-trans retinol occur as pale yellow crystals, practically insoluble in water but freely soluble in alcohol, chloroform, light petroleum, ether and fixed oils. They are produced in the form of esters as acetate, proprionate or palmitate which are present as concetrates in oily form, water dispersible form or as dry powder. Synthetic vitamin A concentrate may contain stabilising agents as antioxidants (European Pharmacopeia, 1975). Commercially available retinoids are

incorporated into gelatin matrix that protect them from oxidation over reasonable periods of time (Olson, 1984).

Vitamin A derivatives are sensitive to oxidation and metals like cobalt and copper. They are unstable in ultraviolet light. They are more stable in basic rather than in acid solutions (Chader, 1984).

Vitamin A is present most commonly in the liver of fish and animals, green leaf such as lettuce and spinach and yellow vegetables such as carrots and pumpkins. They are also present in butter, cheese and egg yolk but to a lesser extent in milk (Chader, 1984).

Carotenoids have similar chemical solvent properties to vitamin A derivatives. They have yellow to orange colour and are present in plants such as carrots, green vegetables as spinach and red palm oil. The β carotene precursor contains at least one β ionone ring that is not hydroxylated in order to show vitamin A activity (Olson, 1984). Natural carotenoids generally possess all - trans configurations. The actual biological activity of carotenoids may be considered higher or lower than their analytical content, depending on the nature of the meal, the presence of fat and the binding of carotenoids within the food. Carotenoids have lower bioavailability than vitamin A. This is due first to the specific requirement for bile salts during in their absorption and second the relative slow rate of cleavage of carotenoids to vitamin A in the intestine and in other tissues.

The biological activity of retinoids has been expressed in International Units (I.U.). One I.U. equates to the activity of

0.300 µg of retinol or 0.334 µg of retinyl acetate derivatives.

In a healthy person, the accepted normal serum level is between 30 -100 µg /100ml. If the serum level is less than 10µg/100ml, it indicates a deficiency of vitamin A in the body. The serum level of vitamin A is not an accurate reflection of its value in the body and particularly of the liver. Vitamin A values of the plasma and the liver in humans are influenced by socio-economic status. In human adults, the values of retinoids in the plasma and the liver stores were not correlated (Suthutvoxavoot and Olson, 1974). In infants, the median values of retinoids in the liver are over 5 µg/gm while in children over one year are over 20 µg/gm (Olson, Gunning & Tilton, 1979).

4-4 Retinoid Identification

A variety of physico - chemical methods are used to determine the concentration of retinoids in chemicals, pharmacological preparations, tissues and food. The main procedures commonly used are: The ultra - violet Absorption Spectrum, The Fluorescence Method, The Colorimetric Method and The High Pressure Liquid Chromatography (H.P.L.C.).

4-5 Metabolism of Retinoids and Carotenoids

Retinoids and carotenoids in the food are released from proteins by the action of pepsin in the stomach and the proteolytic enzymes in the small intestine. In the stomach, the free carotenoids and retinyl esters congregate in fatty globules. The fat globules are broken into small lipid congregates in the duodenum, in the

presence of bile salts. These are digested by pancreatic lipase and retinyl ester hydrolases. The resultant mixed micelles which contain retinol, carotenoids, phospholipids, mono- and diglycerides and fatty acids become finely dispersed in the intestinal epithelium, making contact with the intestinal cell membrane (Glover & Walker, 1964). The components of the micelle enter the mucosal cell mainly in the upper half of the intestine (Olson, 1984). Some of β carotenes are converted to retinoid esters, mainly at the upper two thirds of the intestine (Olson & Herron, 1961). The overall absorption of retinoids is about 80-90% (Olson, 1984)

Carotenoid absorption has an absolute requirement for bile salts while retinoids in any solubilised form are readily absorbed (El-Gorab, Underwood and Loerch, 1975) . Once β carotene and other carotenoids enter the intestinal cells, they are cleaved to retinaldehyde by two enzymes. The retinaldehyde in turn is reduced to retinol and later may be esterified to retinyl esters. Retinyl esters may be hydrolysed to retinol. These reactions may occur in different tissues including the intestine, liver and the adrenal glands. The overall absorption of carotenoids is between 50-60% (Olson, 1984).

Retinoid absorption depends on the integrity of the intestinal mucosa, the stimulating effect of the protein at the intestinal lumen and the importance of fat as a vehicle for retinoids and carotenoids and as a stimulant of bile (Olsen, 1984).

Retinoids enter the lymphatic ducts and the blood vessels as chylomicrons. They are carried to the liver to be degraded by lipoprotein lipase in the liver and other tissues leaving retinoid

esters to be taken up (Chader, 1984). A pre-Retinol Binding Protein (pre R.B.P.) is synthesized in the liver (Soprano, et al, 1981). After its conversion to Retinol Binding Protein (R. B. P.) within the parenchymal cells, it combines with all - trans retinol and is secreted into the plasma as holo-R.B.P. This regulatory process is controlled by the availability of retinoids however it is not blocked by any inhibition in protein synthesis. This indicates the presence of a large pool of apo-R.B.P. ie retinol free protein (Goodman, 1981). The plasma R.B.P. is a single polypeptide chain which possesses one site only for retinol and interacts with plasma prealbumin. The concentration of R.B.P. in plasma is 3 - 4mg /100 ml (Kanai, Raz & Goodman, 1968). Under normal condition about 90% of R.B.P. is saturated with retinol. All-trans retinol is the only type, under physiological conditions, which combines with R.B.P. while retinoic acid is carried in plasma by albumin (Smith and Goodman, 1979). In human, plasma R.B.P. and retinol form a complex with transthyretin (prealbumin) which also binds thyrod hormone in 1:1 molar complex. Within R.B.P., retinol resides in a hydrophobic cleft that protects it from oxidation or destruction during transport. Since R.B.P. is known to be filtered by the kidney, the complex prevents its rapid removal and destruction. This explains the rise in plasma level of retinol and R.B.P. in chronic kidney disease (Goodman, 1981).

The cell surface receptors recognise only the protein of apo R.B.P. and not the retinol. Only the retinol part enters the cell while the holo R.B.P. remain at the cell receptors. The target tissues for the retinol, apart from the eye, are the skin, the

intestine, the adrenal gland, testis and the salivary gland (Goodman, 1981).

Once the retinol is inside the cell, it is quickly bound by a specific binding protein called cellular retinol binding protein (C.R.B.P.). This protects the retinol from oxidation within the cell and serve as a carrier to its intracellular site of action (Ross, 1980). Another specific intracellular protein called cellular retinoic acid binding protein (C.R.A.B.P.), is bound to retinoic acid. This protein is found in the same epithelial tissues that contain C.R.B.P. with the exception of liver, kidney, lung, spleen and muscle. Retinoic acid binding to C.R.A.B.P is very low in the corneal epithelium and is undetectable in the conjunctiva of normal rabbits. In retinoid deficient rabbits, the retinoic acid binding protein increases in corneal epithelium and is detectable in the conjunctival epithelium (Wiggert, van Horn and Fish, 1982). The C.R.B.P. and C.R.A.B.P. are present in foetal tissue but their distribution differ in adult rat. They are present in some tumours as in carcinoma of the lung, breast and in skin papilloma. It is not clear yet if there is a specific binding site for retinol on the cell nucleus (Chytil & Ong, 1979). A third binding protein of retinal in bovine retina has been found which has a preference for binding to 11-cis isomers than to all-trans configurations (Futterman, Saari and Blair, 1977).

In the nucleus of retinoid deficient animals, the ribonucleic acid shows low electrophoretic mobility. This process can be reversed by giving retinoids (Kaufman et al, 1972).

4-6 Storage of Retinoids

More than 90% of the retinoids are stored in the liver. They are stored in the form of lipid-protein aggregates and as retinoids (Sklan et al, 1982). Of these retinoids, 96% are retinyl esters and 4% unesterified retinol. Retinaldehyde may be oxidised to retinoic acid which is an irreversible reaction. Retinoic acid is not stored in the liver or other tissues. Since retinoids are hydrophobic, they tend to associate within the cells with proteins, lipid rich aggregates or membranes. Therefore, the transformation of retinoids from one derivative to another tend to be a complex process.

Some of the carotenoids which are absorbed unchanged by the mucosal intestine, are taken up mainly by adipose tissues whilst small amounts are taken up by the liver.

4-7 Excretion of Retinoids

Of the absorbed retinoids, 20 - 50% are oxidised or conjugated to products that are excreted within one week. The remaining 30-60% of retinoids are stored.

Retinol is partly transformed in the liver to β-glucuronide which is then secreted into the bile.

4-8 Biological Function of Retinoids

Retinoids are necessary for life in higher species. Retinol and retinoic acid are essential for vision.

Retinol and retinoic acid stimulate growth directly in a number of tissues in the body (Zile, Bunge and DeLuca, 1979). The

tissue differentiation affects particularly the trachea, skin, salivary gland, cornea and testes. Retinoids help in cell adhesion and contact inhibition.

Retinoids regulate the synthesis or glycosylation of epithelial glycoprotein in rats. The administration of retinoids increases the glucosamine incorporation in glycoprotein or glycoprotein fragments (Hassell and Newsome, 1981). The synthesis of specific epithelial glycoprotein was directly related to the retinoids level adminstered (Hassell, Newsome and De Luca, 1980). Hassell and Newsome (1981) concluded that corneal epithelial cells have the ability to synthesize goblet cell- like glycoproteins.

4-9 Recommended Daily Requirement of Retinoids

Recommended daily requirement varies from one country to another. In broad terms, an infant's daily requirements, as recommended by both the National Research Council of U.S.(1980) and W.H.O./Food and Agriculture Organisation, are 400 IU and 300 IU respectively. For children between 1-10 years old, the recommendation is 400-700 IU for the National Research Council of U.S. and 250-400 IU for W.H.O./Food and Agriculture Organisation and for adults, it is 800-1000 IU and 750 IU respectively (Olson, 1984).

4-10 Retinoid Deficiency

About 5 million children a year develop clinical xerophthalmia and one half a million develop blindness due to corneal disease. The deficiency is due to inadequate intake of food,

poor digestion and absorption or an increase in the requirements of the body. The changes affect different organs and systems in the body (Sommer et al, 1981).

In animals fed on a retinoid deficient diet, growth is retarded and if such feeding is continued, death follows. The changes in the anterior part of the eye manifest as xerophthalmia, keratinisation and secondary infection while the changes in the outer segment of the retina result in night blindness. The skin is dry with hyperkeratosis while the bone shows defective modelling and cancellous structure. In the urinary tract, there may be calculi and secondary infection. The manifestations in the nervous system shows increase in the cerebrospinal fluid, optic atrophy and ataxia. In the reproductive system, there is degeneration of the testis while in the female, early abortion has been reported (McLaren, 1966).

Mortality increases with the degree of retinoid deficiency (Sommer et al, 1983). Children with mild xerophthalmia, have increased the risk of developing respiratory diseases and diarrhoea, compared to well nourished children of the same age group (Sommer, Katz & Tarwotjo, 1984).

4-11 Retinoid Toxicity

The toxicity may be acute, as in the well documented cases of eating polar bear livers by their hunters. The symptoms are irritability, drowsiness, headache and vomiting due to raised intracranial pressure. The condition is reversible (Olson, 1984).

In chronic toxicity due to prolonged intake of retinoid,

there is enlargement of the liver and spleen, hypoplastic anaemia, leucopenia, itchy skin with pain and stiffness in the spine and the limbs (Moore, 1967). There may also be a mucous metaplasia of normally keratinizing epithelia (Lawrence and Bern, 1963).

The toxic ophthalmic manifestations are blurred vision, diplopia, mild exophthalmos and loss of hair from the eyebrows and eyelashes (Oliver & Havener, 1958).

4-12 Retinoids and the dry eye

The effects of retinoids as essential factors for growth and differentiation of epithelial cells have been well documented. Furthermore the retinoids increase the number of epidermal growth factor (EGF) receptor sites although they have no effect on the affinity of EGF for its receptors (Jetten, 1980).

The use of topical retinoids in the treatment of vitamin A deficiency and secondary dry eye showed a significant improvement in the regeneration of epithelial cells with an increase in the density of goblet cells. The effects of topical retinoids on albino rabbit eyes, showed that more than 50% of retinol was absorbed by the conjunctival epithelium over 2 hours period. The solubilisation of vitamin A by proteins in tears and by cellular lipids and the R. B. P. in corneal epithelium appears to play an important role (Lee & Carson, 1985).

The regenerative effect of topical retinoids was assessed on humans with systemic vitamin A deficiency. The clinical manifestations of dry eyes such as punctate keratopathy and Bitot's spots demonstrated an improvement in the degree of ocular surface

dryness and in vision. It even showed an early corneal healing to eyes treated only systemically (Sommer & Emran, 1978). The application of topical retinoids on dry eyes with severe cases of squamous metaplasia in non-vitamin A deficiency patients showed an encouraging results by some researchers (Tseng, 1985 ; Wright, 1985) while others did not find a significant improvement in the squamous metaplasia (Soong et al, 1988).

CHAPTER FIVE

Standardisation of the Filter Paper Surface for Impression Cytology

5-1 Introduction

Impression cytology is a non - invasive qualitative technique. It is used to examine the conjunctival epithelial and goblet cells by means of cellulose acetate filter paper. The technique is simple, easy to use and causes little discomfort to the patient. The information obtained is accurate and complementary to the clinical tests for dry eye disorders. Moreover the test is as informative and far less damaging to the eye than a conjuctival biopsy.

Although the use of cellulose acetate filter paper in cytological studies has been employed for studies of cancer cells in different fluid specimens since 1959 (Del Vecchio et al, 1959), the impression cytology technique was introduced using "Millipore filter paper", for the study of dry eye in 1977 (Egbert, Lauber & Maurice, 1977). Since then several researchers have used this technique to study the different aspects of epithelial and goblet cells in normal and abnormal conjunctiva. Nelson and Cameron (1980) assessed the goblet cell status in symptomatic and asymptomatic dry eye patients. Natadisastra et al, (1987) employed impression cytology for the detection of conjunctival cells in vitamin A deficiency. In addition, impression cytology has been used diagnostically in chlamydial

conjunctivitis (Maskin, Heitman & Yee, 1986) and when combined with scanning and transmission electron microscopy in a case of mucopolysaccharidosis (Maskin & Bode, 1986).

The technique employed by Egbert et al (1977) is simple and efficient. The filter paper strip is held by forceps and is placed on the ocular surface with one hand and pressed by a glass rod with the other hand for three to five seconds. The filter paper is then peeled off and put into fixative solution prior to staining. Adams (1979) devised an applicator where the "Millipore filter paper" was attached to a plastic strip. This applicator has been adopted in the present experiments.

"Millipore filter paper" has a smooth shiny surface on one side and a dull comparatively rough surface on the other side. Nelson, Havener and Cameron (1983) and Adams (1979) have stated that the dull surface is more suitable in obtaining conjunctival epithelial cells and the smooth surface is more suitable for obtaining mucus. Furthermore, Adams (1979) claimed that the removal of surfactant, which is normally present in the filter paper, gives a better yield of epithelial cells. By contrast, Tseng claimed that both surfaces of the filter paper have been used satisfactorily in obtaining epithelial cells in normal and pathological conditions. He also found no difference in cellular yield between filter papers with and without surfactant (Tseng, 1985).

The aim of this experiment is to assess the adhesive quality of each type of filter paper surface by determining the yield of epithelial cells in both dull and smooth surfaces and with and without surfactant.

5-2 Materials

Nine male and six female normal volunteers consented to the trial. They were recruited from the Tennent Institute of Ophthalmology, Glasgow. The majority of volunteers were overseas students . None had any known history of eye disease, symptoms of dry eyes or arthritis. None of the volunteers was on local or systemic medication. The age range was between 26 and 53 years (mean 33.4 years). The volunteers' anterior segments were normal with slit-lamp microscopy.

5-3 Method

All the materials used in this experiment are listed with the details of the suppliers, in Appendix I.

" Millipore filter paper " is a pure, biologically inert mixture of cellulose acetate and cellulose nitrate. It contains about 5% surfactant (by weight) as a wetting agent. It is plain and very fragile. It becomes transparent when immersed in xylene. The type of filter paper used in this experiment is white with a pore size of 0.45 µm.

Pre- treated filter papers were soaked in tap water for thirty minutes to remove the surfactant then left to dry before cutting the filter paper to the required size (Adams, 1979).

The plastic used in this experiment is a clear, 1mm thick PVC sheet. Adams removed the filter paper from the applicator, at the end of the staining procedure, before immersing it in xylene. Plastics generally are damaged by xylene but not the type used in this experiment. A piece of plastic was left in xylene overnight with

no apparent damage or loss in clarity. Furthermore plastics was also examined by scanning electron microscopy. No abnormalities were found on the plastic surface before or after it was immeresed overnight in xylene solution.

5-3-1 Preparation of the Applicator

This device was designed by Adams (1979). The filter paper was cut into strips, 5mm wide and 15 mm long, which were mounted by a double sided adhesive tape on one end of a plastic strip measured 5 mm wide and 76 mm long (Figure 4). These were sterilized by exposure to ethylene oxide vapour. The length of the plastic strip was designed to fit into slide rack (Figure 5). A large number of Applicators can be processed in the staining procedure at one time.

There are several advantages in using this device over the one used by Egbert et al and later adopted by others (Tseng, 1985; Nelson, Havener and Cameron, 1983). First the ease of handling the applicator with one hand. Secondly, the applicator delivers an even pressure on the ocular surface which is better than Egbert's technique of pressing the filter paper on the ocular surface with a glass rod. Thirdly there is no damage to the fragile filter paper whilst pressing on the ocular surface compared to Egbert's method of handling the filter paper with forceps. Fourthly when the applicators are put in a slide rack, they do not touch any surface throughout the staining process and therefore ensure no damage to the attached epithelial cells. The last advantage is superior to the staining process used by Tseng, where the filter paper strips in a

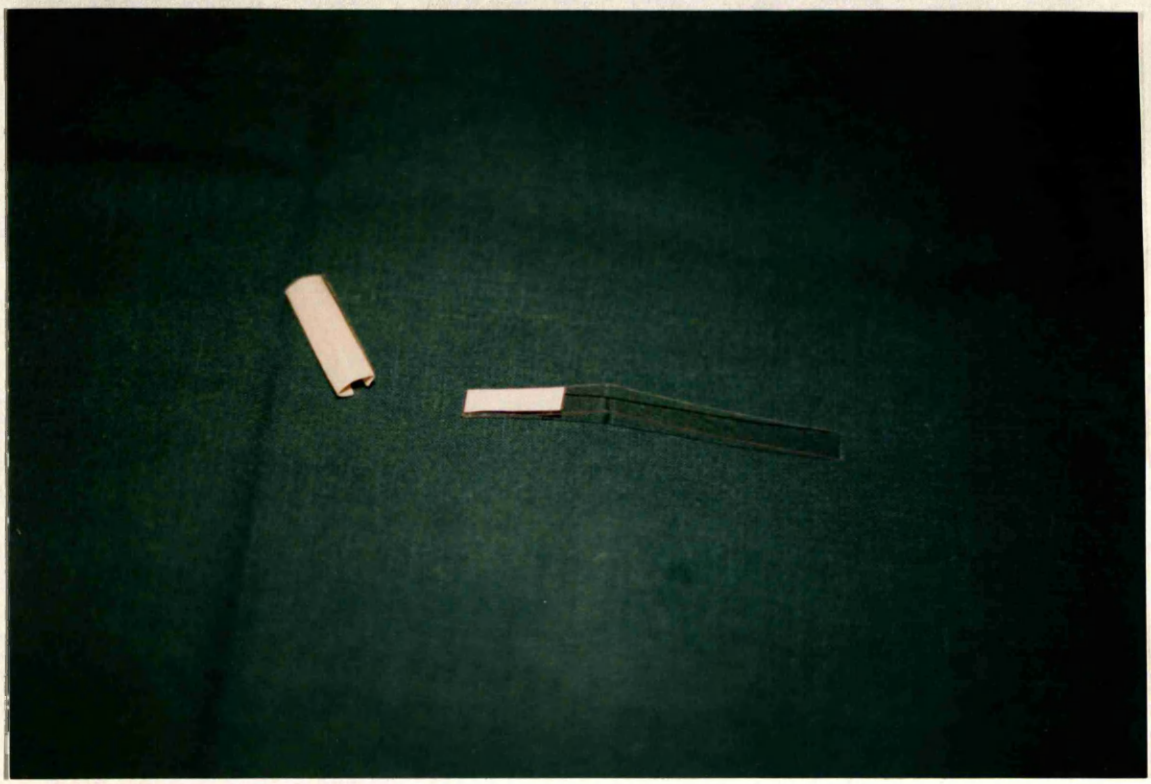


Fig. 4 _ An Applicator

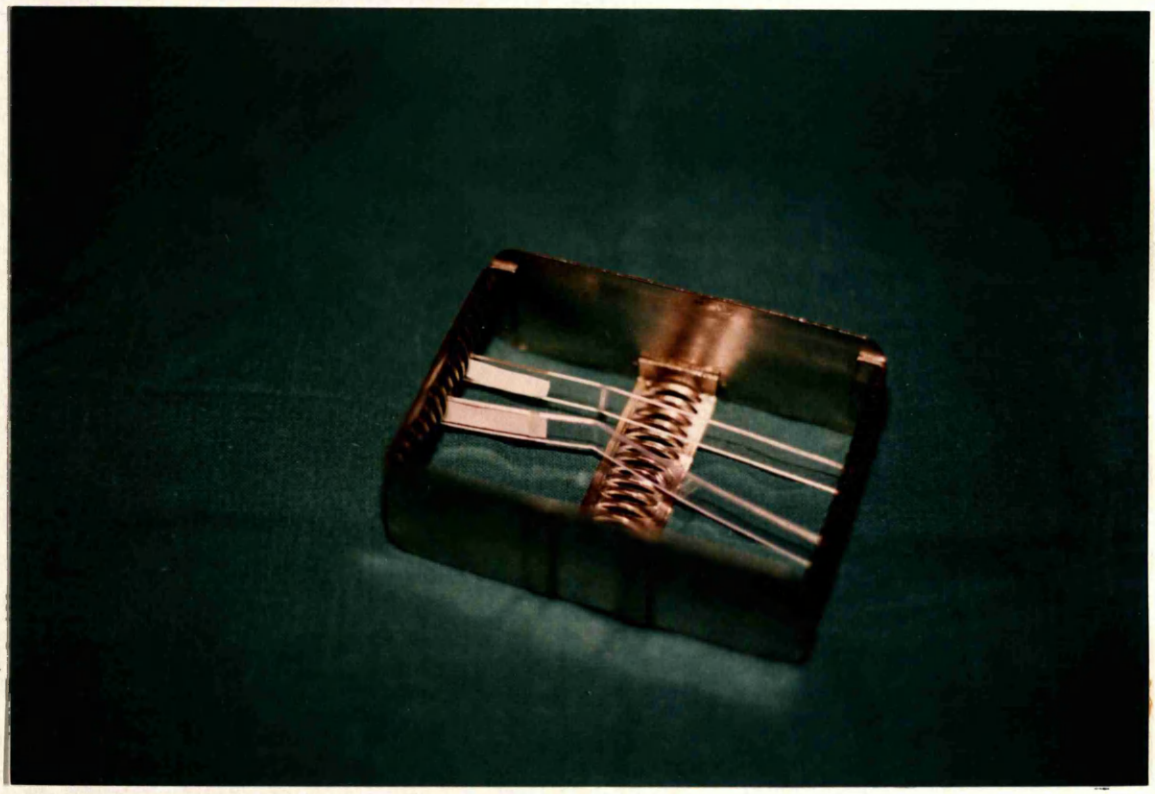


Fig. 5 _ A number of Applicators in a slide rack.

24 -well plastic culture plate, may easily stick to the walls of a well (Tseng, 1985).

5-3-2 Fixative Solution

The solution is formed of one part glacial acetic acid, one part formaldehyde 37% and twenty parts of 70% ethyl alcohol. Fixation with formalin alone is not suited for mucin which has a high water - binding capacity. On the other hand alcohol - formalin solution prevents the goblet cells from taking up water (Moe, 1955).

5-3-3 Preparation of Staining Solutions

Schiff's reagent - This staining reagent is prepared in the sequence listed in Table 4. The final preparation is one part Schiff's reagent to 3 parts of freshly distilled water. It should be kept away from light at a tempreture of 4°C. The solution was changed approximately every month. The Schiff's reagent should be fresh as its staining ability may be varied with time (Kessing, 1986).

Gill's Haematoxylin- Gill, Frost and Miller (1974) formulated a haematoxylin which retains the advantages of Harris' and Mayer's haematoxylins. Gill's haematoxylin stains the nucleus but scarcely tints the cytoplasm. It stains mucus minimally. This solvent system prevents wasteful precipitation of aluminium - haematin, the troublesome scum that forms daily in Harris' haematoxylin. It does not stain the walls of either the glass or plastic storage container. The solution in storage is stable for about one year.

The materials and preparations of Gill's haematoxylin are

1. Boil 200ml distilled water then take it off heat.
2. Add 1 gram. of basic fuchsin.
3. Cool to 50° centigrade.
4. Add and mix 2 gram of potassium metabisulphite .
5. Cool to room tempreture.
6. Add 2 ml of conc. hydrochloric acid .
7. Add 2 gram of activated charcoal .
8. Leave overnight in a dark room.
9. Filter the solution through Whatman filter paper No1.
10. Store at 4° centigrade.
11. Distilled water is added to Schiff's reagent (three volumes of distilled water to one volume of Schiff reagent.

The Schiff's reagent should be freshly prepared as its staining ability may vary with time.

Table 4. Preparation of Schiff's reagent

1. Distilled water 730 ml.
2. Add ethyl alcohol 250 ml.
3. Add Haematoxylin anhydrous powder, certified (C1 No 75290), 2 grams.
4. Add accurate weight of sodium iodate 0.2 grams.
5. Add Aluminium sulphate Al 2(SO4)3 16H2O, 16grams. (The weight was slightly increased by 0.2 grams over Gill's recommendation since the water molecules in this preparation are 16H2O while in Gill's preparation was 18H2O).
6. Add Glacial acetic acid 20ml.
7. Filter in Whatman No1 filter paper.

The above mixture is made up in the order given, andstirred for one hour with a magnetic mixer at room temperature as described by Gill, Frost & Miller (1974).

Table 5. Preparation of Gill's Haematoxylin

listed in Table 5. The materials are mixed in the order given, stirring for one hour using a magnetic mixer at room tempreture.

Scott's Tap Water Substitute- Scott's Tap water is an alkaline solution with a pH around 8. It is made up with 10 Gm. Magnesium sulphate and 2 Gm. Sodium bicarbonate dissolved in one litre tap water. It is changed at every alternate staining procedure.

Modified Orange G.- 2% Orange G powder and 5 % Phosphotungstic acid are added to 100ml of 95 % ethyl alcohol. This is stirred in a magnetic mixer then filtered in Whatman No.1 filter paper .

5-3-4 Impression Cytology Technique and Staining Method.

After instilling one drop of 0.4 % oxybuprocaine hydrochloride (Benoxinate) eye drops in each eye, an applicator with one type of filter paper surface was pressed gently on the bulbar conjunctival surface for about 3 - 4 seconds. The filter paper was peeled off and put in the fixative solution for at least ten minutes.

The optimum pressure applied on the ocular surface has not been assessed. The description in the literature has varied from 'pressure' by Egbert et al (1977), 'a gentle pressure' by Wittpen, Tseng & Sommer (1986) and 'a gentle massage' by Adams et al (1988). Nevertheless only one or two layers' thickness of the conjunctival cells adheres to the filter paper irrespective of the amount of pressure applied on the ocular surface. The staining procedure was facilitated by combining a number of applicators in a slide rack

which fits well into the staining trough. The timing of each step of the staining procedure was adhered to carefully. The staining procedure employed by Tseng (1985) was adopted (table 6). Once the applicator had been lifted from xylene, the filter paper was removed from the plastic, placed on a glass slide and covered with a DePeX mounting solution before applying a glass cover slip.

The conjunctival sites were coded with the letters **A,B,C** and **D** which corresponded with right temporal, right nasal, left nasal and left temporal bulbar conjunctiva respectively.

Under light microscopy, the epithelial cells were identified either as sheets or separate cells. The goblet cells are bigger in size, reddish in colour and therefore more visible and easier to recognise. This is in contrast to Harris' haematoxylin where a dark blue colouration stains the epithelial and goblet cells (Figure 6).

Although the details of the cells are clear, the resolution for the finer parts of the cell is not. This may be due to the thickness of the filter paper compounded by the amount of water present in the filter paper at the end of the staining procedure.

5-3-5 Clinical Tests

The volunteers underwent the following tests in the order described. The scoring code for the history of the dry eye, the clinical tests and the immpression cytology are shown in table 7.

Tear Meniscus - Without touching the eye, the lid margins were inspected with a slit lamp, for the presence, absence or

		Duration
1.	Fixative Solution	10 min.
2.	Ethyl alcohol 70% (rehydration)	2 min.
3.	Tap Water	10 dips
3.	Periodic acid 0.5%	2 min.
4.	Tap Water	10 dips
5.	Schiff's Reagent	2 min.
6.	Tap Water	10 dips
7.	Sodium Metabisulphite	2 min.
8.	Tap Water	10 dips
9.	Gill's Haematoxylin	2 min.
10.	Tap Water	10 dips
11.	Hydrochloric acid 0.05%	30 sec.
12.	Tap Water	10 dips
13.	Scott's substitute tap water	2 min
14.	Tap Water	2 min.
15.	Ethyl alcohol 95% (dehydration)	10 dips
16.	Modified Orange G.	2 min.
17.	Ethyl alcohol 95%	3 min.
18.	Modified Eosin Y.	2 min.
19.	Ethyl alcohol 95%	5 min.
20.	Absolute ethyl alcohol	2 min.
21.	Xylene	5 min.
22.	Mount DePeX	

Table 6. The Staining Procedure of Impression Cytology

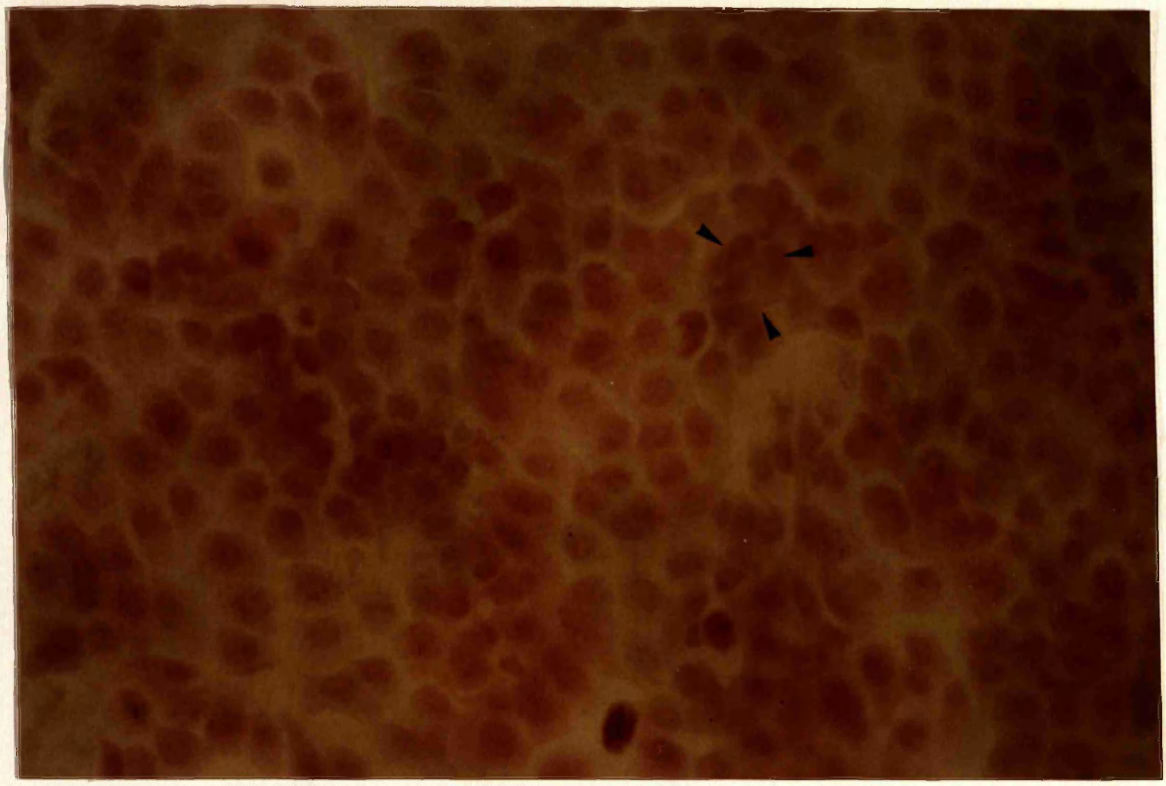


Fig. 6 - Normal superficial conjunctival epithelial cells in Harris' Haematoxylin stain. Goblet cell (indicated by 3 arrows) is not easily recognised.

Name No.
D.O.B.

I History of Dry Eye

<once/week	>once/week	once/day	>once/day
(0)	(1)	(2)	(3)

1. F.B. Sensation
2. Burning Sensation
3. Itchy Sensation

II History of Arthritis -Which joints?
III Drugs

	absent	present
Tear Meniscus-	(1)	(0)

Fluorescein Staining

	absent	¼ of cornea	½ of cornea	>½cornea
	(0)	(1)	(2)	(3)

	<5 sec.	6-15 sec.	16-30 sec.	>30 sec.
Break-up Time	(0)	(1)	(2)	(3)

	<5 mm	6-15 mm	16-30 mm	>30 mm
Schirmer's Test	(0)	(1)	(2)	(3)

Impression Cytology

Table 7. The Symptoms and Clinical Tests of Tear Secretion in normal volunteers.

The values in parenthesis represent the scoring code of each test

discontinuity of the tear meniscus.

Fluorescein Staining- After applying a drop of 1% fluorescein solution to the lower inner lid, the ocular surface was examined by the slit lamp, using cobalt filter light. The examiner is looking for any staining areas on the conjunctival or corneal surfaces. A de-epithelialised stained area gives greenish colour.

Break-Up Time (BUT)- Break-up time is the period between a blink and the appearance of the first break in the tear film. Each volunteer was asked to blink several times before keeping their eyes open for at least thirty seconds. With a small width of normal slit light in a slit lamp, the examiner is moving the slitlight from one side to another, looking for the early break in the tear film and record its time. The procedure was repeated twice in each eye. A mean was taken for each eye.

Schirmer's Test Under Local Anaesthesia- One drop of (Benoxinate) eye drops was instilled into the conjunctival sac. After five minutes, a strip of Schirmer paper was inserted over the lower eyelid of each eye at the junction of the outer and the middle thirds, for five minutes. The volunteer was asked to close his or her eyes for the period of the test. The length of the wetting segment of the strip was measured and recorded for each eye.

Impression Cytology- For each volunteer, four different types of filter paper surface were applied to one of each of four sampling sites. These are the nasal and temporal sides of the bulbar conjunctiva of both eyes. The four filter paper surfaces are the shiny dry surface, the shiny wet (pretreated) surface, the dull dry surface and the dull wet surface. The type of filter paper surface was assigned at random, using tables at random numbers and standard methods. Each volunteer was assigned a random number.

The results were analysed using " Friedman method of non-parametric analysis of variance for randomised blocks" by Colquhoun (1971).

5-4 Results

Two of the volunteers gave a history of **foreign body sensation**. Three volunteers had history of **burning sensation** and a same number of volunteers had **itchy sensation**. All the volunteers had normal **tear meniscus** and none had any **fluorescein staining of the cornea.**

As far as the **Break-up time of the tear film**, eight of the volunteers had tear film break-up more than 25 seconds in both eyes. Three volunteers had their break-up tear film between 16-25 seconds in both eyes. One volunteer had a break-up of the tear film between 6-15 seconds in one eye and between 16-25 seconds in the other eye. Another volunteer had a break-up of the tear film 6-15 seconds in one eye and more than 25 seconds in the other eye (Table 8).

The **Schirmer's test** results showed that three volunteers had more than 25 mm/5 min. in both eyes while four volunteers had

Volunteer	Break-Up Time		Schirmer Test		Impression Cytology	
	Right	Left	Right	Left	Epith. Cells	Goblet Cells
1	3	3	3	3	2	2
2	3	3	3	3	1	1
3	3	3	0	0	1	1
4	3	3	3	3	1	2
5	-	-	-	-	1	2
6	2	2	0	1	1	0
7	1	2	0	1	1	2
8	3	3	0	1	-	-
9	2	2	2	2	1	2
10	3	1	1	0	1	2
11	3	2	2	2	1	2
12	3	3	1	1	1	1
13	3	3	1	3	2	0
14	3	3	2	2	1	1
15	2	2	2	2	1	2

Table 8. The relationship of Break-Up Time, Schirmer's Test and Impression Cytology.

Code for the Break-Up Time

0 = < 5 sec.
1 = 6-15 sec.
2 = 16-25
3 = >25

Code for the Schirmer's Test

0 = < 5 mm
1 = 6-15 mm
2 = 16-25 mm
3 = >25 mm

Code for the Impression Cytology yield for epithelial and goblet cells

0 = absent
1 = minimum yield
2 = moderate yield
3 = good yield

16-25 mm/5 min in both eyes. One volunteer's wetting of the filter paper was between 6-15mm/ 5min. and another volunteer wetting of the filter paper was below 5mm/ 5min. Four volunteers' score in one eye was below 5mm /5min and in the other eye between 6-15mm /5min. Only one person had wetted the filter paper in one eye >25mm /5 min. and 6-15mm/ 5min. in the other eye (Table 8).

There was a correlation in the results of Schirmer's test and break-up time in only seven out of fifteen volunteers (47%). In the other non-correlated eight cases, the break-up time gave a normal result ie 16-25 seconds or better whilst the Schirmer's test results gave wider variations. There was very small difference in the results between the right and the left eyes (table 8).

By analysing the results of the break-up time, the Schirmer's test and the dull wet filter paper for the overall yield by the "Spearman rank correlation coefficient", there was no significant statistical correlation ($p>0.1$).

5-4-1 Results of the impression cytology

The filter papers were examined under light microscopy. Filter papers were assessed according to the overall yield of cells, the amount of mucus, the yield of epithelial and goblet cells.

The histological appearance of the epithelial cells is polygonal in shape. The nucleus occupy most of the cell in a ratio of 1:1 or 1: 2 (Figure 7). They may appear in sheets, small group of cells and single cells (Figure 8). The mucus may appear as indistinct lines separetly (Figure 9) or combined with epithelial cells (Figure 10). The goblet cells are normally grouped in one part of the

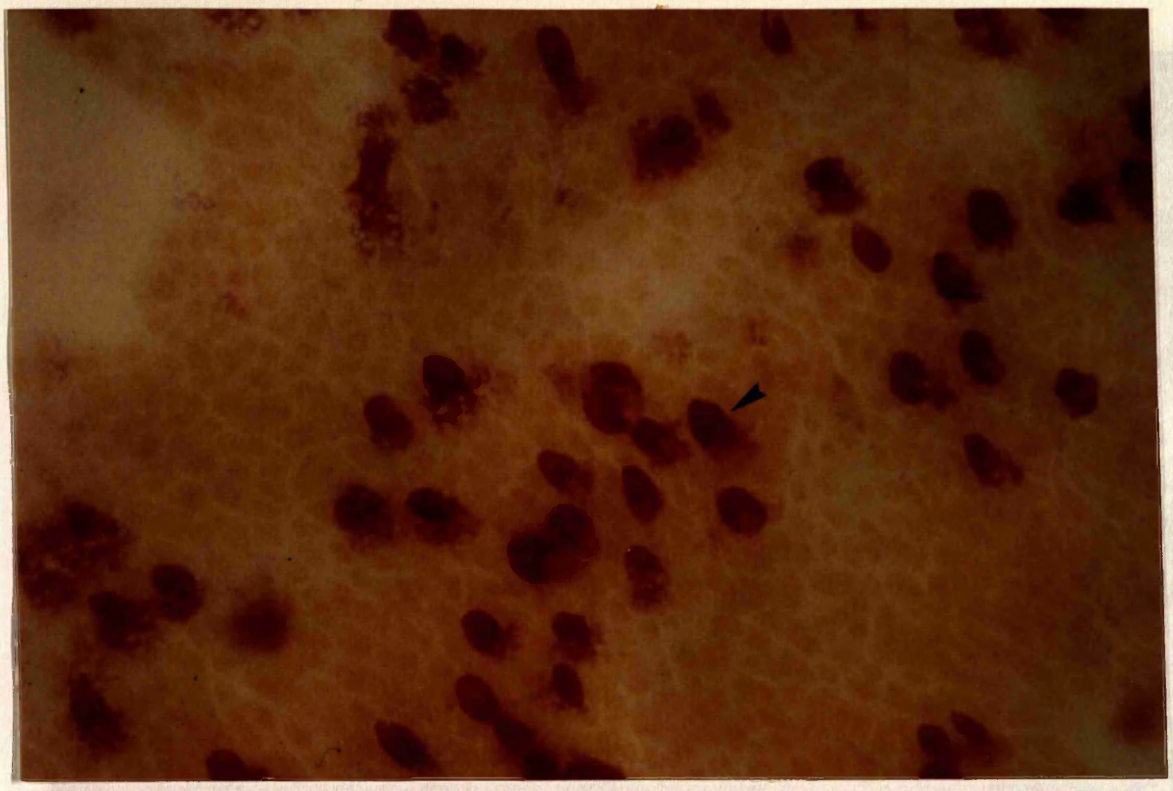


Fig. 7_ Normal conjunctival epithelial cells in Gill's Haematoxylin stain. Goblet cells are clearly seen (arrow)

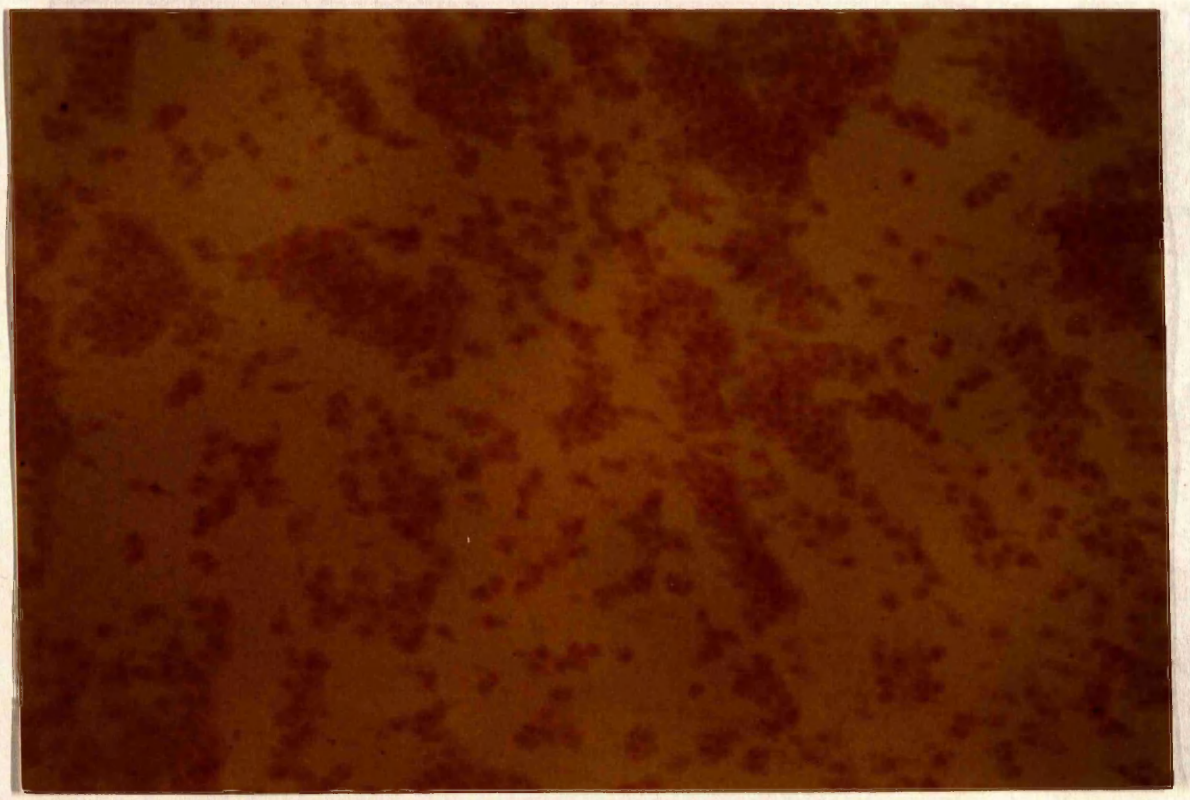


Fig.8 _ Epithelial cell as sheets and group of cells.

Fig. 9_ Mucus as indistinct lines on a filter paper.

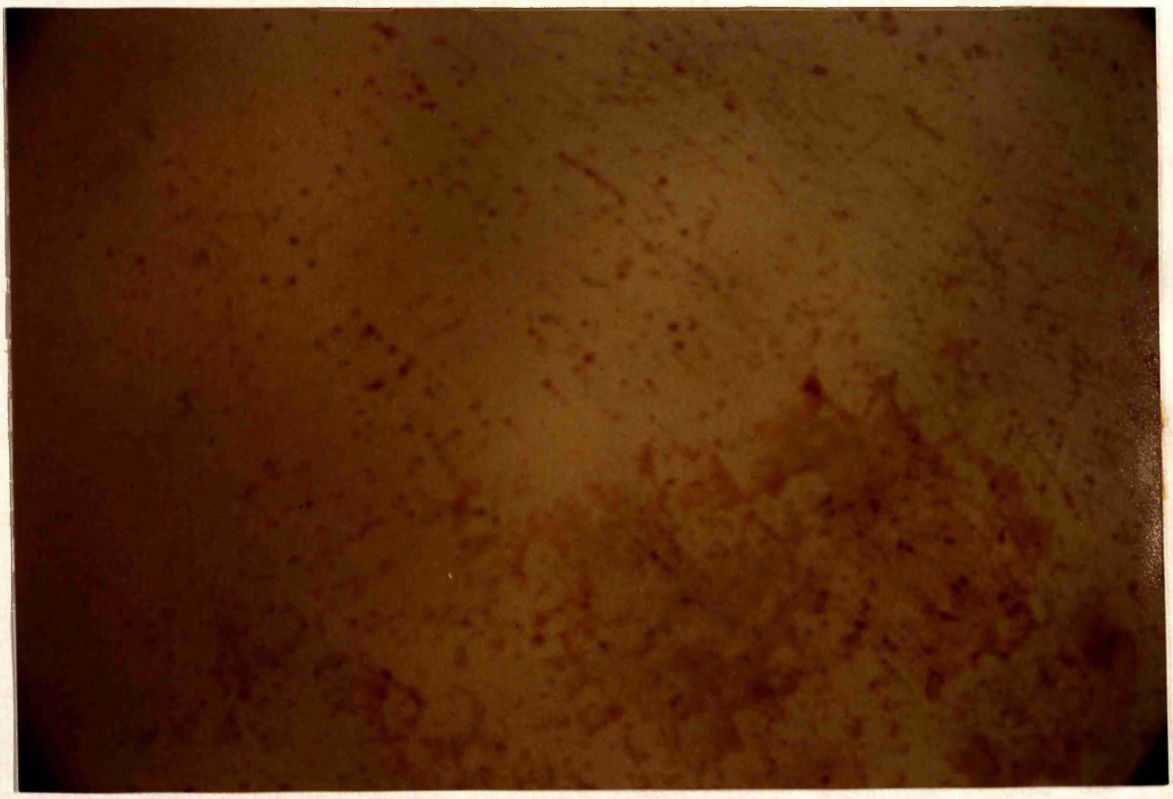


Fig. 10_ Mucus, epithelial and goblet cells.

filter paper and rarely seen as single cells (Figure 11).

The results of the overall yield for the different filter papers are shown in figure 12. The dull surface filter paper gave a better overall yield of cells than the shiny surfaced filter paper ($0.01 < p > 0.05$ for dry filter paper: $p<0.01$ for wet filter paper). The results were statistically significant. However there was no statistical difference between filter papers with and without surfactant ($p>0.05$ for both dull and smooth surfaces).

The results of the mucus yield for the different filter paper strips are shown in figure 13. No apparent statistical significance was found between the different filter paper surfaces ($p<0.5$). There was no significant difference between the pretreated and the untreated surfaces.

The results for the goblet cell yield in the different filter paper strips are shown in figure 14. The pretreated and untreated surfaces of the dull filter paper have better yield than the shiny surfaces. This is statistically significant ($p<0.02$).

The results for the epithelial cell yield are shown in figure 15. It showed no significant difference between the different filter paper surfaces ($p<0.5$).

5-5 Conclusion

Impression cytology is an easy and simple technique which does not require sophisticated equipment. It takes less than an hour to complete the staining procedure. The interpretation of the results are made easy by the use of Gill's haematoxylin which facilitates the recognition of the epithelial and goblet cells. In this experiment,

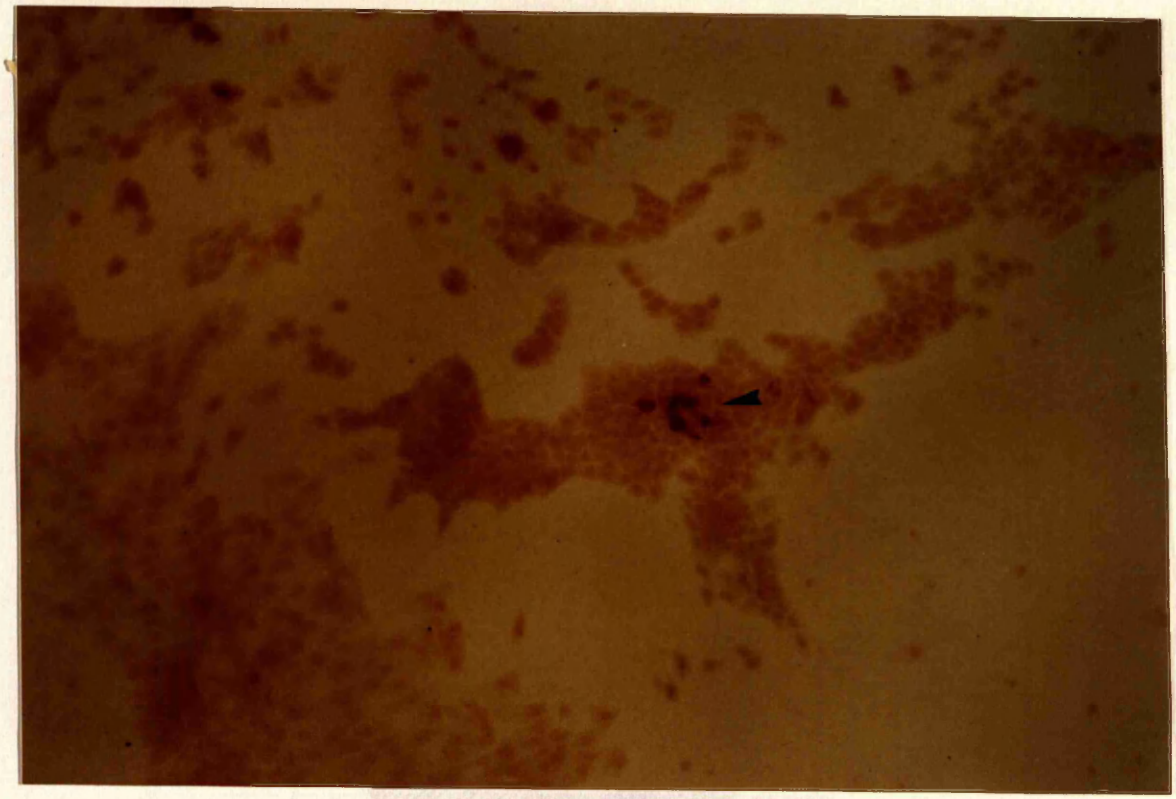


Fig. 11. Groups of goblet cells (arrow).

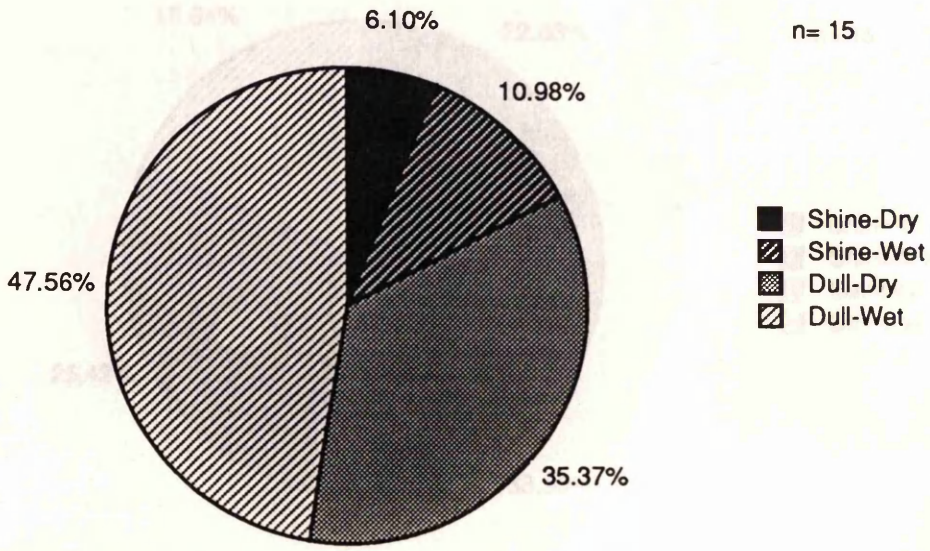


Figure 12 - Overall Yield in the Four Filter Papers

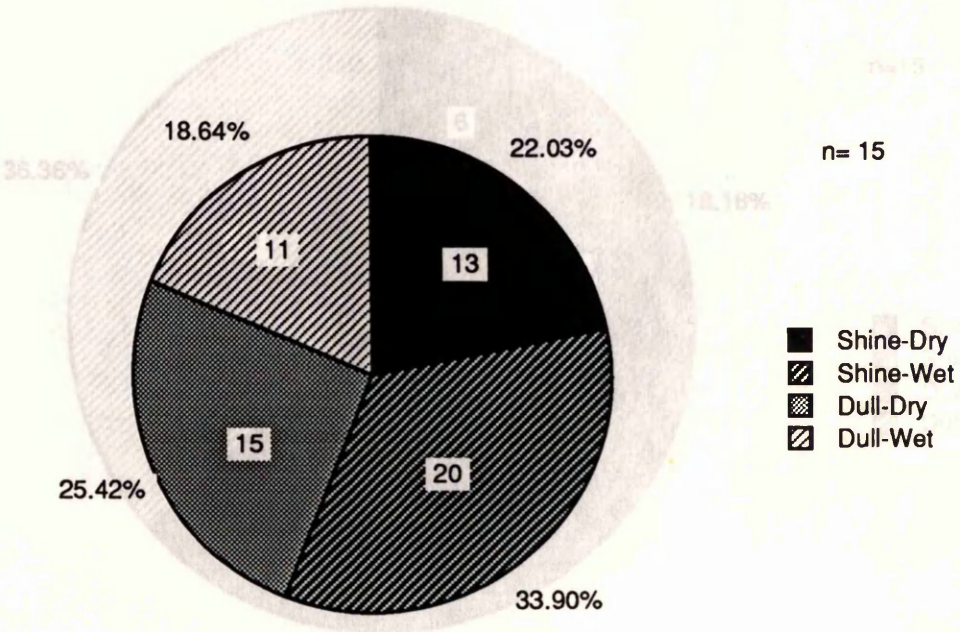


Figure 13. Mucus Yield in the Four Filter Papers

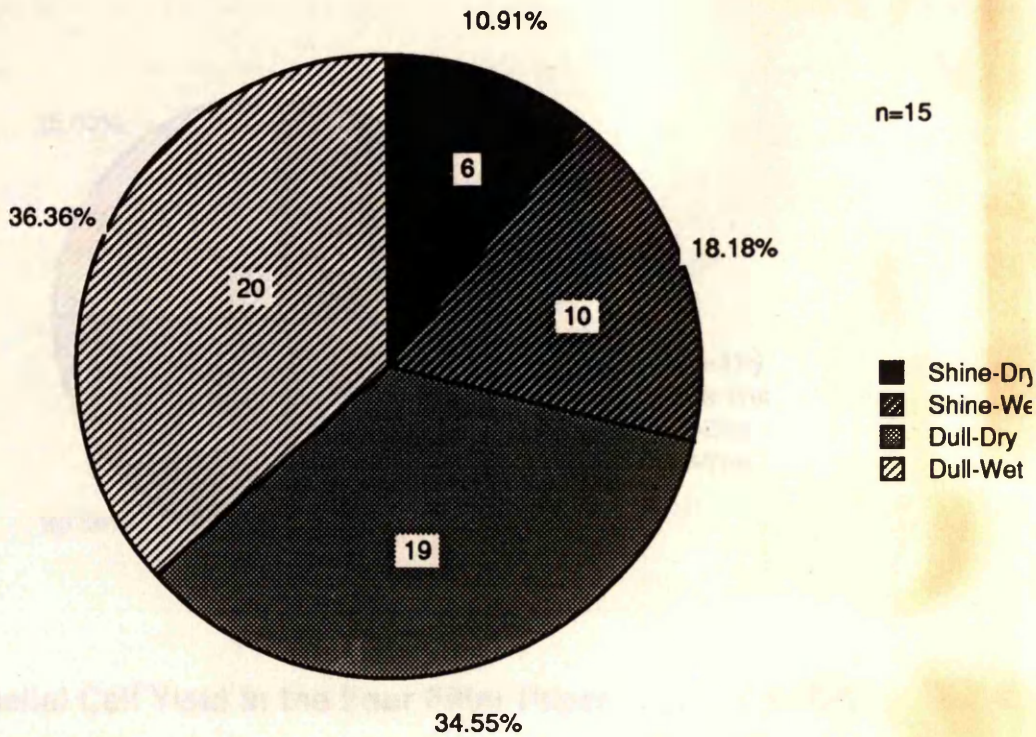
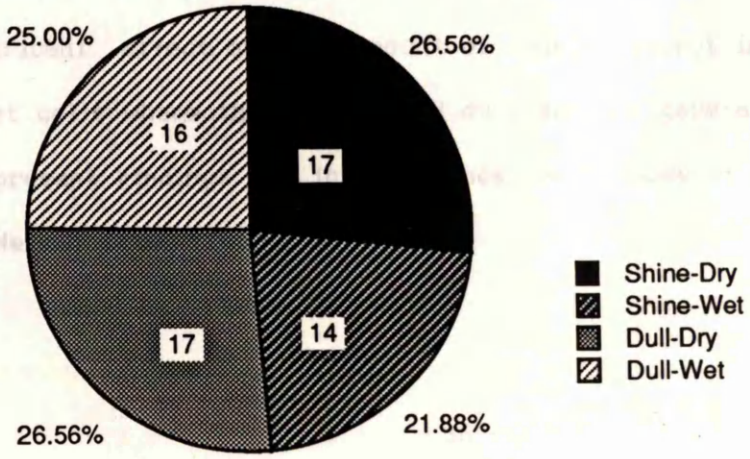


Figure 14. Goblet Cell Yield of the Four Filter Paper



gure 15- Epithelial Cell Yield in the Four Filter Paper quadrants

impression cytology has been employed to asses the relative diagnostic utility of the different surfaces of the filter paper. It is evident from this experiment that the dull surface is more effective in obtaining a higher yield of conjunctival cells. The presence of surfactant in the filter paper does not seem to have any significant effect on the amount of yield except in the case of goblet cells where the pretreated dull surface gave a better yield. The present findings are in accordance with those of Adams (1979) and Nelson et al (1983).

CHAPTER SIX

Topical Retinol Acetate in the Treatment of Sjögren's Syndrome

6-1 Introduction

The management of dry eye disorders is often unsatisfactory. Treatment of dry eyes has been aimed at relieving the symptoms by using topical artificial tears preparations and in severe cases by occluding the puncta to preserve the remaining tears in the conjunctival cul de sac.

Dry eye disorders may give rise to squamous metaplasia of the corneal and conjunctival epithelia. The histopathological study of these surface disorders shows that the substantia propria usually have an intense inflammation in the acute stage and dense scarring in the chronic stage. The conjunctival epithelium exhibits an early loss of goblet cells. A progression of this process leads to an increase in the epithelial cell layers and ends in keratinisation. The two underlying pathological processes in the differentiation of the conjunctival epithelium, the loss of vascularisation and the intense inflammation, have been identified and correlated with the loss of goblet cells, although the exact inter- relationship between the two processes remains unknown. (Tseng et al, 1984).

Topical vitamin A was used half a century ago in Hungary and Russia for the treatment of different dry eye conditions and good results were reported (De Grosz, 1939). During the present decade, several reports have shown marked improvement in the clinical

manifestations and the histological appearances of the conjunctiva following the use of topical all - trans retinoic acid within two months of starting the treatment (Tseng et al, 1985; Wright, 1985b). These two reports were not double masked and the number of patients with each individual dry eye condition was small.

This is a double masked, placebo - controlled trial which was set up to assess the patient's dry eye condition before, during and at the end of a six week period by documenting and analysing subjectively and objectively the clinical manifestations with a histological study of the conjunctival epithelium using impression cytology technique. This trial assesses a commercially available vitamin A product (Retinol Acetate 10 000 IU/Gm eye ointment) for the treatment of biopsy proven Sjögren's syndrome patients who demonstrated definite clinical manifestations of dry eyes and who had not been relieved by their current treatment.

6-2 Materials

All the materials used for the impression cytology, the fluorescein staining and the Schirmer's tests are described in section 5-2 and Appendix I.

6-2-1 Retinol Acetate 10 000 IU/Gm eye ointment

The retinol acetate ointment which was used in this trial, has been available commercially in Europe and the Middle East for many years. It was specially prepared and supplied by ' Dispersa Pharmaceutical Company, Switzerland' for this trial. The eye ointment comprises Retinol acetate, Cetyl alcohol, Adeps lanae, Liquid paraffin

and white petrolatum. The ointment is a clear preparation, packed in unlabelled tubes. An equal number of tubes was labelled "R" for the right eye and "L" for the left eye.

6-2-2 Vehicle containing Eye Ointment

A tube similar in size and shape to the one containing the Retinol Acetate was specially prepared and supplied by Dispersa Pharmaceutical Company (Switzerland) for this trial. It contained the vehicle without the retinol acetate component. The ointment was also clear. The tubes were marked by the company with a red spot. Equal numbers of tubes were labelled "R" for the right eye and "L" for the left eye. These labels covered the red spot on the tube.

An equal number of tubes marked "R" and "L" of a retinol acetate tube and a vehicle containing tube were paired together into a bag. A pair of tubes was picked randomly for each patient. This ensured that an equal number of the two types of tubes was distributed randomly and equally to the right and left eye without the examiner or the patient knowing the content of the tubes.

6-3 Method

A number of patients with Sjögren's syndrome who have been attending the Oral Medicine Department at The Dental Hospital, Glasgow under the care of Dr. P-J Lamey, were invited to join the trial. The diagnostic criteria for selecting the volunteers were based on the following. A biopsy from the accessory salivary glands showing degenerative changes of the acini. Also signs of dry eyes which include grittiness, foreign body sensation, itchy sensation and

burning sensation. Twenty three from twenty five patients who had definite clinical manifestations of dry eyes were accepted for the trial. Their ages ranged between 31 and 80 years with a mean age of 59.6 years. There was one male in the group (4%) and the rest were female (96%). None of the patients wore contact lenses. None of the females of child - bearing age was pregnant or contemplating pregnancy during the period of the trial. All the patients were asked to continue with their present topical artificial eye preparations.

Each patient was examined four times over a six week period, at two week intervals. On the first visit, an explanatory introduction of the aim of the trial, the different clinical tests and the use of topical retinol acetate in the treatment of dry eyes with reference to the known side effects were discussed. Once the patient agreed to the trial, he or she was asked to sign the consent form approved by the Ethical Committee at the Western Infirmary, Glasgow. Then a full history was taken which includes general health, history of dry eyes and mouth and its duration and the history of arthritis if present, and its duration. An assessment of the corrected visual acuity for distance and reading was recorded. This was followed by several clinical tests to assess the degree of dryness in both eyes. These tests comprised the Tear Meniscus test, the Fluorescein Staining test, the Break - Up Time and the Schirmer's Test. They are described in sections 5-3-1 to 5-3-4. The chronological order of these tests as outlined in table 9, was adhered to at every visit. The scoring code for the tests is listed in table 9.

Name No.

D.O.B.

HISTORY Systemic diseases

- of Arthritis and its duration
- Dry mouth and its duration
- Dry Eye and its duration

	<once/week	once/week	once/day	>once/day
1- F.B. Sensation	(0)	(1)	(2)	(3)
2- Burning Sensation	(0)	(1)	(2)	(3)
3- Itchy Sensation	(0)	(1)	(2)	(3)

4- Photophobia

Absent	Bright light	Day light	Room light	Any Light
(0)	(1)	(2)	(3)	(4)

5- Drugs- Systemic
Local

	None	od-tid	qid-3hr	2hr-1hr	<1hr
Artificial tear eye drops	(0)	(1)	(2)	(3)	(4)

CLINICAL MANIFESTATIONS

1- Visual Acuity Distance Right Left
(with glasses) Near Right Left

2- Tear Meniscus Absent (0) Present (1)

3- Fluorescein Stain

Absent	Staining of coni.	Staining of ¼ cornea	Staining of ½ cornea	Staining > ½ cornea
(0)	(1)	(2)	(3)	(4)

4- Break-up Time

<5 sec	5-15 sec	16-25 sec	>25 sec
(0)	(1)	(2)	(3)

5- Rose Bengal

Absent	Staining of coni.	Staining of ¼ cornea	Staining of ½ cornea	Staining > ½ cornea
(0)	(1)	(2)	(3)	(4)

6- Schirmer Test (using Benoxinate 0.4% minims)

<5 mm	5-15 mm	16-25 mm	>25 mm
(3)	(2)	(1)	(0)

Table 9. Clinical symptoms and tests that each patient underwent at each visit with their scoring code.

6-3-1 Rose Bengal Test

1% Rose Bengal dye stains the degenerative and dead epithelial cells. One drop of Rose Bengal was instilled into the conjunctival sac. The extent of staining on the ocular surface was recorded according to the scoring code as shown in table 9.

6-3-2 Impression Cytology

The impression cytology test was performed after the clinical tests on the first and the last visits. Two sites in each eye were chosen. These are the temporal and nasal bulbar conjunctiva of each eye about 4mm from the limbus and coded with the letters A,B,C, and D. For each patient, the four applicators were marked on the plastic strips with a number and for each of the four strips with a letter indicating the site of the impression cytology. Then the applicators were put into fixative solution immediately after the impression cytology. The same staining method used in chapter five and described by Tseng (1985) has been adopted in this test. It is a combination of periodic acid Schiff's reagent (PAS) and Gill's modified Papanicolaou stain. PAS preserves the original staining features of the goblet cells and the mucus patterns. Gill's modified Papanicolaou stain distinguishes the epithelial cellular differentiation by providing pictures of clear cytoplasm with differential colours for non, partial and full keratinisation with distinct nuclear chromatin pattern for nuclear degenerative changes. Batches of applicators were stained and analysed at the same time and therefore no individual case was examined separately. The classification and coding of the results are outlined

Code	Goblet Cells	(Nucleus/Cyto.)	Changes in Nucleus	Other Changes
0	abundant	(1/1)		
1	present	(1/2 -1/3)		
2	absent	(1/4)		
3	absent	(1/6)	Pyknotic changes	Visible keratin filaments
4	absent	(1/8)	Pyknotic changes	Dense keratin filaments + Keratohyaline granules
5	absent	Shrunken cytoplasm	Pyknotic changes + Pyknolytic changes	

Table 10 _ Impression cytology test. Classification of the histological grades of the epithelial cells and its coding.

in table 10.

At the end of the first visit, two tubes, one tube containing 0.1% retinol acetate and the other containing the vehicle only were given to the patient. The patient was instructed on how to apply the ointment into the conjuctival sac. Twenty patients were instructed to use the ointment twice a day at regular intervals while three patients (one patient in group II and two patients in group I) used the ointment once a day as their symptoms were not severe. The retinol acetate and the placebo eye ointments were allocated randomly to each eye.

The patients were asked to contact the hospital during the six week period if they developed irritation, discomfort or if they have any query regarding the treatment. A letter to the patient's general practitioner was sent after the first visit, informing him of the patient's acceptance in the trial, the aim of the trial and of the known side effects of topical vitamin A.

On the following visits, each patient was asked first specifically if he or she noticed any symptomatic changes in their eyes or general condition including the dry mouth and arthritis if present. The patient gave the degree of severity of their symptoms as detailed in table 9 from number 1- 4. The frequency of using the artificial eye drops and the assessment of the visual acuity were recorded. Then the clinical tests were done in the same order as shown in table 9.

On the last visit, the impression cytology test was the concluding test. A new code number was given to the 4 applicators used for each patient. After leaving the applicators in fixative

solution for at least ten minutes, the applicators were stained and examined under light microscopy as described in section 5-3-5. The descriptive stages of the degeneratng epithelial cells in dry eyes as suggested by Tseng (1985) and their scoring codes are detailed in table 10. The statistical analysis of the results of each test were performed using the t-test while the impression cytology results were tested by means of the Wilcoxon signed - rank test for the non-parametric and paired test.

6-4 Results

The patients' results were divided and analysed into three groups. Group I comprises of 13 patients who finished the trial. Group II consists of 3 patients (13%) who failed to attend after the first visit. Group III comprised of 7 patients who developed pain and redness following the use of the ointments.

Group I consisted of 13 patients (57%) who attended the whole trial and did not develop allergic reactions. All the patients were female with a mean age of 58 years. Sjögren's syndrome had been diagnosed between one and 18 years earlier. Arthritis was present in 12 patients in this group (92%). All the patients have had the symptoms of dry mouth for a minimum period of 2 years and a maximum period of 20 years. The symptoms of dry eyes had been present in all the patients for at least one year and a maximum period of 12 years. Of the 13 patients in this group, 3 patients have had systemic lupus erythematosus and one patient had Raynaud's phenomena, skin dryness and itchy sensation.

In group I, the results of each symptom were compared,

over the four visits, between the groups of eyes receiving retinol acetate eye ointment and the control treated eyes.

Retinol acetate 10 000 IU/ Gm eye ointment has shown a statistically significant improvement in relieving the symptoms of foreign body sensation ($p< 0.031$) and of photophobia ($p< 0.048$) over the placebo treated eyes. The results of the clinical tests and the impression cytology showed no statistically significant improvement in these dry eyes between the retinol acetate and the control treated eyes (table 11).

Group III consisted of 7 patients (30%). All the patients in this group developed pain (100%) whilst only four developed redness (57%). The pain and redness were present in both eyes and occurred after using the eye ointments. The symptoms were mild to moderate and they disappeared within 48 hours after discontinuing the ointments. Generally the onset of the symptoms occured, in the majority of cases (70%) in the first week whilst the remaining 2 cases (30%) appeared after one month. The results are listed in table 12.

Five patients developed **pain** within a week of starting the eye ointments while the other two patients developed the pain after one month. Although the pain and discomfort were in both eyes, the eyes treated with topical retinol acetate were more painful in four of the seven patients. One patient had more pain in the control eye than in the retinol acetate treated eye and two patients had the pain equally in both eyes.

Four of the seven patients developed **redness** in both eyes.

Patients No.	Time stopped Treatment (days)	Degree of Pain with		Redness with	
		Vit.A	Control	Vit.A	Control
17	30	++	+	present	None
18	6	++	+	All the eye	Nasal side only
19	35	+	+	None	None
20	7	++	+	None	None
21	4	++	+	++	+
22	7	+	+	+	+
21	4	+	++	None	None

Table 11. Group III patients' analysis of the degree of pain and redness

++ = more pain than the other eye
+ = less pain than the other eye

	p- value
1- F.B. Sensation	0.031 (significant)
2- Burning Sensation	0.39
3- Itchy Sensation	0.17
4- Photophobia	0.048 (significant)
5- Artificial Eye Drops	0.11
6- Tear Meniscus	0.34
7- Fluorescein Staining	0.88
8- B.U.T.	0.79
9- Rose Bengal Stain	0.98
10- Schirmer's Test	0.37
11- Impression Cytology	0.059

Table 12. The results of the patients in group III showing the p-value of each of the symtoms, the clinical tests and impression cytology over the four visits.

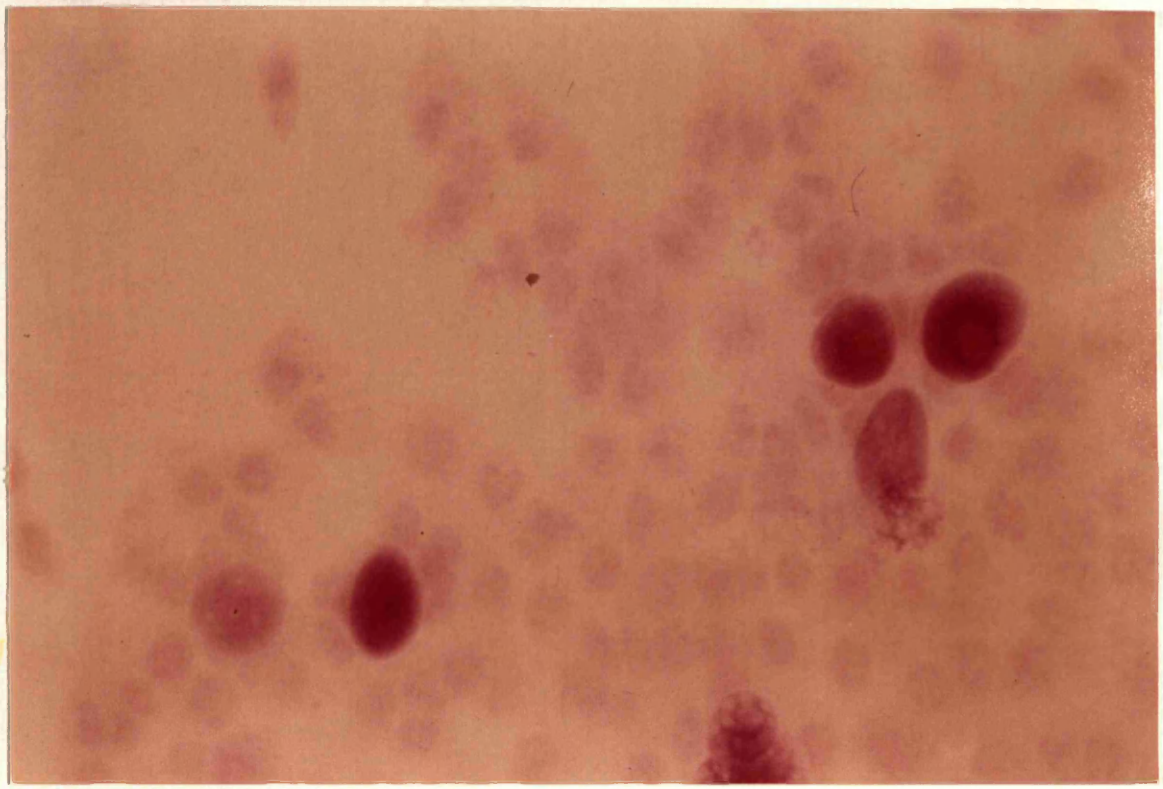


Fig. 16 _ Group of normal epithelial cells.

The redness was more pronounced in the eyes treated with topical retinol acetate than the control eyes.

6-4-1 The results of Impression Cytology

The filter papers were examined under light microscopy. A general view showed a high variation in the size and shape of cells from small normal groups of cells (Fig 16) to separate irregular single cells (Fig 17). The cells were larger than normal with a nucleus / cytoplasm ratio between 1: 2 and 1: 5 (Fig 18). The shape of the cells was irregular (Fig 19 and 20). The goblet cells in general were few in number in some filter papers and completely absent from others. Mucus was present in few specimens. Some filter papers showed rounded group of cells which may indicate an active growth of the epithelial cells similar to the growth and spread of the epithelial cells in the cornea (Fig 21).

6-5 Discussion

The results in this trial have shown that topical retinol acetate 10 000 IU / Gm reduces the foreign body sensation and the photophobia in two third of the patients with Sjögren's syndrome and dry eye conditions. However, the results also show that retinol acetate has no significant improvement in the burning or itchy sensations. Furthermore it did not show any statistical clinical improvement on the tear meniscus, the tear film break - up time or the conjunctival epithelium as tested by fluorescein and rose bengal stainings. The impression cytology of the conjunctival epithelium showed no statistical significant change between the retinol acetate

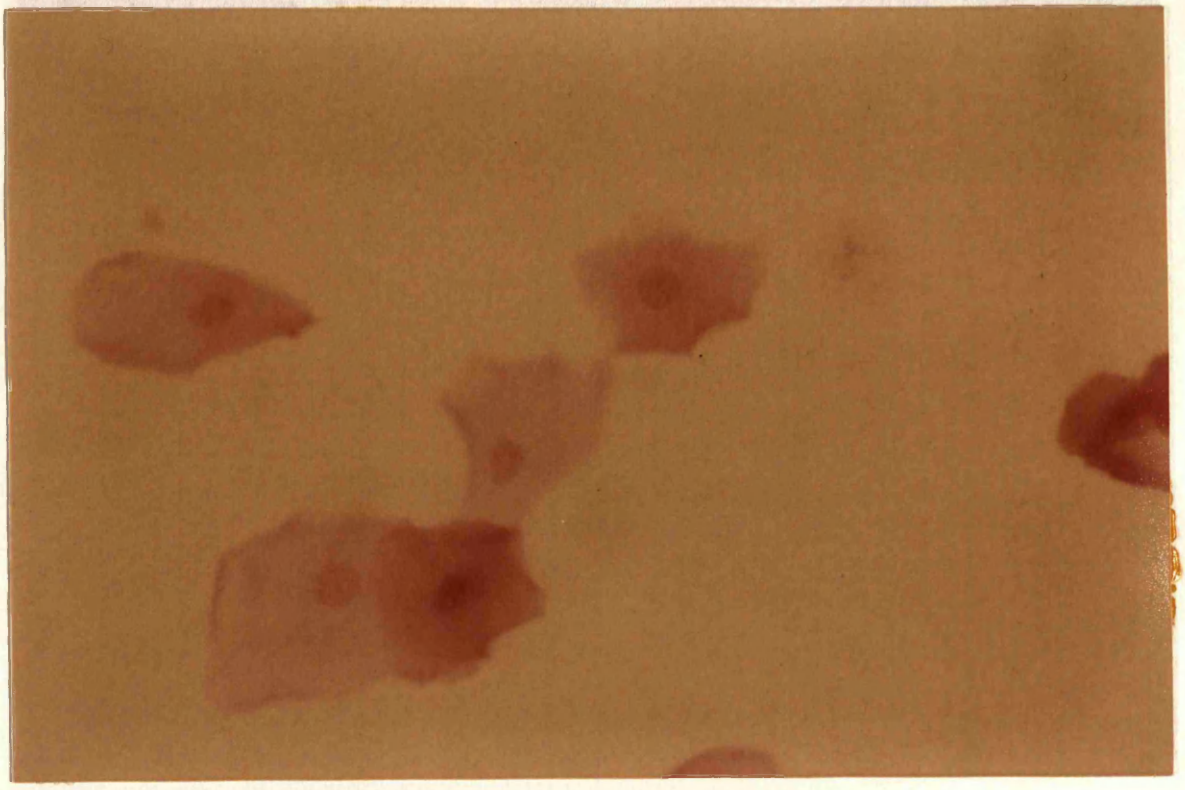


Fig. 17_ Single irregular large cells.

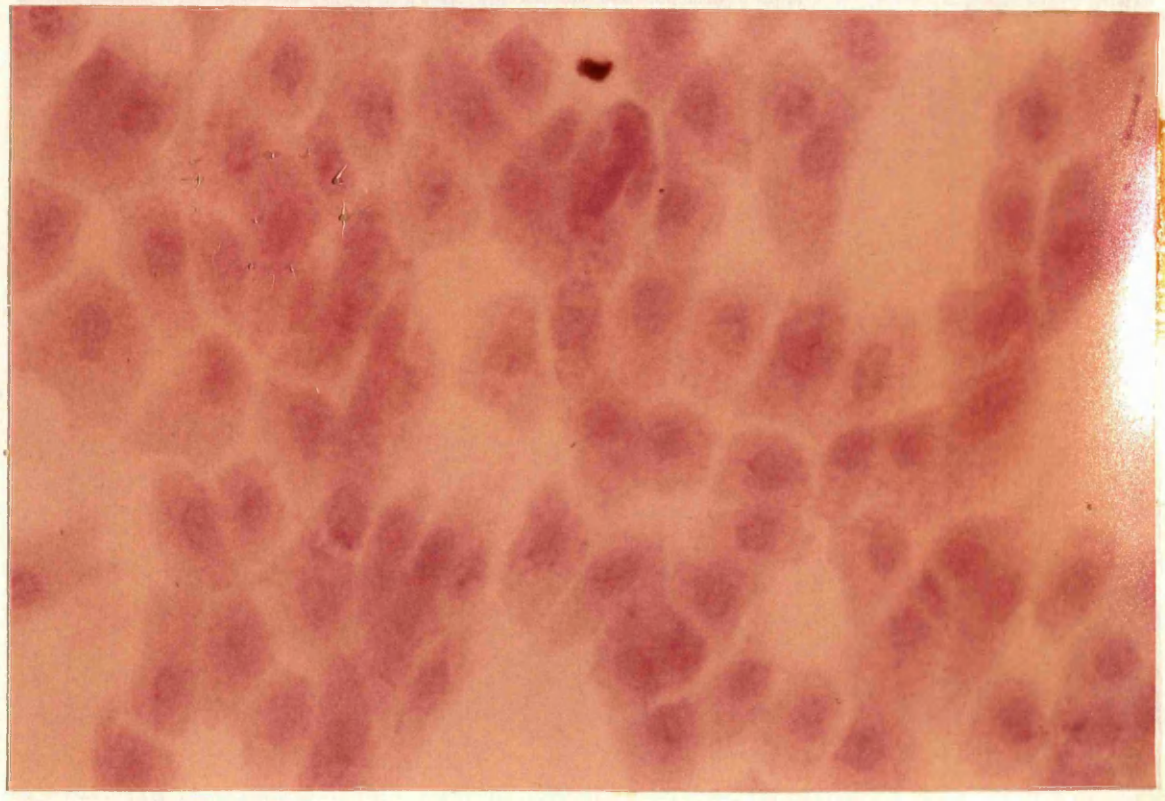


Fig. 18_ Large irregular epithelial cells.

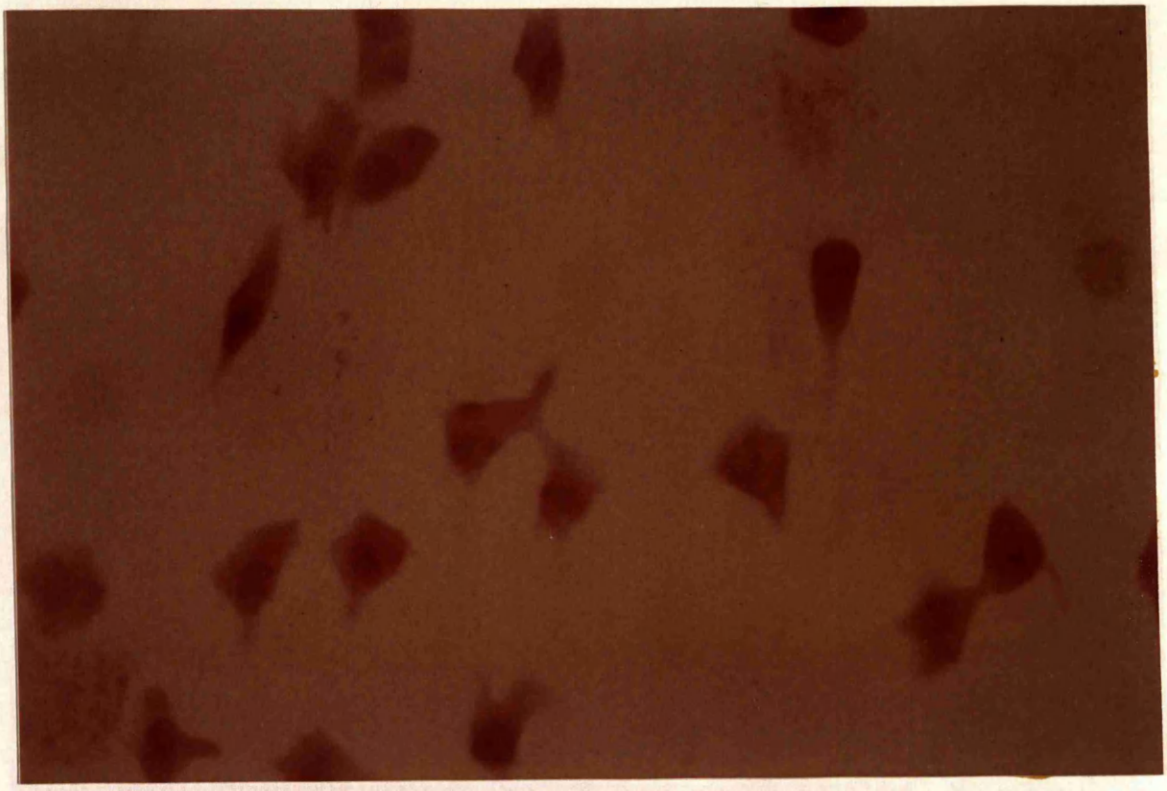


Fig. 19_ Degenerative irregular epithelial cells.

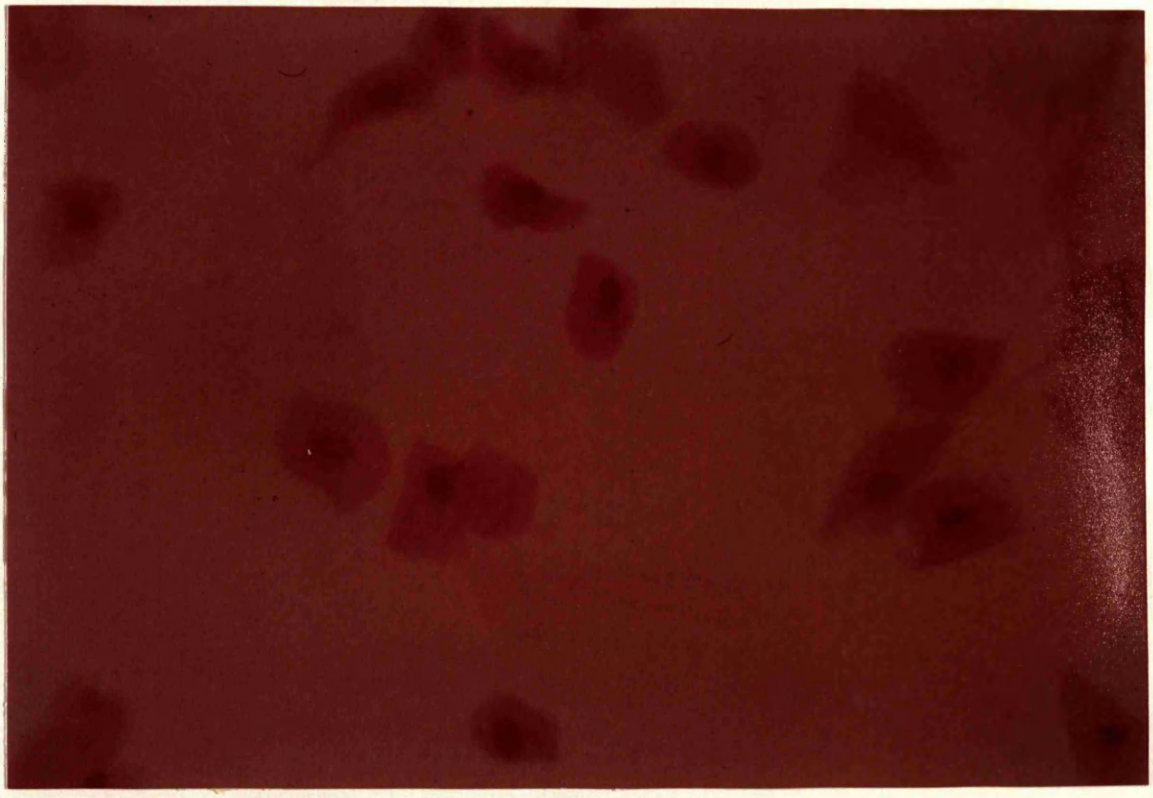
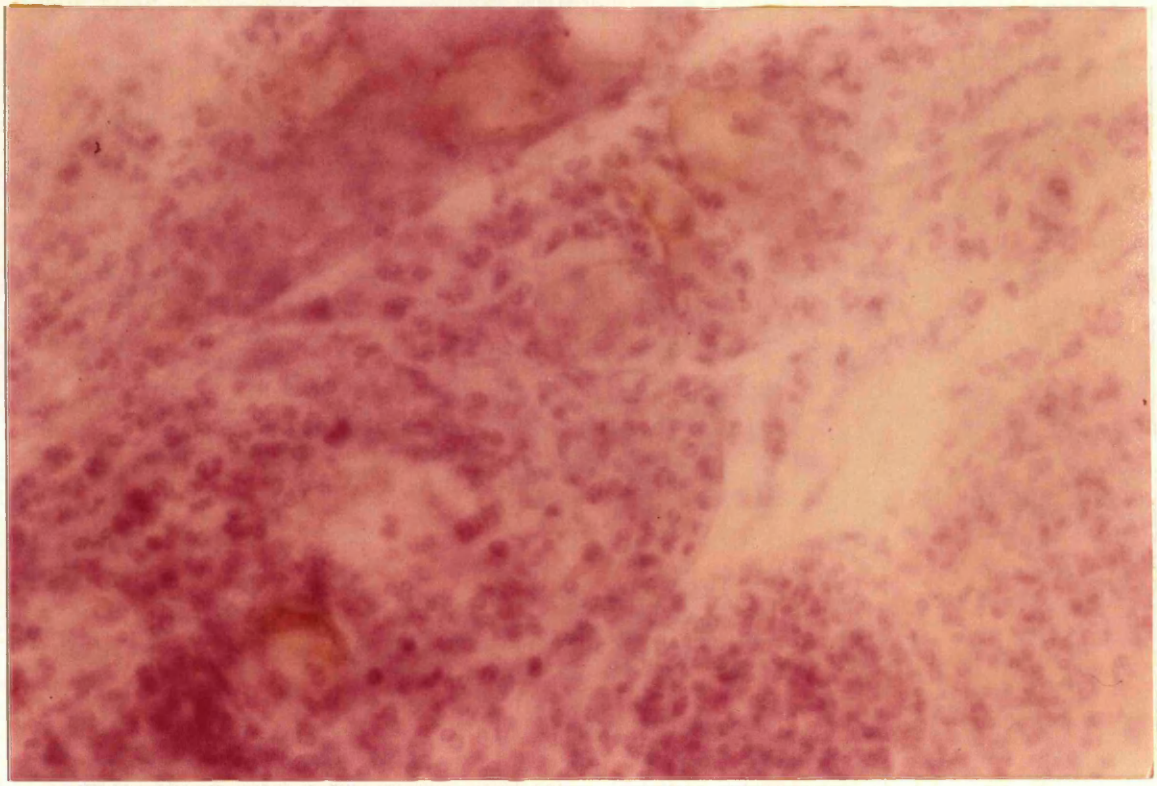


Fig. 20_ Large cells with high nucleus/ cytoplasm ratio.



Fig, 21 _ Mucus stain over the epithelial cells which were forming rounded groups.

and the control treated eyes, before and at the end of the 6 weeks treatment. These results are in accordance with the report by Soong et al (1988) in which an analysis of the adjusted mean changes of Kerato- conjunctivitis sicca patients showed no statistically significant difference between all-trans retinoic acid and the placebo treated eyes.

There was a high incidence of allergic or toxic reactions to the eye ointment (30%) manifested by pain, discomfort and redness in both eyes. In most cases, the symptoms were felt more in the retinol acetate treated eyes than in the control eyes. The pain and discomfort occurred in all the patients of this group whilst about half the cases developed redness. No explanation could be given for the high percentage of side effects. The redness in these cases was not confined to the lid margins as reported in cases who received toxic dosage. Also the twice daily applications were not considered excessive. Soong et al (1988) reported a double masked study, a 17% drug-related side effect of all-trans retinoic acid treated eyes and a 14.1 % adverse reaction in the placebo treated eyes.

The concentration of retinol acetate measured by international units is more accurate in assessing the efficiency of the concentration of the active vitamin A in a preparation compared to a preparation of vitamin A made as weight/weight which was used by Wright (1985b), Tseng (1985), Soong et al (1988) and others. These preparations did not reflect the effectiveness of vitamin A but only its content. Nevertheless in experimental xerophthalmia due to vitamin A deficiency, the effects of topical

retinol in 0.2% and 0.1% concentration and all-trans retinoic acid 0.2% have effective action on the reversal of corneal keratinisation. Moreover the action of all-trans retinoic acid has an effective reversal action on the following untreated eye, which topical retinol did not have (Hatchell, Faculjak and Kubicek, 1984).

In summary, topical retinol acetate produces few significant improvements in the symptoms of patients with Sjögren's syndrome and dry eyes. There was no demonstrable statistically significant data indicating an improvement in the tear film layer as tested by several clinical tests including the tear film break-up time, which has not been tested in previous trials. Moreover, this trial has shown no statistical improvement on the epithelial cells treated by retinol acetate ointment when tested by impression cytology.

Topical vitamin A preparations may be effective in treating severe keratinised ocular surfaces but they have not been proved to be effective in the treatment of mild or moderate dry eye conditions. However, a longer duration of treatment ie, more than six weeks, may give encouraging results.

CHAPTER SEVEN

Cell Culture of the Conjunctival Epithelium

7-1 Introduction

Cell culture has been established as an important laboratory technique. Its main advantage is the study of cell division within a controlled environment. The cell culture is relatively an easy procedure however the strict use of aseptic equipments in an aseptic environment by using Laminar Air Flow cabinet is important. The establishment of primary cell culture which is the first line cell division of host tissue, requires a suitable culture medium which needs the addition of serum, buffering solution, L-glutamine and antibiotics. A primary cell culture has a limited life span. The uses of cell culture in research and diagnostic procedures cover a wide variety of disciplines and is important in the understanding of cell behaviour in different conditions and stimuli.

The ability to isolate and grow conjunctival and corneal epithelial cells, without the presence of any connective tissue cells, has been a goal for many investigators. The present process of obtaining corneal epithelial cells is to remove most of the corneal stroma and then eliminate the remaining connective tissue cells during the cell culture procedure. Sun and Green (1970) inhibited the fibroblasts in the cell culture, either by planting the cells on an irradiated 3T3 cells or by the addition of 0.02% versene, once the

epithelial colonies grew to an appreciable size. Microdissection techniques for the preparation of epithelial cell, keratocytes and endothelial cells in corneal rabbits were first described by Stocker et al (1958) who identified the cells by light microscopy. The same technique was used by Cook, Aitken and Brown (1987) on rabbit cornea who concluded that the potential cellular contamination existed during the seperation of the epithelium from the underlying stroma. Baum et al (1979) using the same method, cultured human corneal endothelial cells. Gipson and Grill (1982) prepared an epithelial cell culture by using an enzymatic method.

In this chapter a series of cell culture tests is described. Two separate groups of experiments were attempted. The first group included several trials for culturing conjunctival epithelial cells which were obtained directly by using "Millipore filter paper". This direct approach ensured that known and specific layers of the conjunctival epithelium were obtained without the presence of unwanted fibroblasts in the cell culture. The second group of these experiments was to evaluate the effects of therapeutic and toxic concentrations of retinol acetate on conjuctival epithelial cells cultures.

7-2 Materials and Method

The experiments described below, have been carried out in a chronological order. Several unsuccessful attempts have been made to culture conjunctival epithelial cells in vivo. Each experiment was tried more than once in three or four petri- dishes at each attempt.

Applicators containing the "Millipore filter paper" were prepared as described in section 5-2, by using the dull- wet type of filter paper surface which showed its efficiency in obtaining the maximum number of conjunctival epithelial cells and the minimum amount of mucus.

The dishes employed for cell culture, are made of modified polystyrene substances. They are sterile, gamma irradiated and disposable. The surface of the dishes are flat for optimum cell growth. They also have an excellent optical clarity for examination and photography. The growth of the epithelial cells was photographed regularly through a phase- contrast inverted microscope using Kodak coloured slide film (Ektachrome 400).

All the photographs were taken at two magnifications, a low magnification of X 40 for generalised views and a high magnification of X 200 for the details of the cells. These magnifications give a uniform outlook to the continous changes in the cell growth.

Glasgow growth medium (table 13) was used in the early experiments. It has a wide general use in cell culture with good results however Dulbecco's growth medium (table 14) was found in the later experiments to be more suitable for the growth of conjunctival epithelial cells.

All the experimental work was done in a Laminar Air Flow Hood under aseptic conditions. The details of the different cell growth dishes, pipettes, needles, syringes etc, are listed in Appendix I.

1. Put 10 ml of 10× concentrated Glasgow Modified Eagle Medium in a Gibco sterile bottle.
2. Add 10 ml of Tryptose Phosphate Broth.
3. Add 50 ml of sterile water.
4. Add 3 ml of $NaHCO_3$ 7.5% to make the pH alkaline.
5. Add 1 ml of L-glutamine.
6. Add 1 ml of Antibiotic-Antimycotic solution.
7. Add NaOH drop by drop while shaking the bottle all the time until the colour of the solution change from pink to cherry red colour. This colour indicates slightly alkaline solution.
8. Add sterile water till the solution is 100 ml.
9. Add 10 ml of Foetal calf serum.

The solution was kept at 4°C.

Table 13- Preparation of Glasgow Modified growth medium

1. Put 10 ml of 10× concentrated Dulbecco's Modified Eagle Medium in a Gibco sterile bottle.
2. Add 10 ml of Tryptose Phosphate Broth.
3. Add 50 ml of sterile water.
4. Add 3 ml of $NaHCO_3$ 7.5% to make the pH alkaline.
5. Add 1 ml of L-glutamine.
6. Add 1 ml of Antibiotic-Antimycotic solution.
7. Add NaOH drop by drop while shaking the bottle all the time until the colour of the solution change from pink to cherry red colour. This colour indicates slightly alkaline solution.
8. Add sterile water till the solution is 100 ml.
9. Add 10 ml of Foetal calf serum.

The solution was kept at 4°C.

Table 14- Preparation of Dulbecco's Modified growth medium

7-2-1 Removal of conjunctival epithelial cells by Millipore filter paper

The conjunctival cells were removed by "Millipore filter paper" from the author's bulbar and palpebral conjunctiva. Throughout these experiments the following steps had been carried out first.

After instilling one drop of Guttae oxybuprocaine hydrochloride 0.4% in the conjunctival sac, a sterile Applicator was pressed gently on the bulbar or palpebral conjunctival surface for about 3-4 seconds. Then under aseptic conditions, the filter paper was removed immediately from the plastic part of the applicator by a sterile blade and placed in a petri-dish containing a growth medium.

7-3 Unsuccessful attempts of culturing conjunctival epithelial cells

Several unsuccessful attempts were made to achieve a first line cell culture of monolayered conjunctival epithelial cells, by using "Millipore filter paper" with the aid of growth medium in one experiment or the addition of collagen which encourages the cell growth in another experiment. In the third experiment, the trypsinizing solution was employed to detach the cells from the filter paper. None of the above experiments gave a satisfactory result.

7-3-1 The Culture of Epithelial Cells in Glasgow growth medium

After removing the filter paper from the plastic Applicator as described in 7-2-1, it was placed in 60mm petri-dish containing 3ml Glasgow growth medium. The filter paper surface containing the epithelial cells, was put downward in few dishes and upward in

others. The dishes were put in a humidified incubator at 37°C and 4% CO2 atmosphere.

Few epithelial cells appeared at the edges of the filter paper after only 3- 4 days (Fig 22). At the end of the second week, very few cells were seen floating in the growth medium but none was attached to the floor of the petri dish (Fig 23). On the third week, there was no increase in the number of cells at the edges of the filter paper or floating in the growth medium. On the fourth week the cells disappeared from the filter paper and the growth medium.

Conclusion - The few floating epithelial cells did not attach to the floor of the dish and consequently no cell division was possible.

7-3-2 The Culture of Epithelial Cells in Collagen and Glasgow growth medium

Collagen, as part of the extracellular matrix, encourages the epithelial cells to attach to them for their growth and proliferation (Gospodarowicz, Vlodavsky and Savion, 1980). The following experiments employed collagen for the growth of epithelial cells. These experiments used first a single then double layered collagen in 60mm dimension petri-dishes.

Preparation of Collagen Gels: Sterile collagen was kindly provided by the Cell Biology Department at Glasgow University. The stored sterile collagen at -20°C was brought to 4°C over 24 hours. In a universal glass container, one part of collagen solution was mixed to nine parts of 10× BHK21 Glasgow modified growth medium. To obtain a uniform mixture the universal glass container was put on ice and

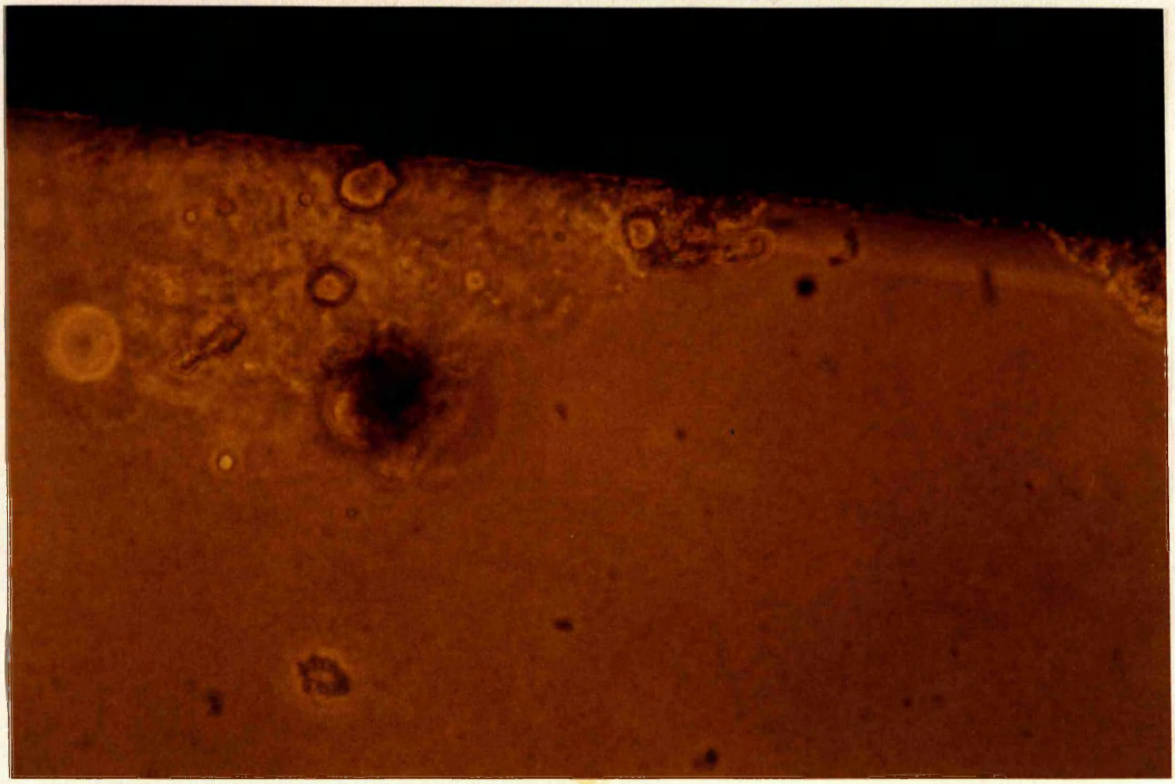


Fig. 22 _ After 3 days, few cells are seen at the edge of the filter paper. (Mag.X200)

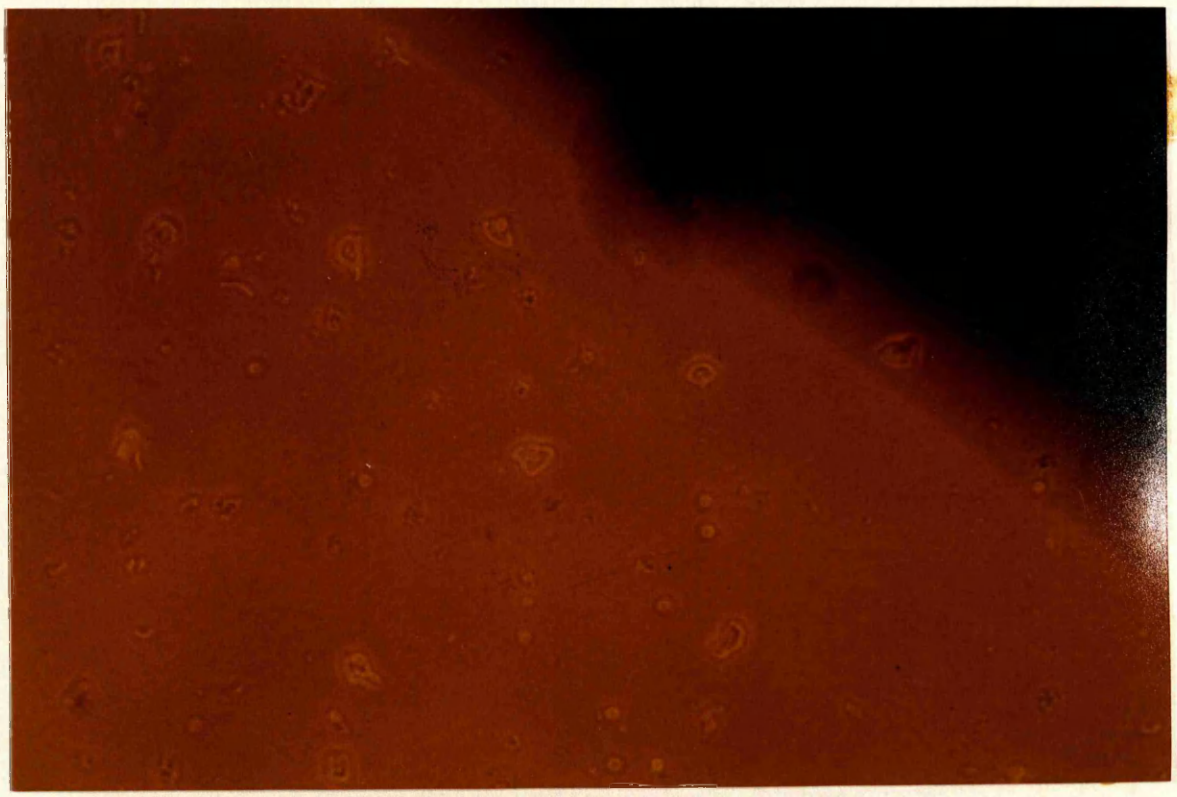


Fig. 23 _ After two weeks, few conjunctival epitheial cells are floating in the growth medium. (Mag. X200)

the mixture was stirred with a spatula. The pH was adjusted by adding NaOH, drop by drop until the colour of the mixture turned cherry red. The collagen gel was poured into a 60mm petri-dish and occupy about one third of the depth of the petri-dish.

Preparation of single layered collagen dishes - After pouring the collagen gel into 60mm petri-dishes, the "Millipore filter paper" was placed with the epithelial cells surface downward in some dishes and with the epithelial cells surface upward in other dishes. The dishes were left to set at 37°C for one hour before adding 3ml of Glasgow modified growth medium. Then the dishes were kept in an incubator at 37°C and 4% CO2 atmosphere.

Very few cells were seen at the edges of the filter papers in the first week and none was seen in the collagen gel. In the second and third weeks, the cells diappeared gradually from the edges of the filter papers.

Preparation of double layered collagen dishes The filter paper was sandwiched between two layers of collagen gel. The collagen surrounded the growing cells on the filter paper so the cells could attach to, proliferate and spread in the dish.

Procedure - A single layer of collagen was placed into a 60mm petri-dishes. Over the layer of collagen, a filter paper with the epithelial cell surface upward was placed in few dishes but other filter papers with the epithelial cell surfaces downward were placed in other petri-dishes. Then collagen gel layer was placed over each filter paper and left to set for about one hour before adding 3ml of

Glasgow modified growth medium. The dishes were kept in an incubator at 37°C and 4% CO2 atmosphere.

Very few cells were seen at the edges of the filter papers in the first week and none was seen on the collagen gel. The cells at the edges of the filter papers decreased in number gradually over the following two weeks.

Conclusion - The filter papers placed in single or double layered collagen dishes showed that the epithelial cells remained at the margin of the filter paper but did not attach to the collagen to multiply. No obvious advantage was seen in the use of collagen for the growth of the conjunctival epithelial cells in this test.

7-3-3 The Culture of the Epithelial Cells with Trypsinisation of Monolayered Specimens

The trypsinisation solution is a mixture of 0.05% (w/v) trypsin and 0.02% (w/v) EDTA. The mixture is available ready made or could be prepared by adding trypsin and EDTA. The trypsin, a protein enzyme, breaks down tissue into the constituent amino acids. The EDTA helps to detach the cells who are strongly adhered to the filter paper surface.

The epithelial cells have been growing on the filter paper as seen by the increasing number of cells at the edges of the filter paper in the first week. Nevertheless, very few cells were seen detached from the filter paper. The tryptinising solution was employed to remove the epithelial cells from the filter paper.

In order to assess the optimum time for the trypsinisation solution to detach the epithelial cells from the filter paper with

the minimum damage to the cells, six petri- dishes were prepared where the trypsinising solution was added to each dish at different periods of time.

The Dulbecco's Modified Eagle growth Medium was employed in the following procedures. It has the advantage that epithelial cells grow well in it. L-glutamine, foetal calf serum and Antibiotic-Antimycotic preparations were added. The Antibiotic - Antimycotic solution contains 10 000 units of Penicillin, 10 000 µg Streptomycin and Fungizone 25µg per ml. This preparation has a wide spectrum against bacteria, fungi and yeasts. The detailed preparation is described in Table 14.

Procedure - Six filter papers, containing conjunctival epithelial cells, were placed each in petri- dish containing 3ml Dulbecco's growth medium. The dishes were kept in an incubator at 37°C in 4% CO2 atmosphere for one week when cells at the edges of the filter paper were seen multiplying. Then each filter paper was put in a 60mm dimension petri-dishes. 1ml of trypsinizing solution was added for 0 sec., 1 sec., 2 sec., 3 sec., 5 sec. and 10 sec. respectively in the six dishes before the addition of 4 ml of Dulbecco's medium which neutralises the action of the trypsinizing solution (in the case of 0 second, the Dulbecco's medium was added with the trypsinizing solution). Then each filter paper surface was pipetted vigorously by a 1 ml sized plastic pasteur pipette, to detach the epithelial cells. The resulting cell suspensions were removed to another petri-dish. The dishes were kept in an incubator at 37°C and 4% CO2 atmosphere. All the dishes were examined for three weeks. The floating cells were multiplying in the first two

weeks but in the third week the cells were reduced in number gradually.

These results showed a reasonable number of epithelial cells however there was a lot of debris. The debris was caused by particles from the damaged filter paper and damaged cells. The number of normal cells were relatively higher in the first three dishes ie 0 sec, 1 sec and 2 sec. The highest amount of debris was found in the sixth dish when the neutralising solution was added after 10 seconds.

Conclusion - The experiment was encouraging in that a number of surviving cells were multiplying however the extent of debris was equally high.

7-4 The Culture of the Conjunctival Epithelial Cells

The following experiment used the above procedure of "pipetting" the filter paper without the addition of trypsinizing solution which produced excessive debri.

7-4-1 Procedure

Six sterile 60mm dimension petri- dishes were prepared. In each dish, there were 2ml Dulbecco's growth medium and two sterile filter papers containing conjunctival epithelial cells. The presence of two filter papers in eachn dish would double the number of cells that will multiply at the start of the experiment. The dishes were kept in a humidified incubator at 37°C and 4% CO2 atmosphere. During the first week, the epithelial cells increased in number as clearly seen at the edges of the filter papers (Fig 22). Then the filter

papers were pipetted vigorously several times with the aid of 1 ml sized plastic pasteur pipette, to detach the epithelial cells. The suspension formed from each dish, was transferred to a 35.7mm well in a 24 well multi-dishes. The small sized wells proved to be more efficient for the growth of conjunctival epithelial cells compared to the 60mm dimension petri dishes. The multi-dishes were kept in a humidified incubator at 37°C and 4% CO2 atmosphere. On the following days, the epithelial cells were seen multiplying fast with the appearance of mitotic cells (Fig 24). The increase in the growth of the epithelial cells in the first two weeks led to the formation of a mass of cells (Fig 25). After about three weeks, the cells showed early signs of cell degeneration, manifested by an increase in their size and irregularity in their shape (Fig 26).

No exchange of the growth medium was done nor the addition of L-glutamine throughout the 3 week period of the experiment. Photography of the cell culture was taken twice weekly for three weeks.

7-4-2 Results

The results showed that a cell culture of the conjunctival epithelium removed directly by means of "Millipore filter paper" is possible. The conjunctival epithelial cells grew on the filter paper in the first week but they were adherent to the filter paper however odd cells did detach from the filter paper and floated in the growth medium but it did not attach to the floor of the petri-dish.

The higher the number of filter papers used in each petri-dish before incubation gave a bigger size of cell culture mass. The 24

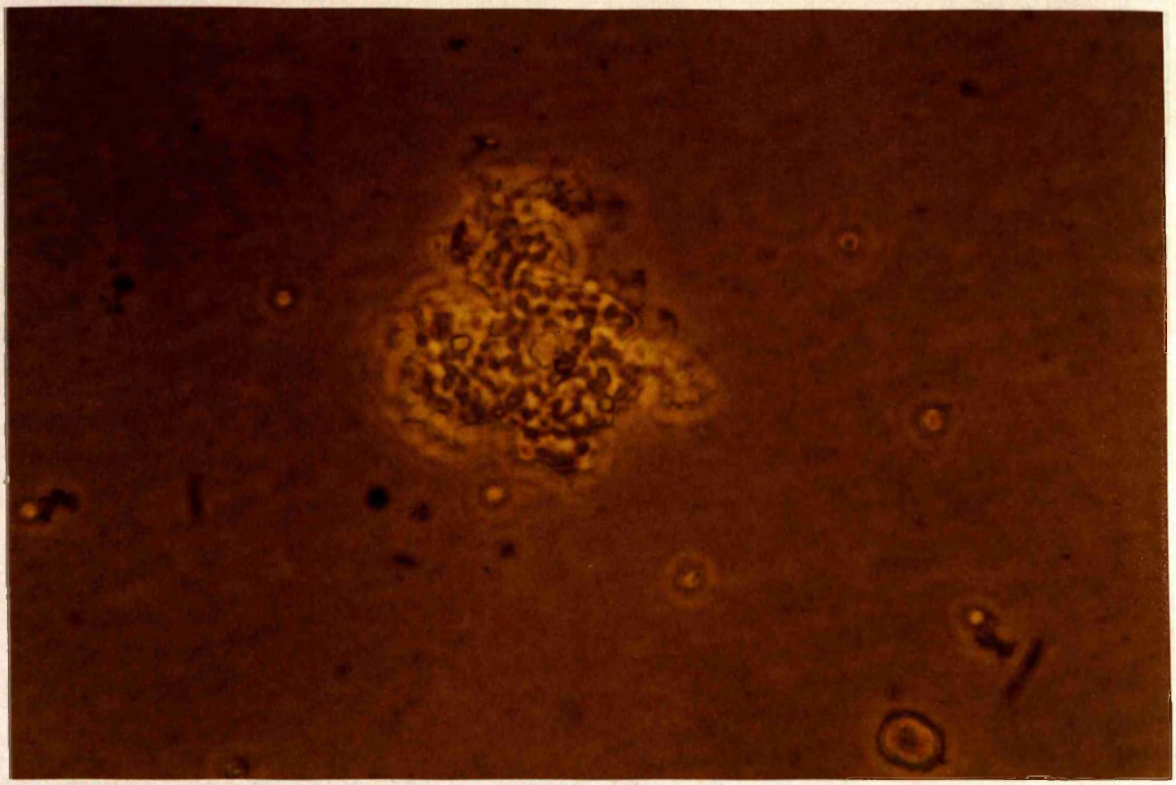


Fig. 24 _ Mass of cells is floating in the the growth medium. (Mag. X200)

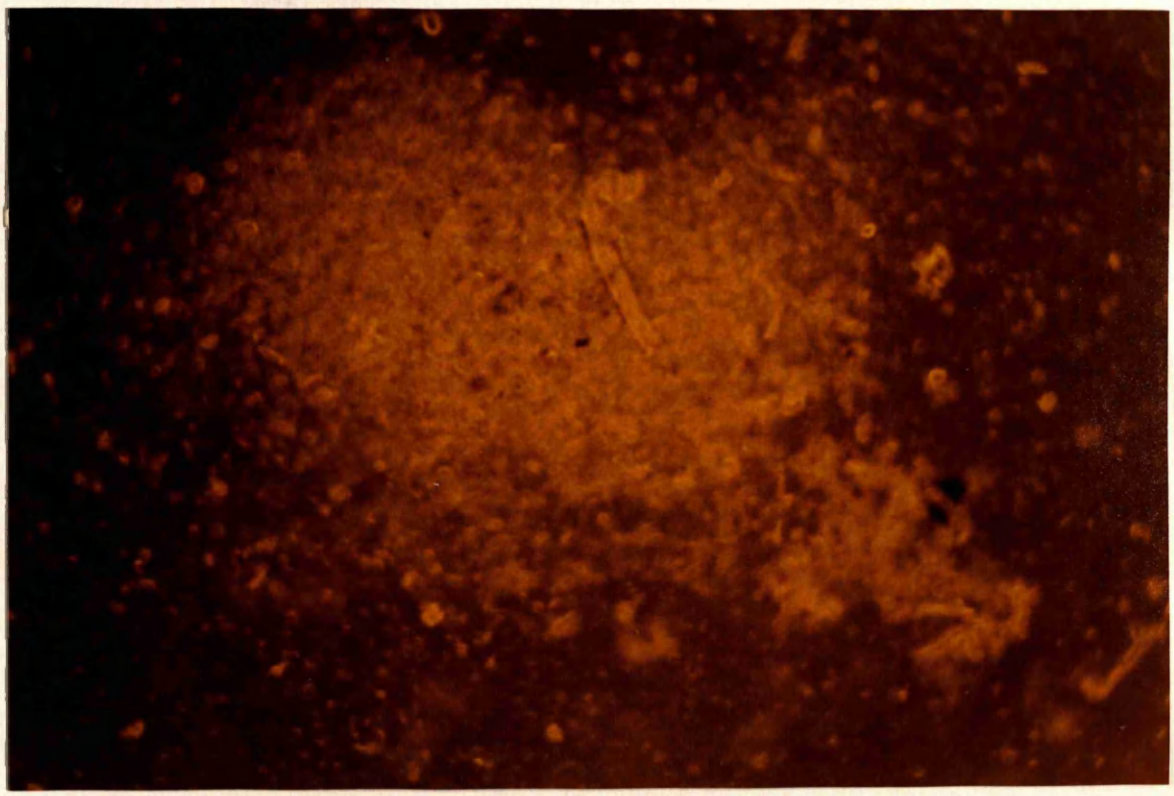


Fig. 25 _ After 2 weeks, a big size mass of cells. (Mag X40)

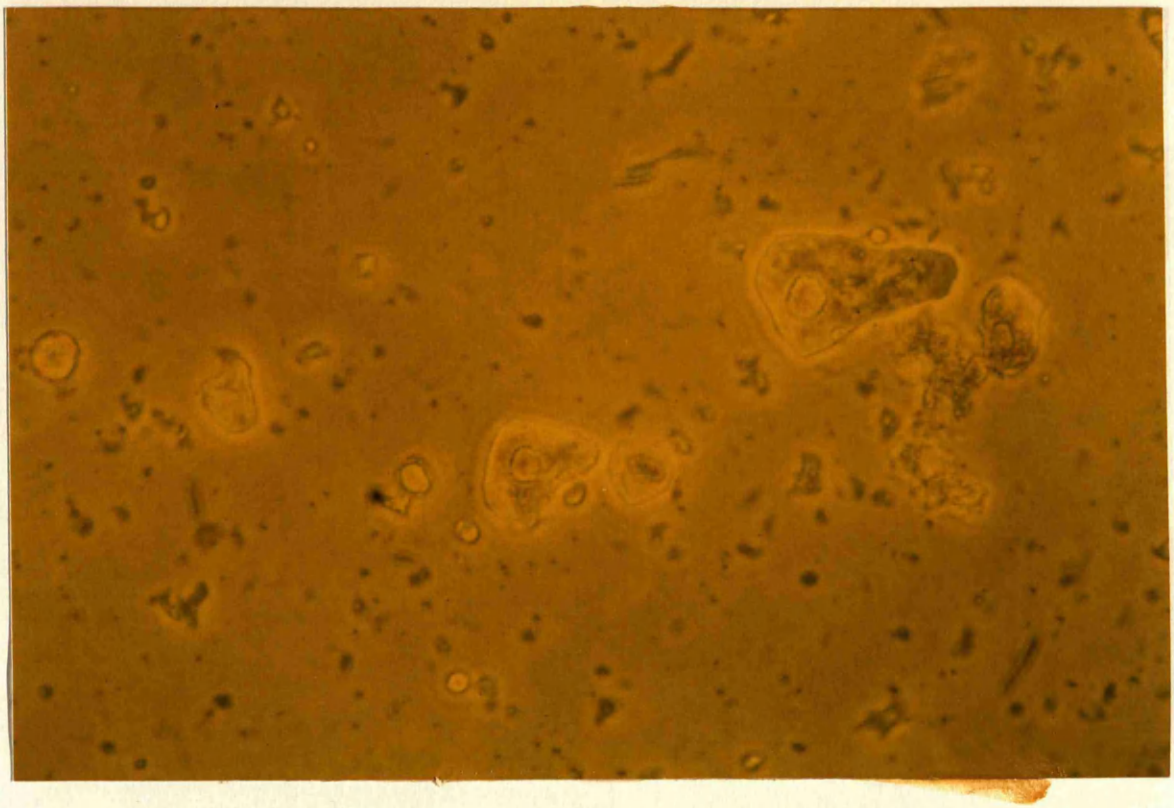


Fig. 26_ Degenerating cells with irregular shape and low nucleus/ cytoplasm ratio. (Mag. X200)

multi-dish wells produced a better response in the proliferation of the conjunctival epithelial cells than the 60mm dimension petri-dish.

7-5 The effects of Therapeutic and Toxic concentrations of Retinol Acetate on normal epithelial cell cultures

In this experiment retinol acetate in both therapeutic and toxic concentrations was employed to assess its effect on the conjunctival epithelial cell culture as described in section 7- 4. Although foetal calf serum, which is normally added to the Dulbecco's growth medium, contains traces of vitamin A, some investigators found that the final vitamin A concentration in the growth medium was about 2% of that in normal human plasma and yet an absence of this concentration will lead to changes in the cellular and molecular properties of the cell cultures (Fuchs and Green, 1981).

7-5-1 All-trans Retinol Acetate Preperations

Retinol acetate crystals were prepared and sterilised in different concentrations at the Pharmaceutical department in the Western Infirmary, Glasgow. Arachis oil was added to transform the retinol crystals to a soluble solution. The concentrated solutions prepared by the Pharmaceutical department comprised of 5µg/ml, 10µg/ml, 100µg/ml of the retinol acetate and a "control solution" which contained arachis oil without retinol acetate. The preparations were kept in the dark at 4°C.

The therapeutic concentration was calculated according to the normal serum level of vitamin A in healthy indivituals which is between 30-100µg/ 100ml. The toxic concentration of X50 was taken on

arbitrarily to be 50µg/ml.

Sterile therapeutic doses were prepared by diluting the concentrated solutions provided by the Pharmaceutical department into one tenth of retinol acetate and nine tenths of the Dulbecco's medium. Therefore the prepared therapeutic solutions at 1/10th of the original concentration, were the arachis oil (control No.1) and retinol acetate at three different concentration of 0.5µg/ml, 1µg/ml and 10µg/ml.

The toxic concentration of 50µg/ml was prepared by mixing equal amounts of 100µg/ml retinol acetate solution and of Dulbecco's growth medium. This concentration necessitated the preparation of another control solution called (control No.2) with comparable concentration (ie a 50% of arachis oil and 50% of the Dulbecco's medium).

7-5-2 Procedure

Cell cultures were prepared in 6 petri-dishes from 2 filter papers in each dish as described in section 7-4. The suspension from each dish was put in a well of a 24 multi- well dishes. In the first well, 2 ml of arachis oil without retinol acetate (control No.1) was added. 2 ml of Retinol acetate at a concentrations of 0.5µg/ml, 1µm/ml and 10µm/ml were added to the second, third and fourth wells respectively. In the fifth well, 2 ml of the higher concentration of arachis oil solution (control No.2) was added whilst in the sixth well, 2 ml of the toxic concentration of retinol acetate at 50µg/ml was added.

The 24 well multi - dishes were kept in a humidified

incubator at 37°C and at 4% CO2 atmosphere. Throughout the preparation, the retinol acetate exposure to direct light was kept to a minimum.

The high concentration of arachis oil in the last two wells led to an interference in the follow up assessment of the changes in the cell culture and in the photography.

The high magnification photographs were taken at the edges of the dense cell culture mass to give a clear details of the cells.

7-6 Results

On the first week, the first four wells which included Control No.1 and the three therapeutic concentrations, showed an early formation of cell mass as seen in low magnification photographs with Control No.1 (Fig 27) and in retinol acetate concentration of 1µg/ml (Fig 28). The high magnification showed normal size epithelial cells with a nucleus /cytoplasm ratio of 1: 2 with increase in cell divisions as seen in retinol acetate concentration of 0.5µg/ml (Fig 29) and retinol acetate concentration of 10µg/ml (Fig 30).

On the second week, the size of the cell mass increased in length and depth as seen in Control No.1 (Fig 31) and in retinol acetate concentration of 1µg/ml (Fig 32). The epithelial cells showed areas of increased activity as seen in Control No.1 well (Fig 33) and in retinol acetate concentration of 0.5µg/ml (Fig 34).

On the third week, there was little change in the size of the cell culture masses however the epithelial cells began to show

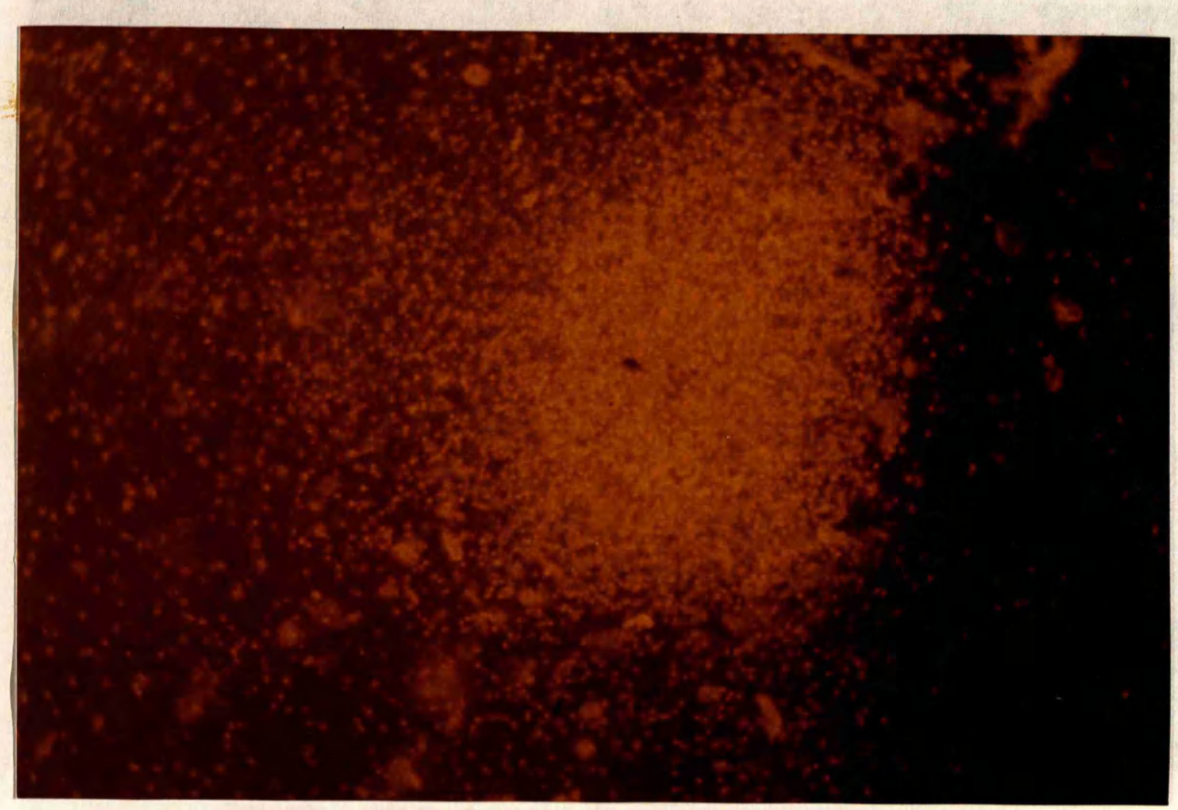


Fig. 27_ After 1 week, cell mass formation in a Control No. 1 well. (Mag. 40)

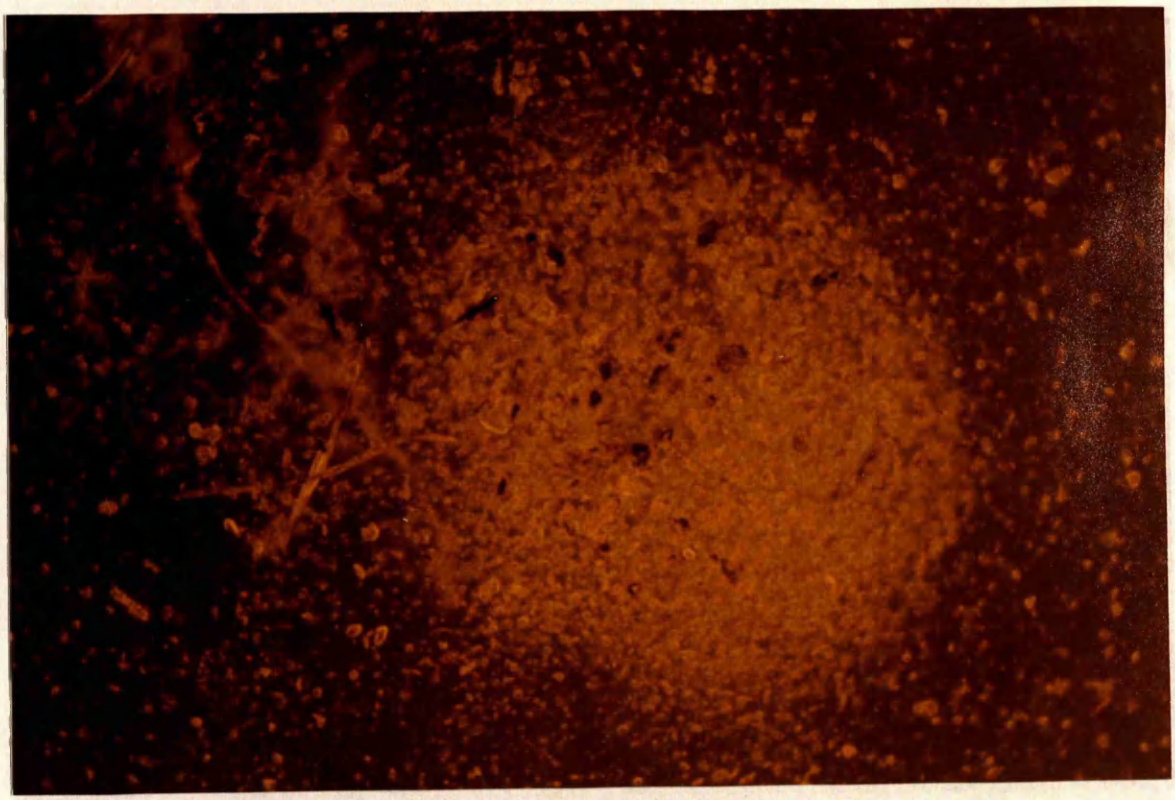


Fig. 28_ After 1 week, cell mass formation in a well containing retinol acetate 1µg/ml. (Mag. X40) 90

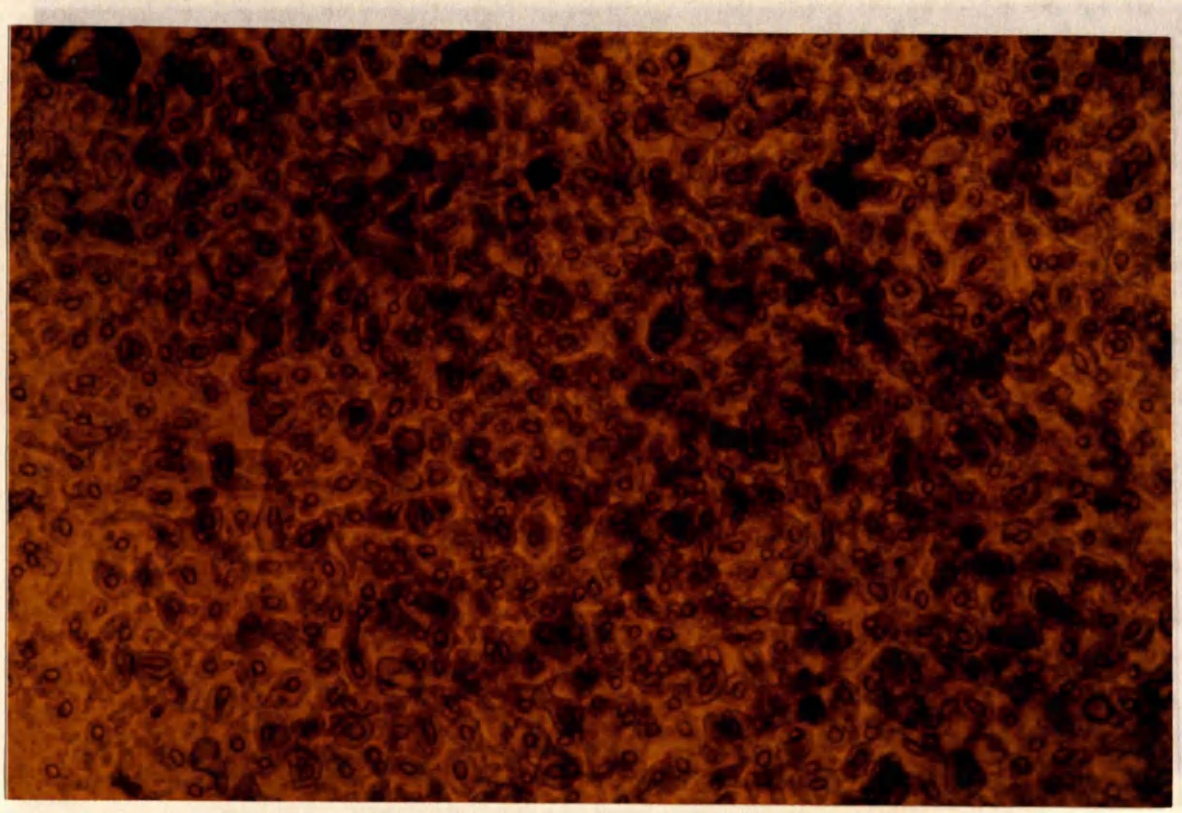


Fig 29 _ After 1 week, increase cellular activity is seen in a well containing retinol acetate 0.5µg/ml. (Mag. X200)

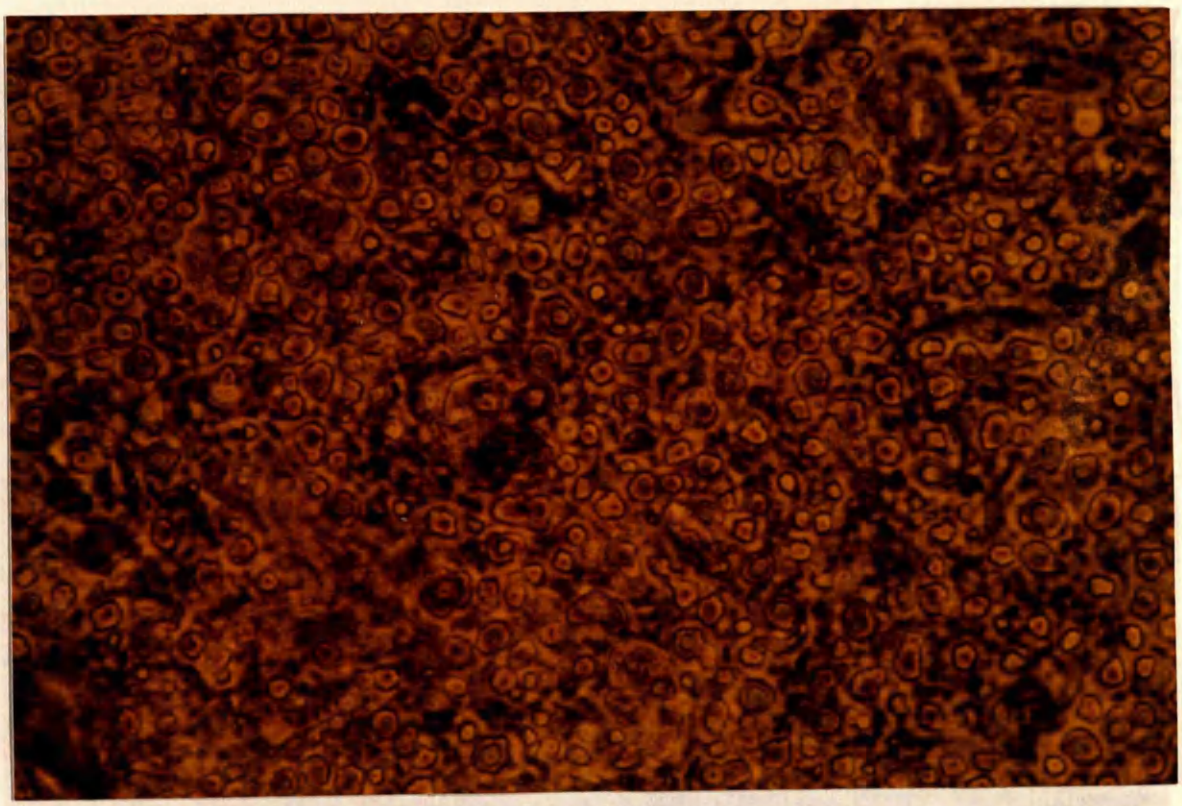


Fig. 30. After 1 week, increase cellular activity is seen in a well containing retinol acetate 10µg/ml. (Mag. X200)

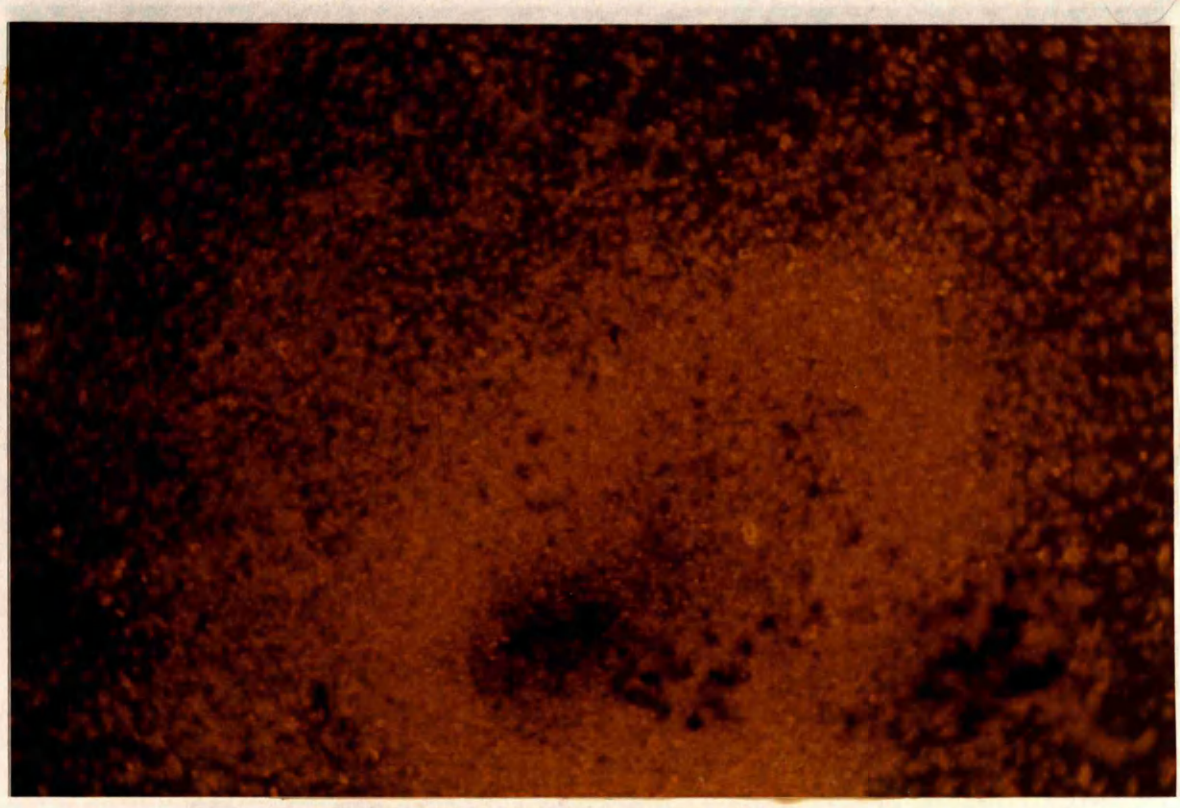


Fig 31. After 2 weeks, there is increase in the size and depth of the cell mass in Control No.1 well (Mag. X200)

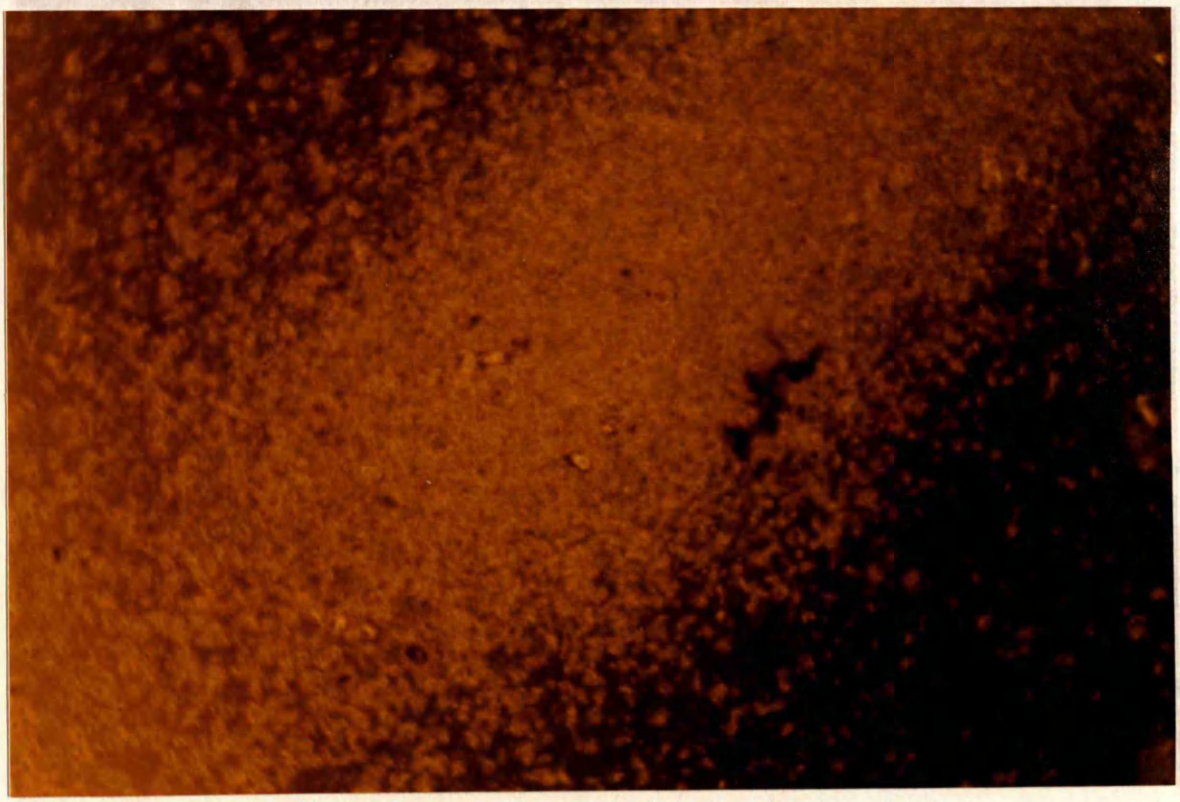


Fig. 32_ After 2 weeks, there is increase in the size and depth of the cell mass in the retinol acetate 1µg/ml. (Mag. X200)

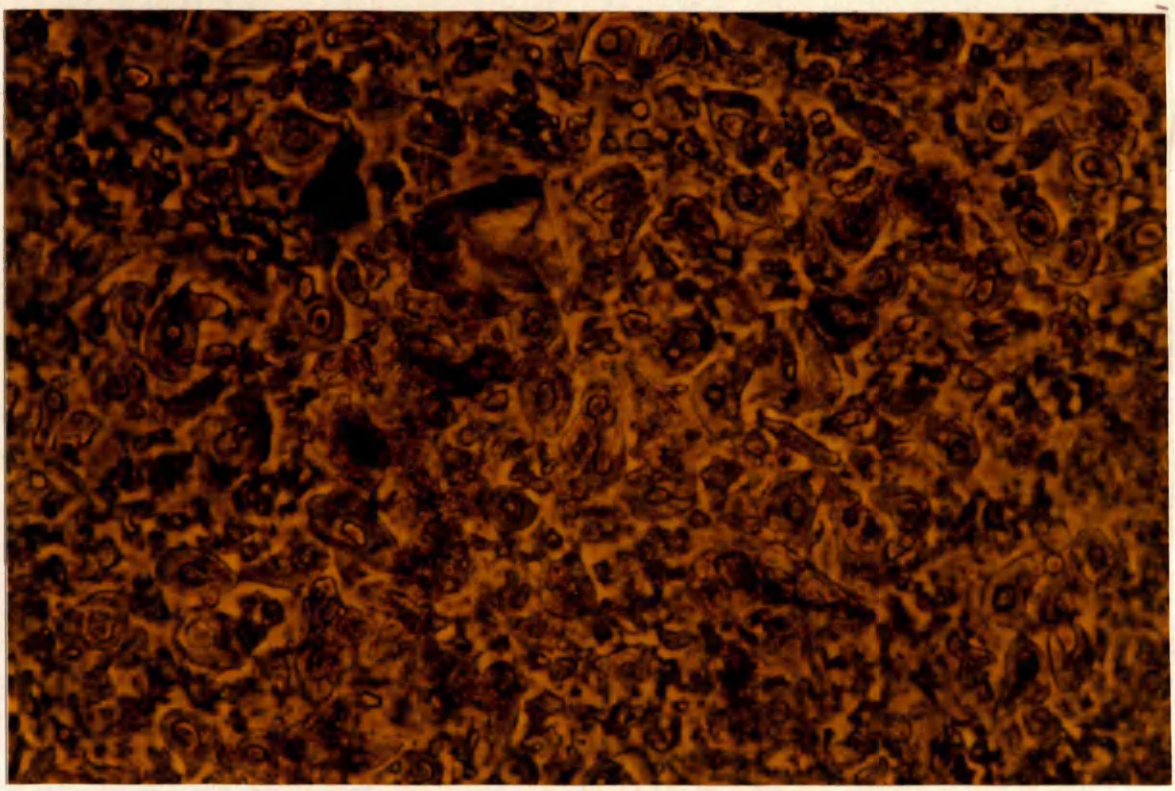


Fig. 33_ Mitotic activity of epithelial cells in Control No.1 well (X200)

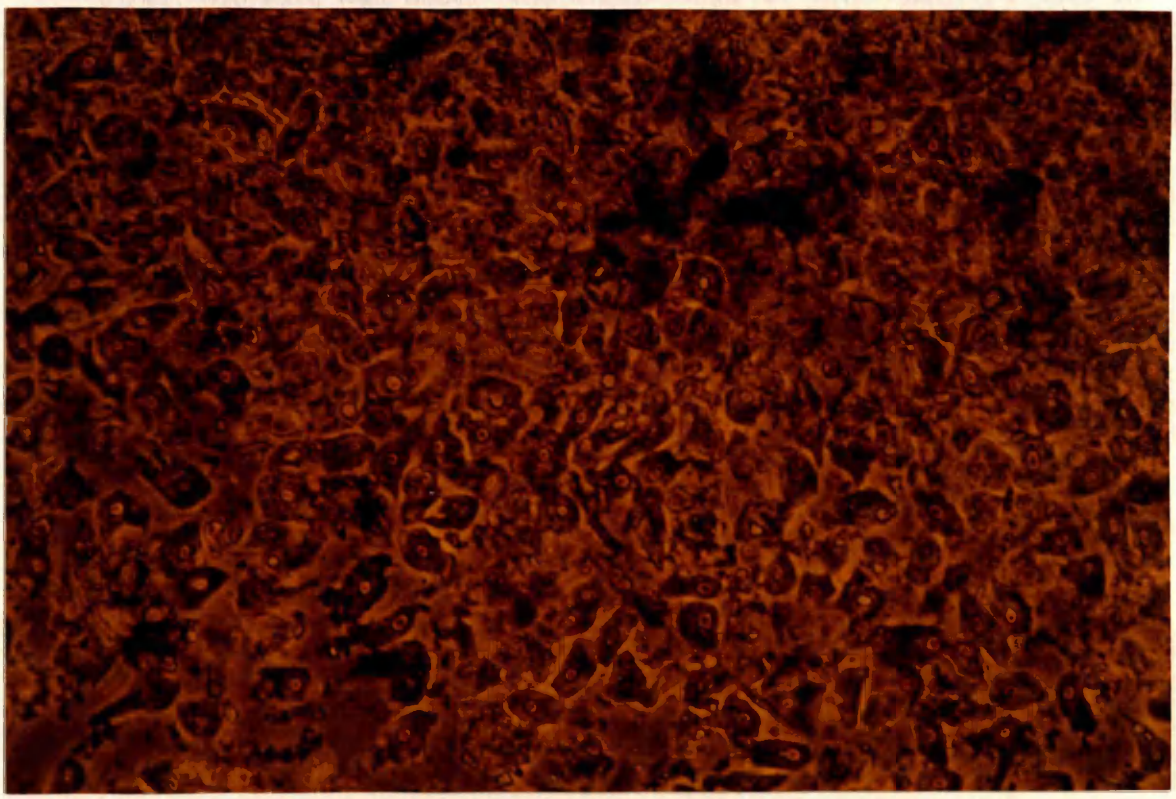


Fig. 34_ Mitotic activity of epithelial cells in retinol acetate 0.5µg/ml. (X200)

sign of degeneration.

The fifth well which contained the toxic concentration of retinol acetate showed small sized mass in the first week (Fig 35) which decreased in size over the following two weeks. The sixth well which contained Control No.2 was difficult to assess and photograph because of the high concentration of arachis oil.

Although the estimation of the number of cells was not made, the gradual increase in the size of the cell culture mass was a clear qualitative estimate of the growth of the cells.

7-7 Discussion

A new method for culturing conjunctival epithelial cells by direct approach was made. The significance of this method relies on its simplicity and provides a guarantee that only epithelial cells were present. Furthermore, the conjunctival epithelial cells removed in layers ie superficial, middle and deep layers, makes the study of each specific layer more important in the understanding of the developmental structure. Also the study of the behaviour of the epithelial cells to changes in the pH, osmolarity or the addition of a drug is very important.

Our knowledge of the factors, intrinsic or extrinsic, that control the development of the basal layer is incomplete (Sun and Green, 1977 ; Doran and Sun, 1979). A cell culture with a known layer of the epithelium is an important step in the understanding of the behaviour and maturation of the conjunctiva. Newsome et al (1974) showed that the cultured epithelial layers retained their structural specialisation of their tissue of origin. They also showed that HL-A

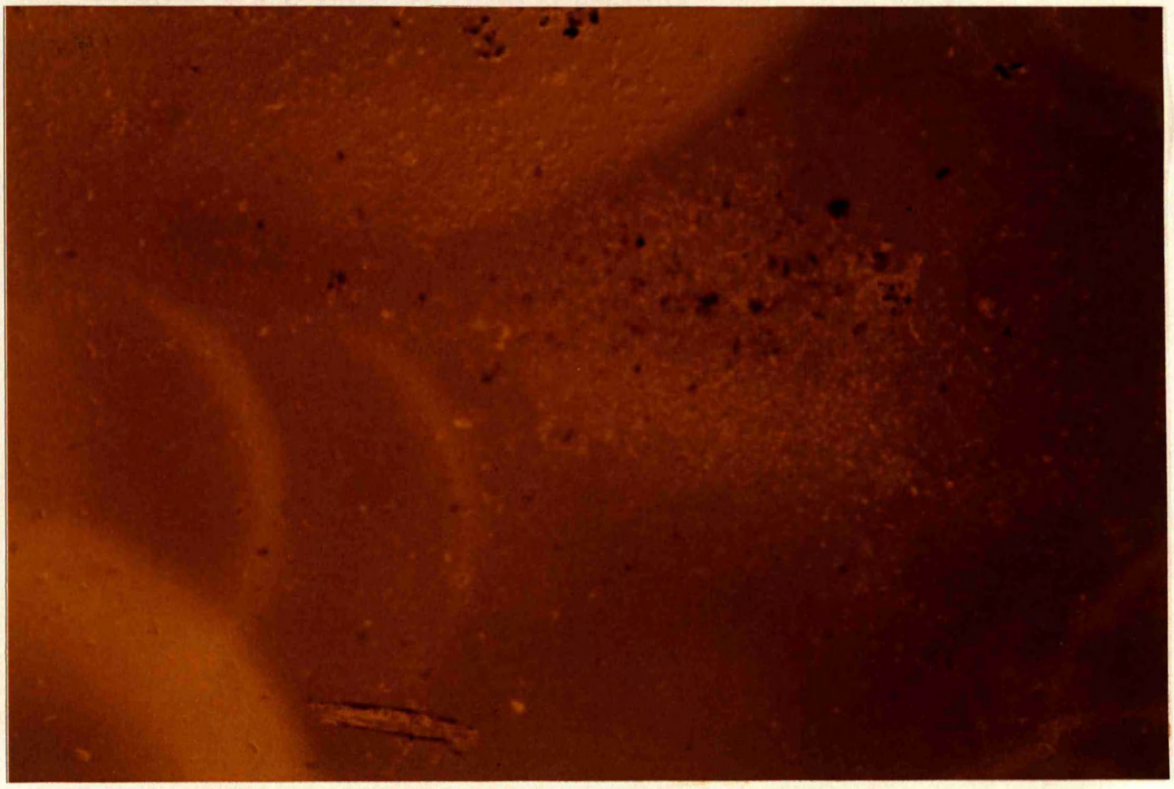


Fig. 35. After one week, a small sized mass in a well containing a toxic concentration of retinol acetate (Mag. X40)

antigens are present in cultured corneal cells which may prove useful in the study of the different immunological processes.

CHAPTER EIGHT

Morphological Study of Conjunctival Epithelium using "Millipore" Filter Paper by Scanning Electron Microscopy

8-1 Introduction

The epithelial ocular surface has been studied by scanning electron microscopy (SEM) in the case of the conjunctiva in human (Greiner, Covington and Allansmith , 1977), and in the rabbit (Pfister,1975) and of the cornae of rabbit, cat, dog and monkey (Pfister, 1973). Another study examined the cellular corneal changes in dry eye rabbit (Pfister and Renner, 1977).

This study examines successive layers of normal epithelium using scanning electron microscopy, by peeling off eight layers of the lower bulbar conjunctiva with the help of " Millipore filter papers". The study also looked at the superficial epithelial layer in patients with Sjögren syndrome. The unique opportunity of examining the deeper layers of the conjunctiva has not been reported before. The views examined are those of the posterior surfaces of the epithelial cells as the anterior surfaces are adherent to the filter papers.

8-2 Materials

This study looked first at eight consecutive impression specimens of normal conjunctival epithelium taken from the lower bulbar conjunctiva of two normal volunteers. The first volunteer is

a twenty nine year old female and the second volunteer is forty four year old male. Neither of the two volunteers has any history of cular allergy or infection. The study also looked at impression specimens taken from the superficial layer of the lower bulbar conjunctiva of seven patients with biopsy proven Sjögren's syndrome. These patients had shown clinical manifestations of dry eyes. Their ages range, between thirty eight to seventy four years of age (mean 56 years). One patient is male and the remaining six are female.

8-3 Method

Sterile applicators were prepared, using the dull wet "Millipore filter paper" strips, as described in section 5-3-1. After instilling one drop of 1% Oxybuprocaine into the conjunctival sac, an applictor was pressed on the lower bulbar conjunctiva, about 3-4 mm away from the limbus, for about 3-4 seconds. Then the applicator is "peeled off" from the conjunctival surface.

In the case of the two normal volunteers, eight consecutive impressions were taken from the lower bulbar conjunctiva. In one normal volunteer, a radial full thickness conjunctival biopsy was taken from the bulbar conjunctiva, including an area of the conjunctival impressions. The biopsy was done under local anaesthsia without touching the centre of the biopsy. The histological sections show the defects in the epithelial layers to vary from one area in the section to another. Therefore the specimens examined under scanning electron microscopy of the eight epithelial layers show an overlapping of the epithelial cells.

All the respective materials and their sources of supply are included in Appendix I.

8-3-1 Fixation of Specimen

After peeling the filter paper from the bulbar conjunctiva, the filter paper was removed from the plastic applicator and put immediately in a sterile test tube containing 3.5% Glutaraldehyde solution in 0.2 M cacodylate buffer for at least 30 minutes. The specimen is then rinsed in 0.2 M cacodylate buffer solution which washes out the aldehydes which may react with Osmium tetroxide and cause precipitations (McDowell, 1978). The details of the procedure are seen in table 15.

Sabatini, Bensch and Barrnett (1963) recommended the use of Glutaraldehyde solution as a primary fixative for its rapid reaction with proteins and the Osmium tetroxide as a secondary fixative for its value in preserving the fine structures of the tissue. They also proposed the use of cacodylate as a buffer solution for it is ease of preparation, stablility during storage for long periods and its resistance to the growth of micro-organisms. Its main disadvantages are the presence of arsenic, which is toxic and its unpleasant smell. Therefore the above procedures were done in a fume cupboard.

8-3-2 Chemical Dehydration

The filter paper was put in successive concentrations of ethanol at 25%, 50%, 75% and 100% for 10 minutes period in each concentration. Ethanol was used to dehydrate the specimen prior to

1 - After removing the filter paper from the plastic applicator, put the filter paper in a sterile test tube containing 3.5% Glutaridehyde solution in 0.2 M cacodylate buffer for at least 30 minutes.

2 - Wash the filter paper X4 in 0.2 M cacodylate buffer solution for one hour.

3 - Put the filter paper into a solution of equal parts of 2% Osmium tetroxide and 0.2M Cacodylate buffer, as a secondary fixative, for 30 minutes.

4 - Wash the filter paper X4 in 0.2M cacodylate buffer solution over one hour period to remove any residual Osmium tetroxide.

5 - Dehydrate the filter paper in graded alcohol.

25% alcohol .....15 minutes
50% alcohol .....15 minutes
75% alcohol .....15 minutes
100% alcohol 3 washes at 15 minutes each.

Table 15 - Fixation and Dehydration of the filter paper specimen

critical point drying. It was chosen as the dehydrating agent as it is miscible with the liquid carbon dioxide as it is described in the next process.

8-3-3 Critical Point Drying Method

To examine a biological material by Scanning Electron Microscopy, it is necessary to dry the specimen completely. Critical Point Drying method preserves the architecture of the specimen by reducing the deformation to a minimum. This is in contrast to the air drying method where exposure to surface tension forces leads to deformation and collapse of the structure of the specimen.

The Critical Point Drying method involves replacement of the dehydrating liquid, ethanol, by a miscible liquid carbon dioxide. Using a Critical Point Drying apparatus, the carbon dioxide liquid in the specimen, which has a comparatively high density is transformed instantaneously, to a gaseous phase with a comparatively low density. This transformation happens at its critical temperature (31.1°C) and pressure (74 bar). The transition from liquid to gas takes place without an interface because the densities of gas and liquid are equal at the critical point (Watt, 1985). Carbon dioxide liquid has been chosen because of its low critical temperature (31.1°C) and pressure (74 bar). The details of this procedure, which are seen in table 16, apply to specific equipments listed in Appendix I and used in the laboratory at a certain period of time.

8-3-4 Sputter Coating

The basic requirement of coating, is to provide a

1- Reduce the temperature, in the chamber of the "Emscope" machine, to 4-5°c by adding 1 ml. of absolute alcohol to produce a moist atmosphere.

2- Place the filter paper in the chamber then seal it tightly.

3- Open an inlet valve to introduce liquid carbon dioxide and at the same time open an exhaust valve to flush the alcohol out. This takes about one minute.

4- Allow the chamber to fill with liquid carbon dioxide. Close the exhaust and inlet valves and leave the specimen to soak for 15 minutes.

5- Flush and refill the chamber with liquid carbon dioxide. All the alcohol should now be removed from the specimen.

6- Switch the temperature to "Heat". The temperature will increase gradually to above 31°C and the pressure will stabilise above the critical point. This is the stage of the critical point.

7- The pressure in the machine will reduce gradually to zero.

Table 16. Critical Point Drying Stages.

continuous electrical path between the stub surface and any point on the specimen likely to be exposed to the electron beam. Also the coating film enhances the emission of electrons from the surface of the specimen (Watt, 1985).

Cathode sputtering is a process whereby particles, in a vacuum chamber, are ejected from a surface by bombardment with positively charged ions. Two plane electrodes, the cathode being a gold foil target, are separated by a gap of several centimeters in a vacuum chamber filled with an inert gas like argon, at a pressure of between 0.1 to 0.01 mbar. When a potential difference of 1000 volts or more is applied across the electrodes, a glow discharge is set up between them. Some of the argon gas is ionised into electrons which are attracted towards the anode while the positive ions are attracted toward the cathode with the gold plate. The positive ions causes neutral atoms of cathode material to be ejected by the sputtering process. The sputtered atoms arrive at the anode from a range of directions and condense to form a thin film of cathode material. The random arrival direction of sputtered material and the fact that gold can be deposited very economically makes this form of deposition attractive for coating the SEM specimen (Watt, 1985). The details of the technique are seen in table 17. Again these details are general guidelines which apply to specific equipments listed in Appendix I and used at a certain period of time.

1- The filter paper was mounted, with a colloidal silver paste, on a 10mm × 10mm cylinder aluminium stubs .

2- Place the stub with the filter paper in the coating unit.

3- Close the lid tightly

4- Evacuate the air in the unit.

5- Controlled Argon gas was leaked to the unit.

6- Bring HT to 1.2 mA.

7- Keep the Argon gas leaking for about 1.5 minutes.

8- Switch off Argon gas.

9- KV to 0.

Table 17. Sputter Coating Technique

8-3-5 Scanning Electrom Microscope

In scanning electron microscopy, the electron beam, travelling in a vacuum, is focused to a very small spot. The beam controlled by deflecting magnetic fields, traverses the specimen in a series of sequential lines. Bombardment of the metal-coated specimen produces secondary electrons which are collected, translated into light, and subsequently converted into a series of corresponding sequential lines on an oscilloscope, much as is done on a television screen. The pictures produced on screen, are photographed on film. The pictures give a three dimensional enlargement of a surface with a great depth of field and direct resolution of about 3.5 nm can be achieved in optimum conditions (Fine and Yanoff, 1972). However the technology available and the nature of the specimens studied has limited the observation to a magnification range of X50 - 10 000 which is more than adequate for this study.

8-4 Results

The first subsection will concentrate on the morphological changes of the epithelial cells in eight consecutive layers of normal conjunctiva. The second subsection considers the cellular changes of the superficial conjunctival layer in Sjögren syndrome patients. The named first layer is the superficial epithelial layer while the eighth layer is the deepest epithelial layer.

Conjunctival epithelial cell sheet as well as individual and small groups of cells have been obtained on one filter paper (Figure 36) although at times two layers of epithelial cells are

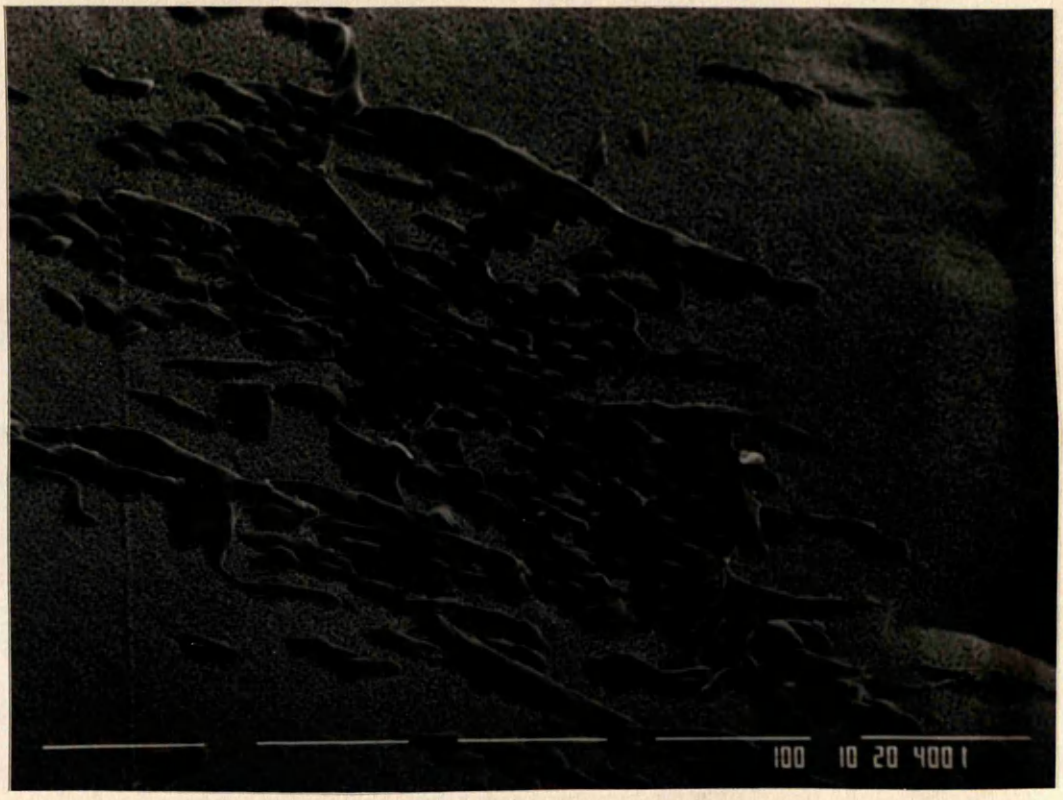


Fig. 36 Panoramic view of posterior surface of normal conjunctival epithelial cells. (Mag. X200)

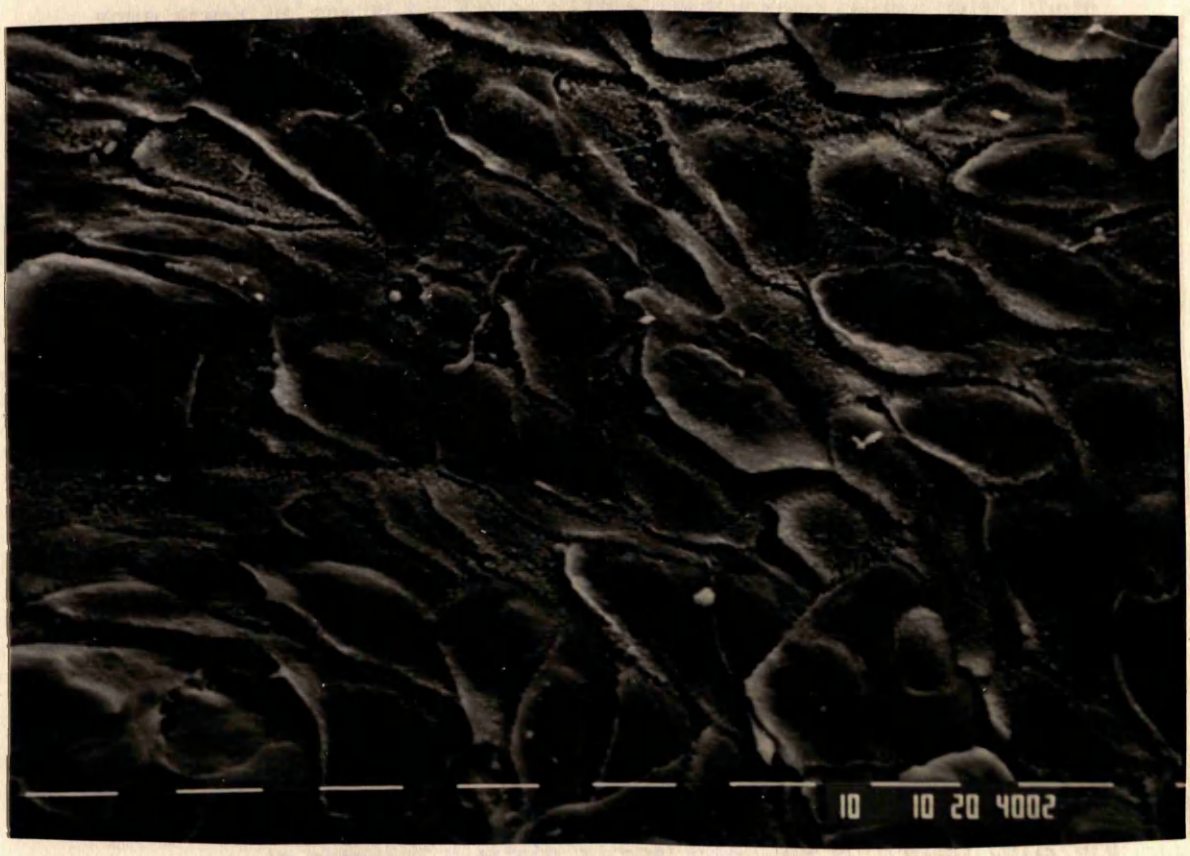


Fig. 37 Superficial epithelial layer of normal conjunctival epithelium showing posterior surface of polygonal epithelial cells with microprojections. (Mag. X1000)

found in the same filter paper (Figure 37). This degree of yield was also present in the specimens taken from Sjögren's syndrome patients.

8-4-1 SEM of eight consecutive conjunctival layers in normal volunteers

The first layer shows flat polygonal epithelial cells with a central elevated nucleus. The cells are generally similar in shape and size (approximately 10μ ×20 μ) (Figure 37). Epithelial cells are connected to each other and to the underlying cells by microvilli (Figure 38) with microprojections extending over the adjoining cells.

The epithelial cells show an early elongation in shape, at some areas of the third layer to cuboidal shaped cells, with their nuclei remaining at the centre of the cell (Figure 39). At the fifth layer, the predominant shaped cells are longitudinal in shape with tapered ends and a central nucleus (Figure 40). Columnar cells with their nuclei situated at one end of the cell, are seen first in the seventh layer (Figure 41). The flat borders of the epithelial cells are well marked at the superficial layers. These flat borders get smaller in size gradually as we examine the deeper layers. At the seventh layer, the flat borders are seen only at one end of the columnar cells (Figure 41).

Goblet cells are seen in the superficial layer (Figure 42) with crystal shaped secretion covering the opening of the cells (Figure 43).

In scanning electron micrographs, the epithelial cells are classified according to their brightness to light. The light cells

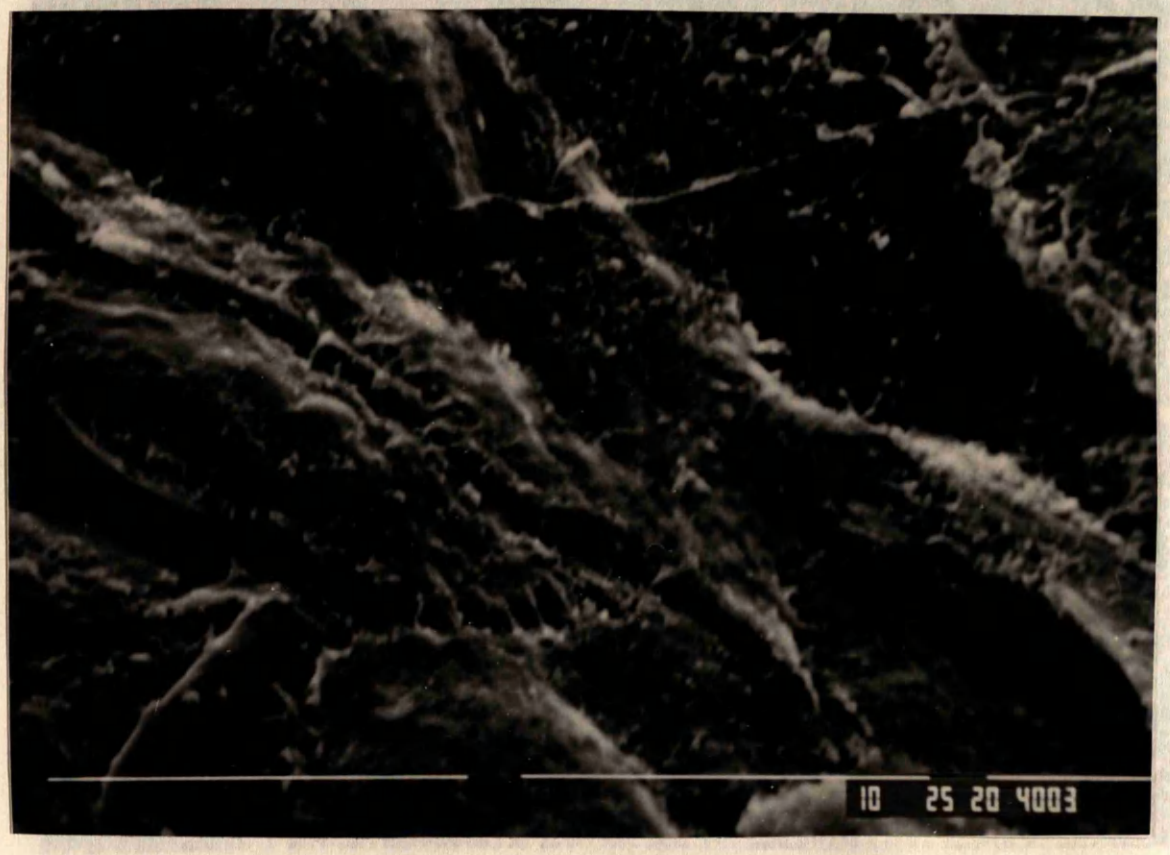


Figure 38 - Posterior surface of polygonal epithelial cells with microvilli extending between adjoining cells. (Mag 5000).

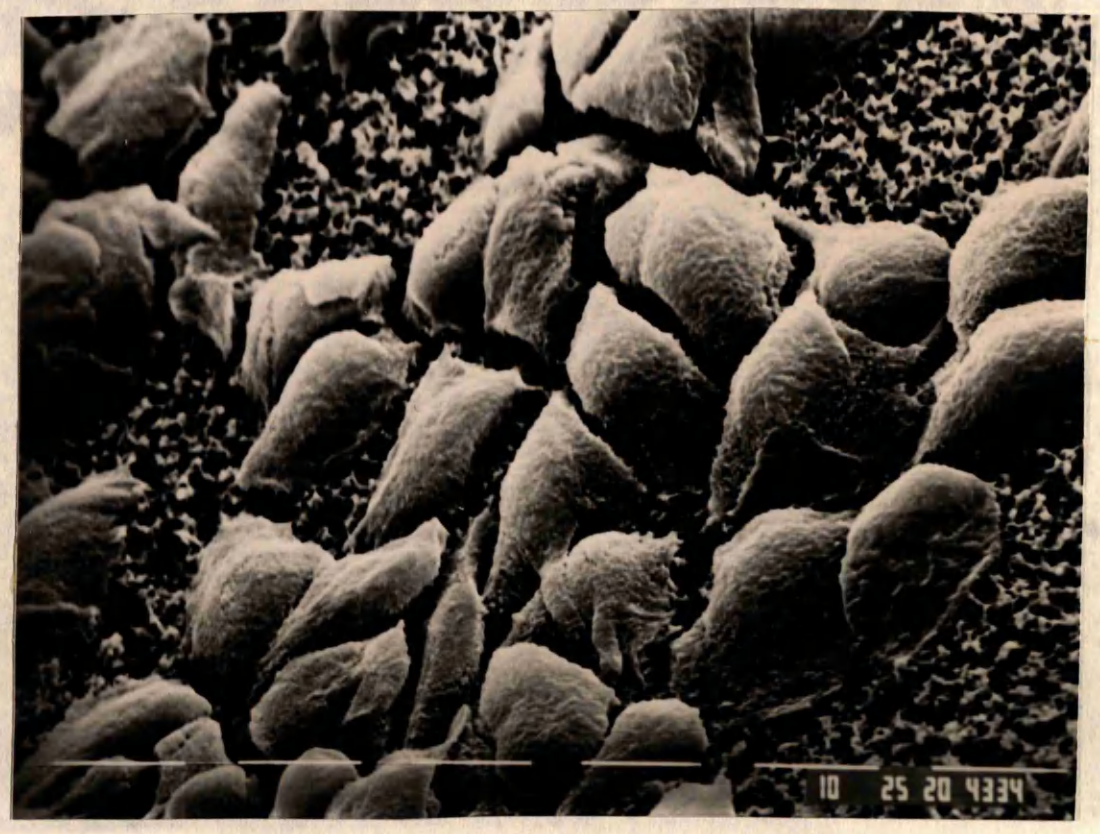


Fig. 39 _ Early changes from longitudinal to cuboidal shaped epithelial cells (posterior surface) at the third layer. (Mag. X1500)

Fig. 40 _ At the 5th layer, the predominant cells are longitudinal in shape. (Mag. X1500)

Fig. 41 _ Columnar cells with the nucleus at one end. (Mag. X3500)

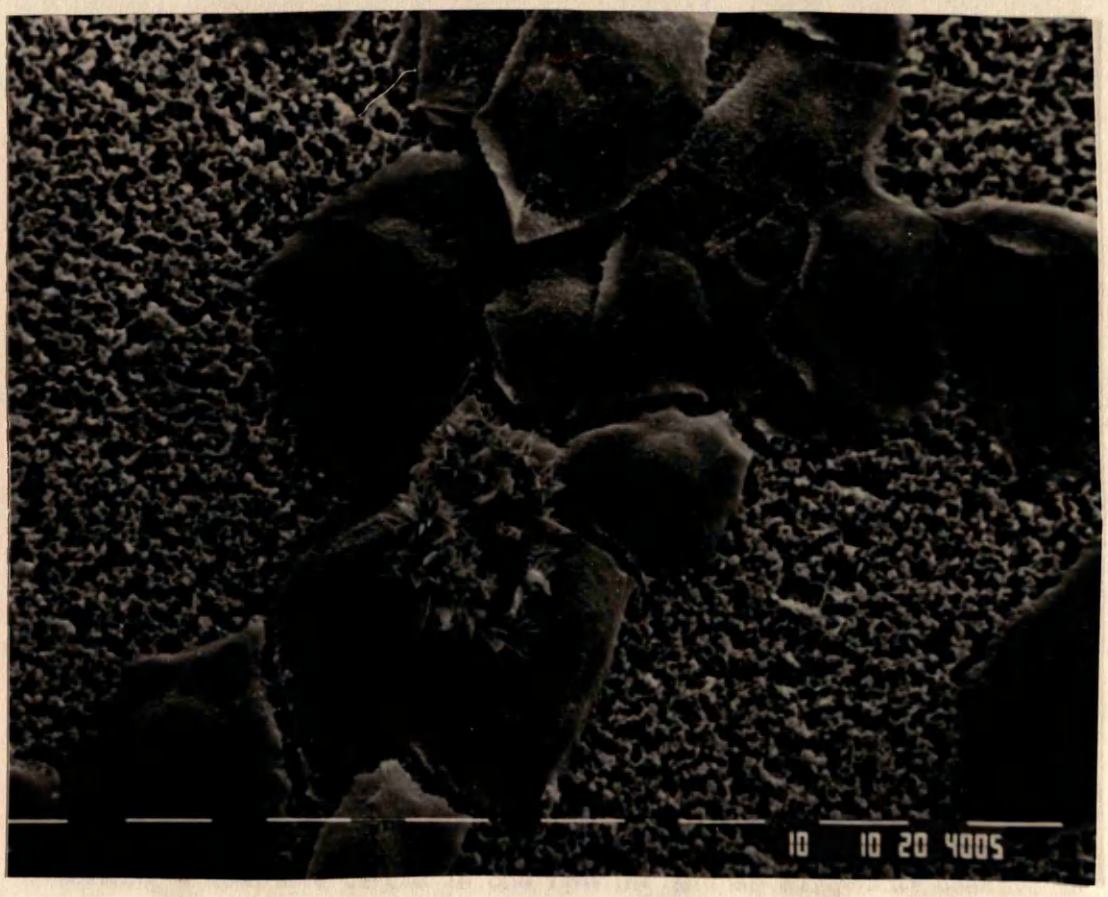


Figure 42- Mucus from an opening in a Goblet cell at the superficial epithelial layer. (posterior view) (Mag. 1000).

Figure 43 - Magnified goblet cell opening. (Mag. 3500)

which are the predominant type, are smaller in size with a relatively smoother surface whilst the dark cells are fewer in number, irregular in shape, larger in size (approx. 50μ in length) with a wavy surface. The dark cells are seen in the first layer, the third layer (Figure 44), the fourth layer (Figure 45), the seventh layer (Figure 46) and the eighth layer (Figure 47).

There are two kinds of openings which are confined to the superficial epithelial layer. These are the intercellular openings and the central holes. In figure 48, the intercellular openings are placed between dark cells. The openings have an elevated margin forming a complete circle in one opening and an elliptical opening in another. The openings width range between 5-7 μ. Thick mucus strands are present near the intercellular openings. The central holes are present in the adjoining cells. The holes are located centrally at the cell. They are fenestrated by membranes still adherent to the interior surface of the cell (Figure 49).

Globular shaped cells are seen in two layers of the conjunctiva. In the eighth layer, the globular cell is about 10 μ in size with a smooth surface and few microvilli (Figure 50). In the fourth layer, the globular cell is slightly larger, about 12-14 μ in size, with higher number of microvilli on the surface (Figure 51).

A dendritic cell is seen at the fifth epithelial layer (Figure 52). It is longitudinal in shape with processes at each end of the cell. The nucleus is situated at the middle of the cell. It has a smooth surface with no microvilli. It has long processes which contain granules with a size of 1μ each (Figure 53).

Melanocytes, Langerhan cells and nerve fibres are normally

Fig. 44_ At the 3th layer, dark flat cells larger in size than light cells. (posterior view) (Mag. X750)

Fig. 45 _ Dark cells at the 4th epithelial layer. (posterior view) (Mag. X1000)

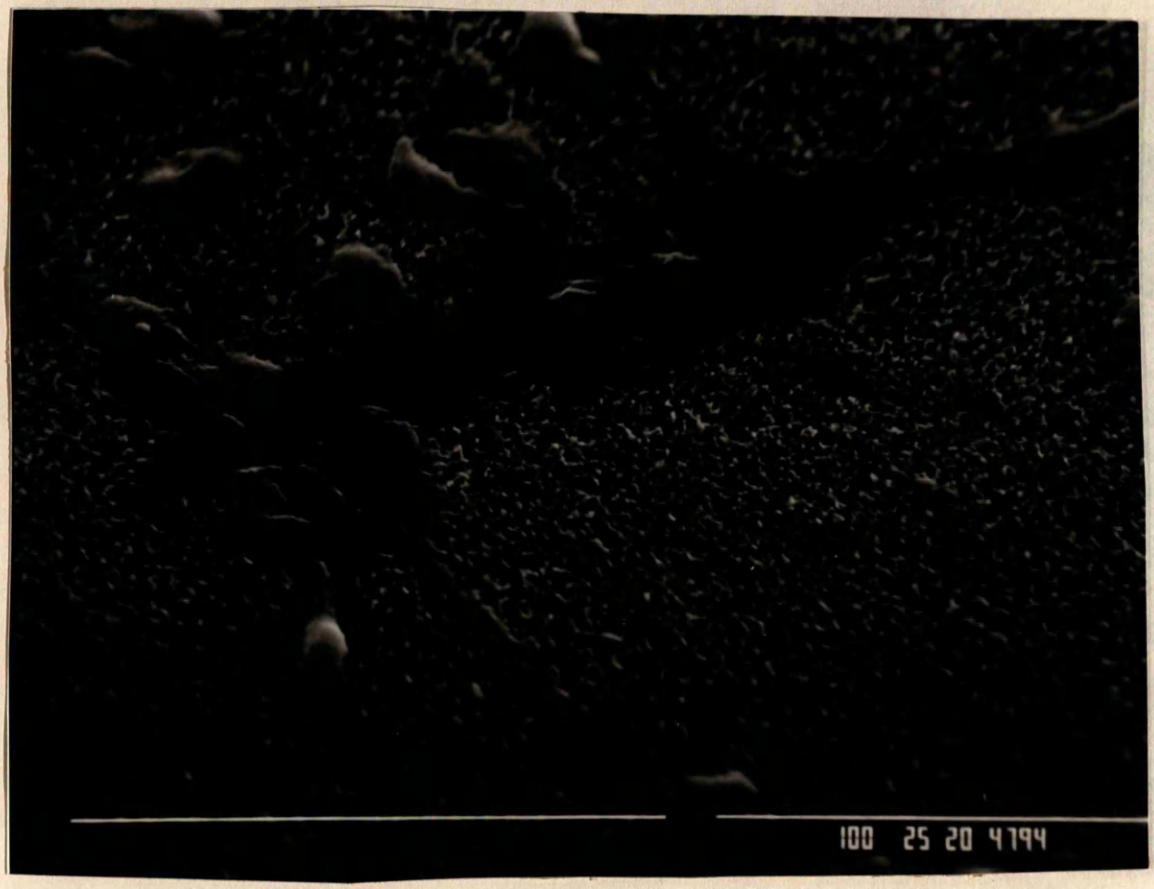


Fig. 46 _ Dark cells from the 7th epithelial layer.
(posterior view) (Mag. 750).

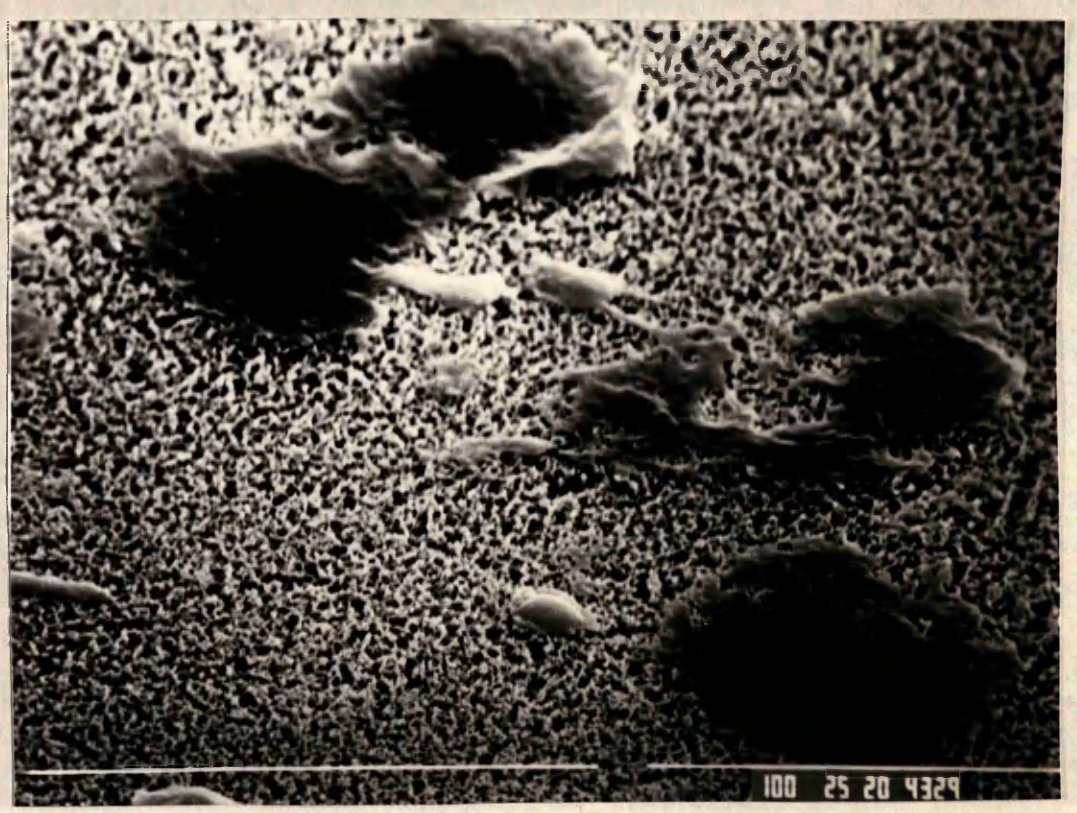


Fig. 47 _ Dark cells from the 8th epithelial layer.
(posterior view) (Mag. X3500)

Fig. 48_ Intercellular openings with mucus strands
(Mag. X2000)

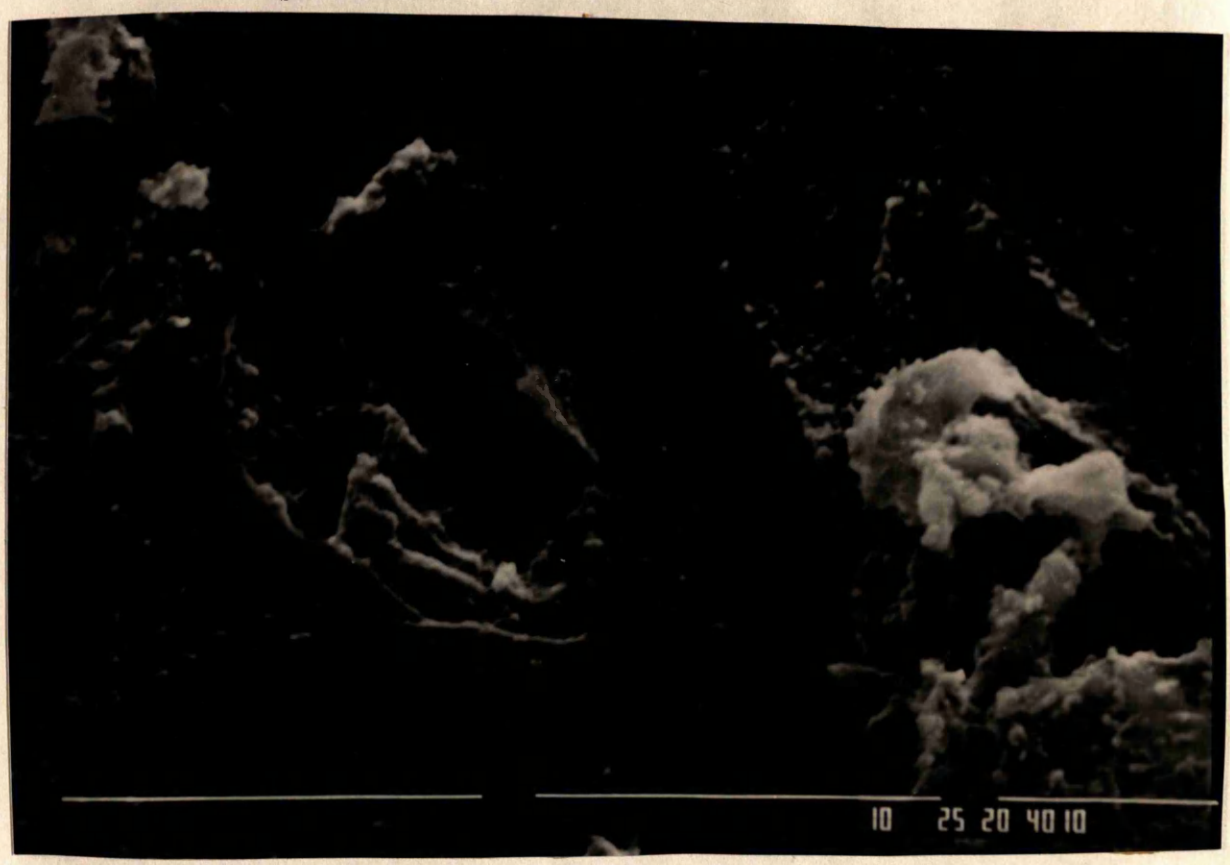


Fig. 49 _ Central opening with fenestrations.
(Mag. X5000)

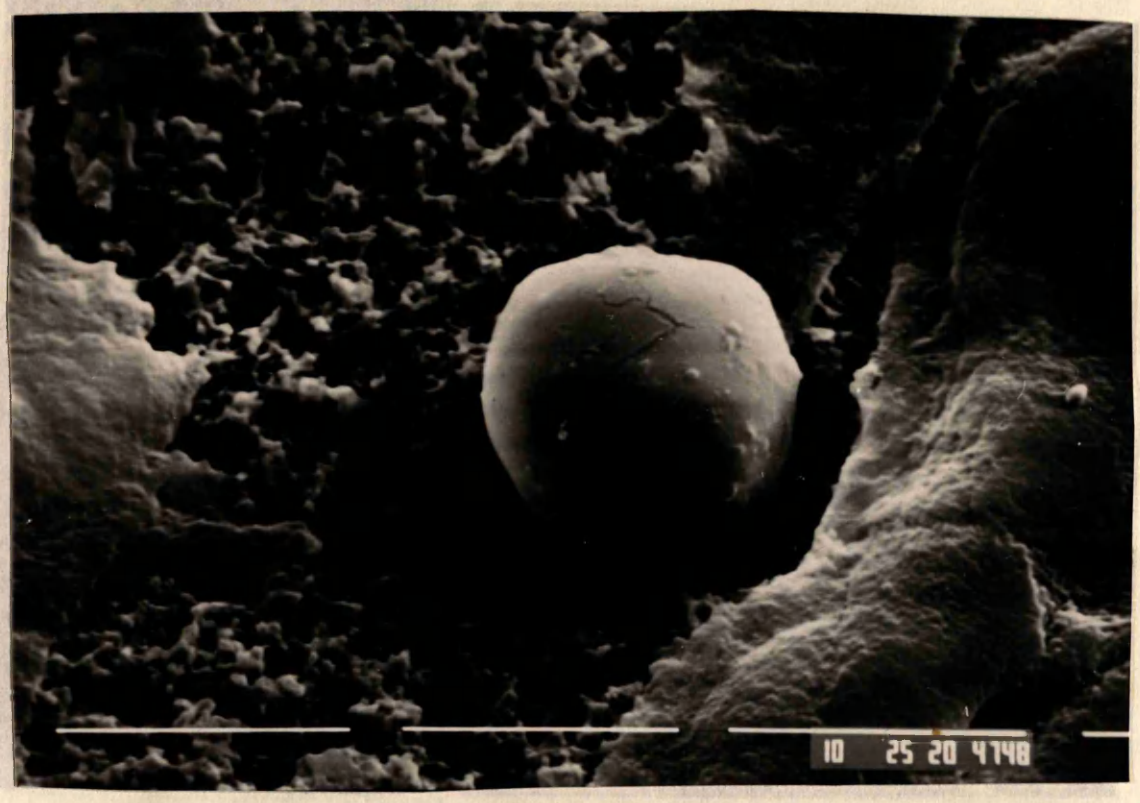


Fig. 50 _ Globular cells with smooth surfaces.
(Mag 3500).

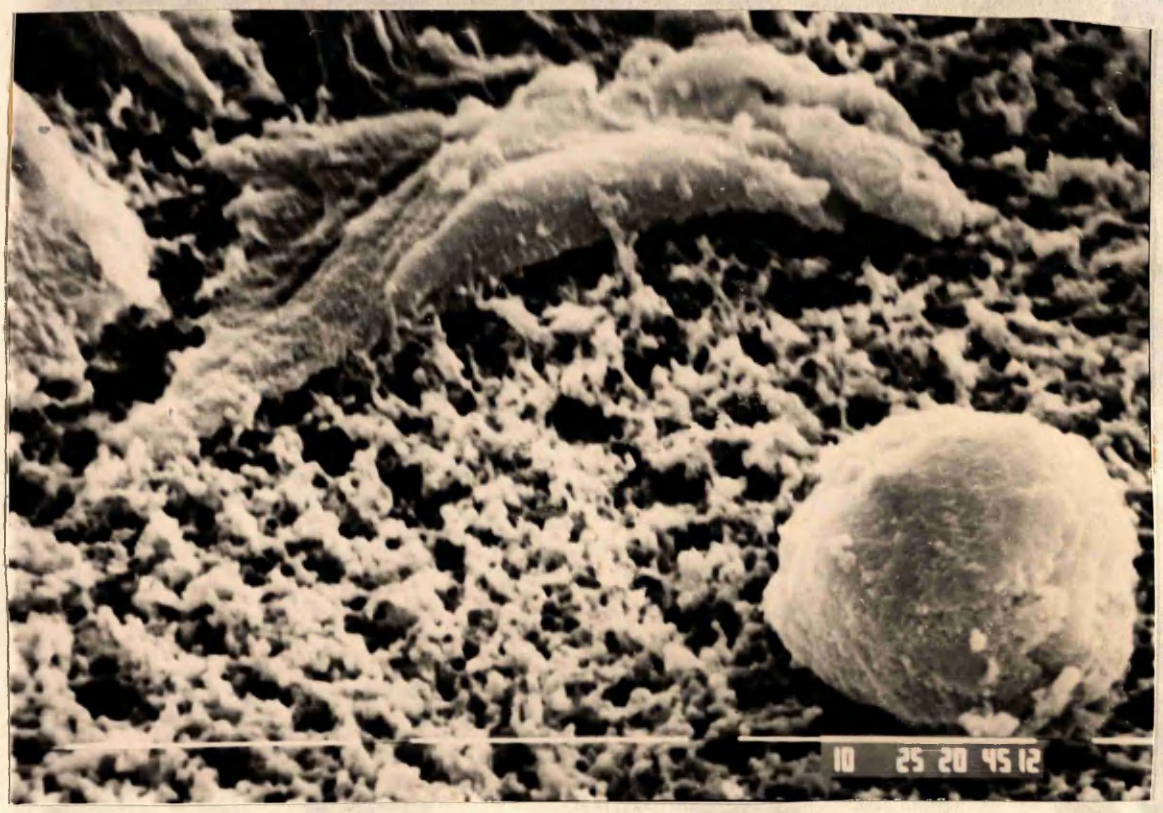


Fig. 51 _ Globular cells with a number of microvilli.
(Mag. 2000).

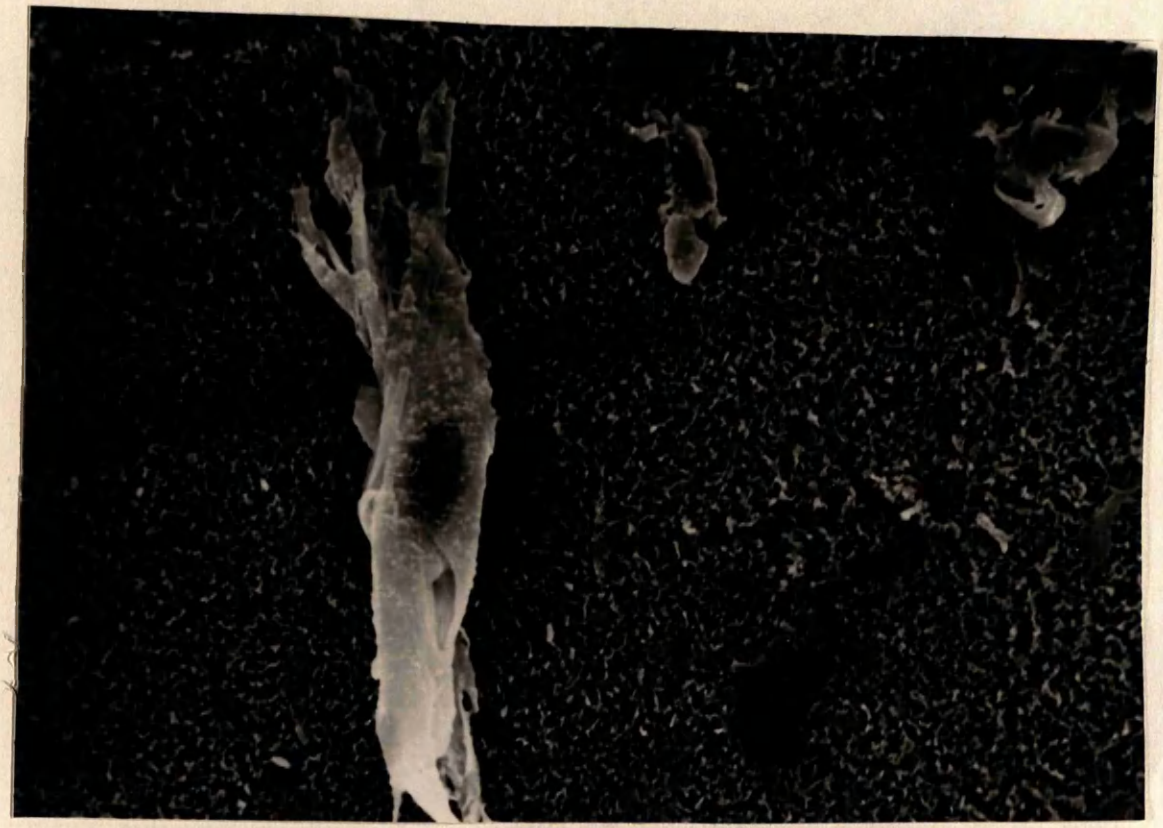


Fig. 52 _ Dendritic cell at the 5th epithelial layer. (Mag. 750)

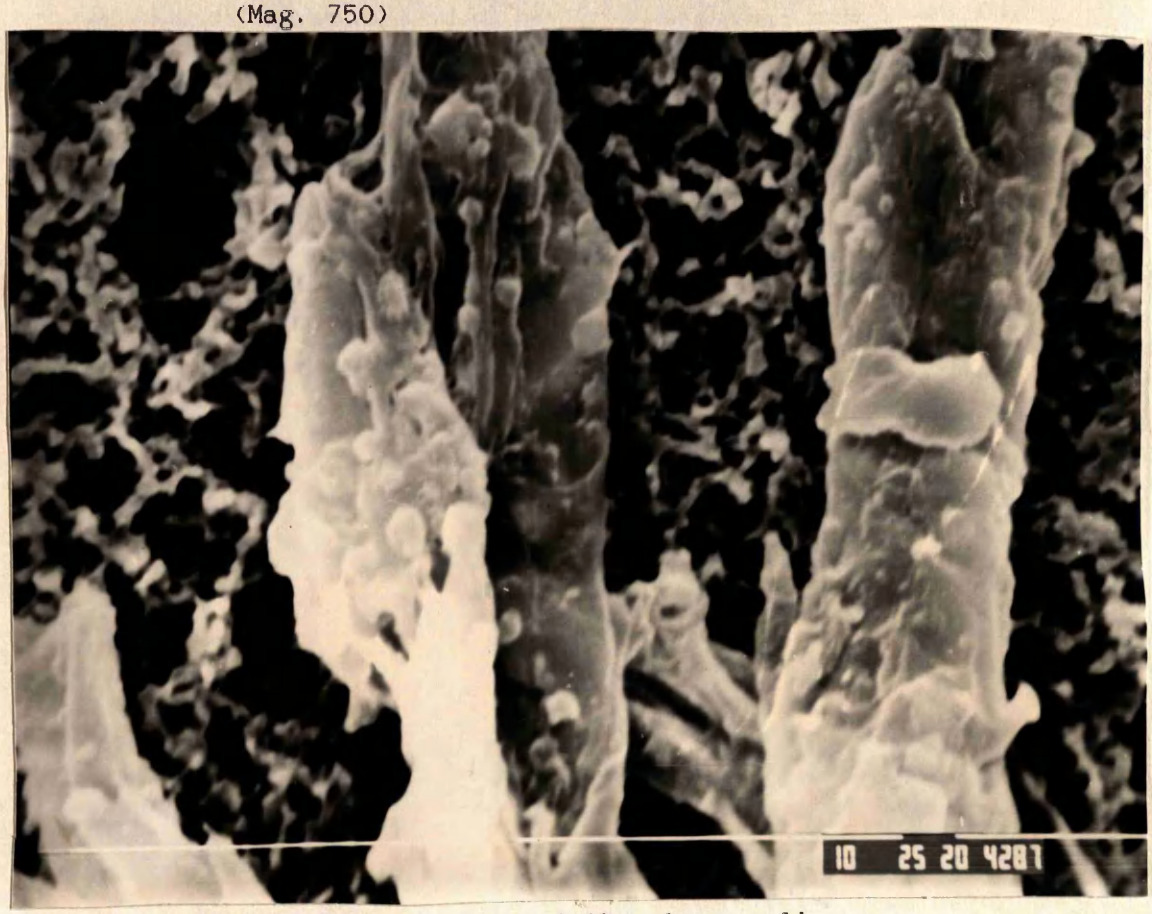


Fig. 53 _ Higher magnification of the above cell showing granules. (Mag. 5000).

present in the epithelial layers. However, it is difficult to differentiate between a nerve fibre and a young melanocyte process with no melanin granules (Wanko et al, 1964). In the first layer, nerve fibres and / or dendrites are crossing an epithelial cell (Figure 54). In the sixth layer, dendrites are criss-crossing each other and other epithelial cells (Figure 55) whilst in another specimen they are attached to one end of the cells (Figure 56).

8-4-2 SEM of the superficial epithelial layers of dry eye patients with Sjögren's syndrome.

In general the shape of the cells is flat polygonal which is similar to that of the normal superficial layer (Figure 57). However, there is a wide range of variation in the size and shape of cells in the same specimen. These variations range from cuboidal shaped cells (Figure 58), to columnar cells with their nucleus at the middle of the cell (Figure 59) and columnar cells with their nucleus at one end of the cell (Figure 60). Microprojections are seen in flat cells but not in cuboidal or columnar cells. Dark and light cells are also seen in these specimens. The light cells are smaller than the dark cells. Goblet cells are seen at the epithelial surface although their number is markedly reduced (Figure 61). Debris is also present in a number of specimens (Figure 62).

8-5 Discussion

In this study, the morphological changes of the epithelial cells from longitudinal shaped cell at the basal layers to flat cells at the superficial layer have been confirmed. However the changes in

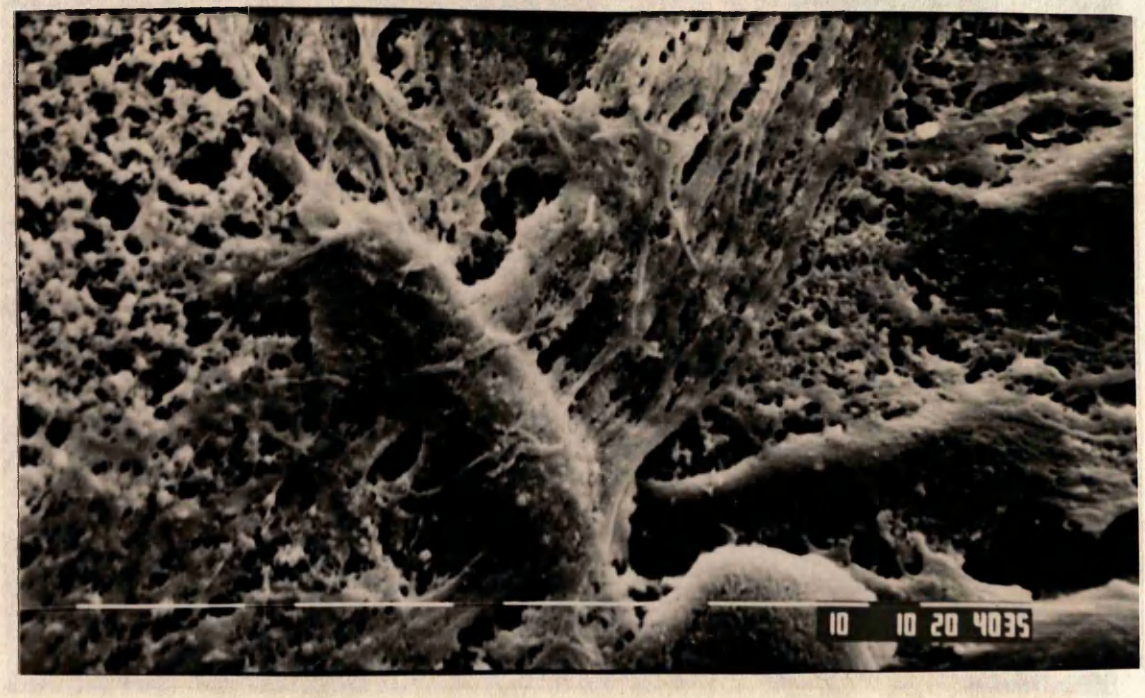


Fig 54_ Nerve fibres or Dendrites crossing epithelial cells. (Mag. X2000)

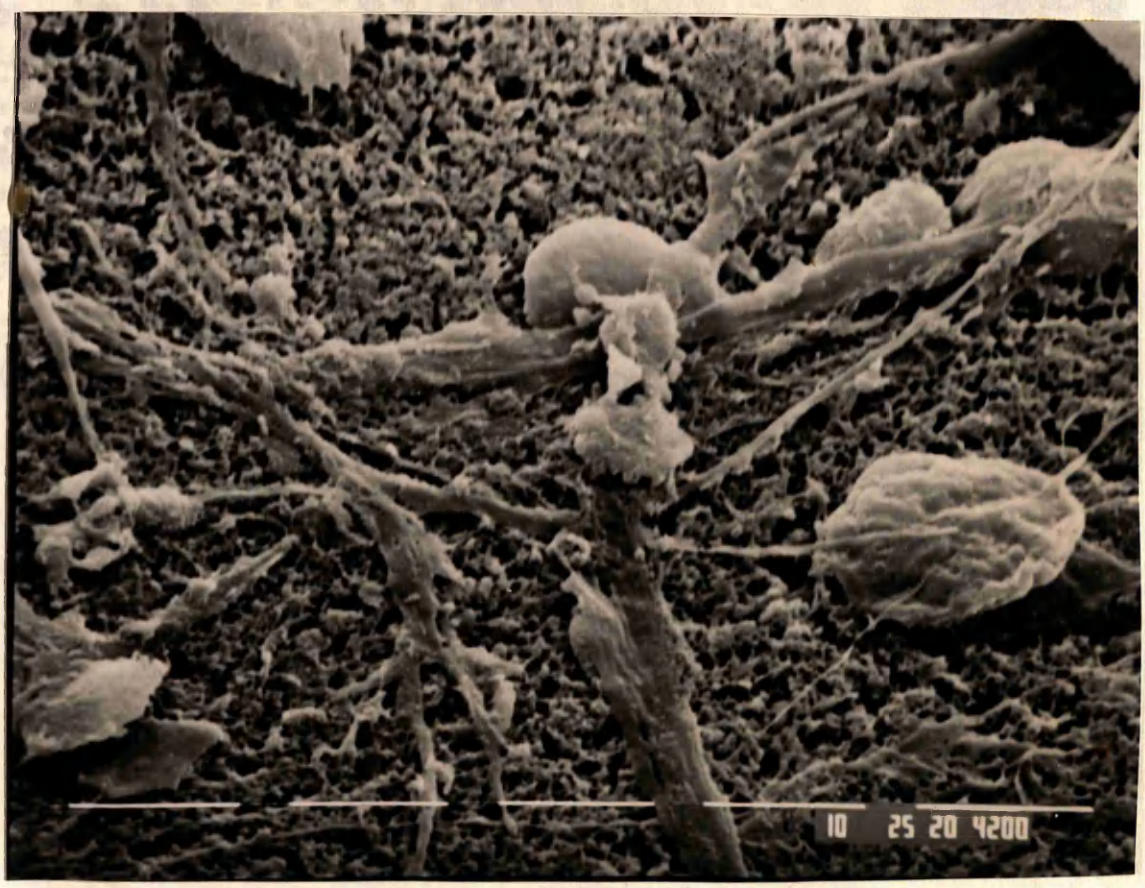


Fig. 55_ At the 6th layer, dendrites are crossing an epithelial cells. (Mag. X2000).

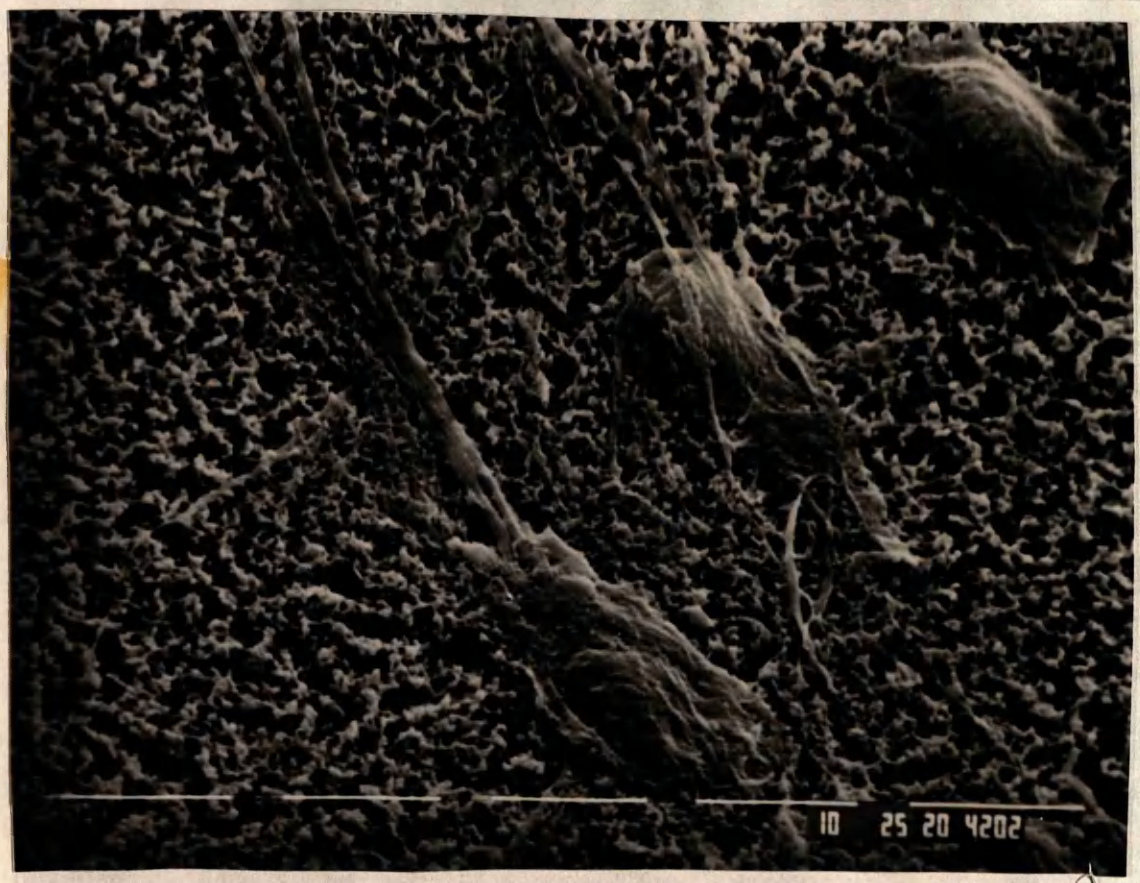


Fig. 56_ Dendrites attached to one end of a cell. (Mag. X2000)

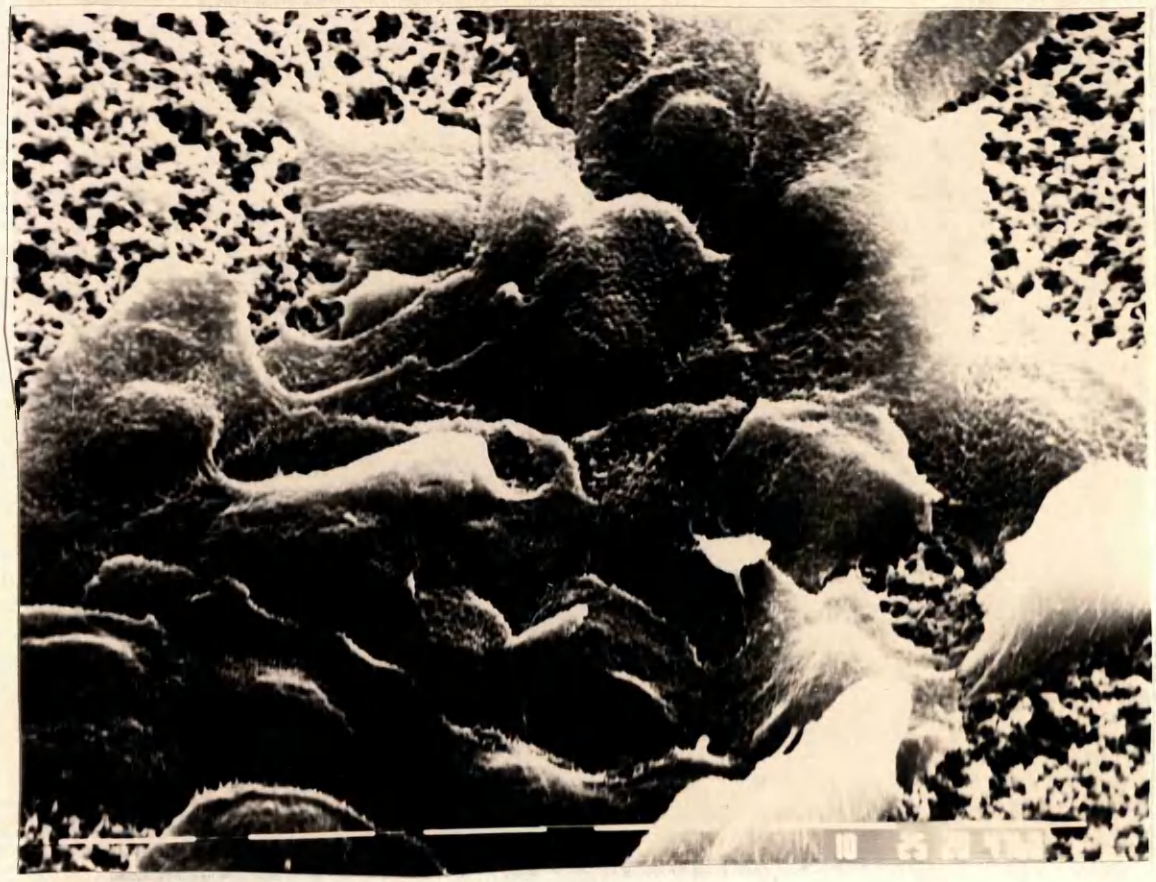


Fig. 57 _ Flat polygonal cells in Sjogren's syndrome patient. (Mag X1500)

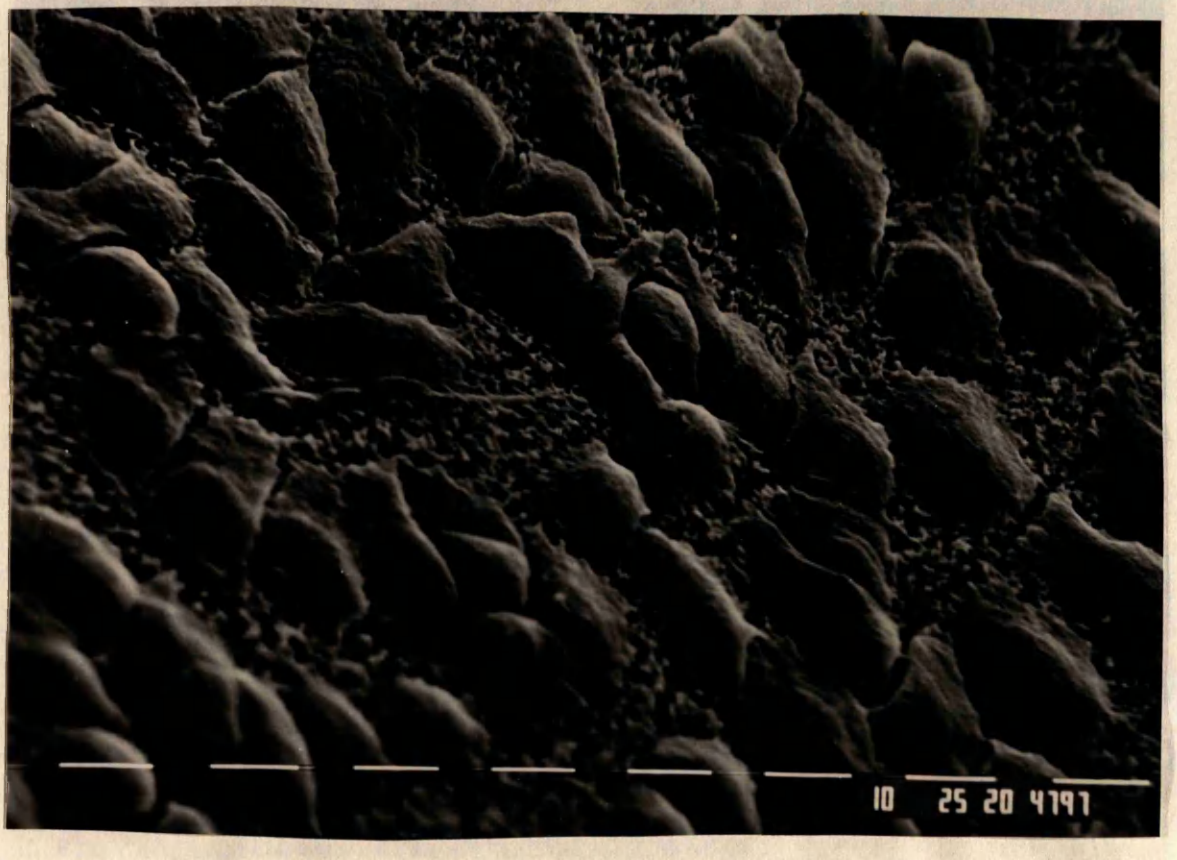


Fig. 58_ Cuboidal cells in the superficial layer. (posterior view) (Mag. X1000)

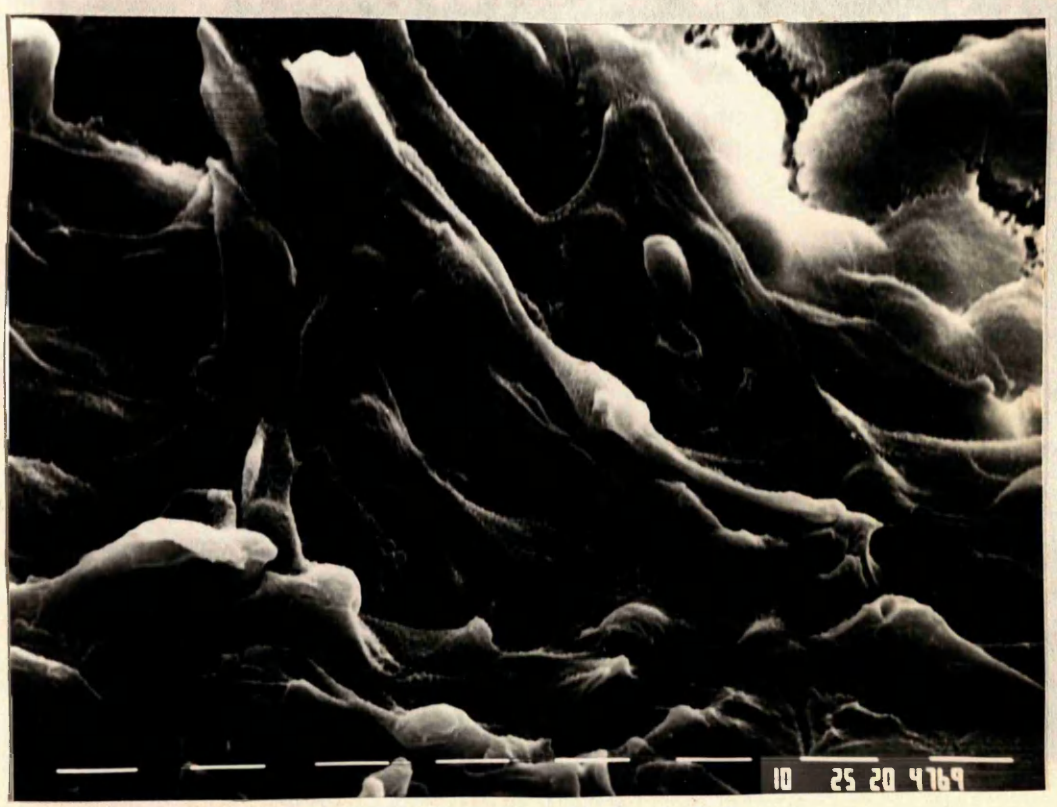


Fig. 59- Columnar cells with the nucleus in the middle of the cells. (posterior view) (Mag. X1000)

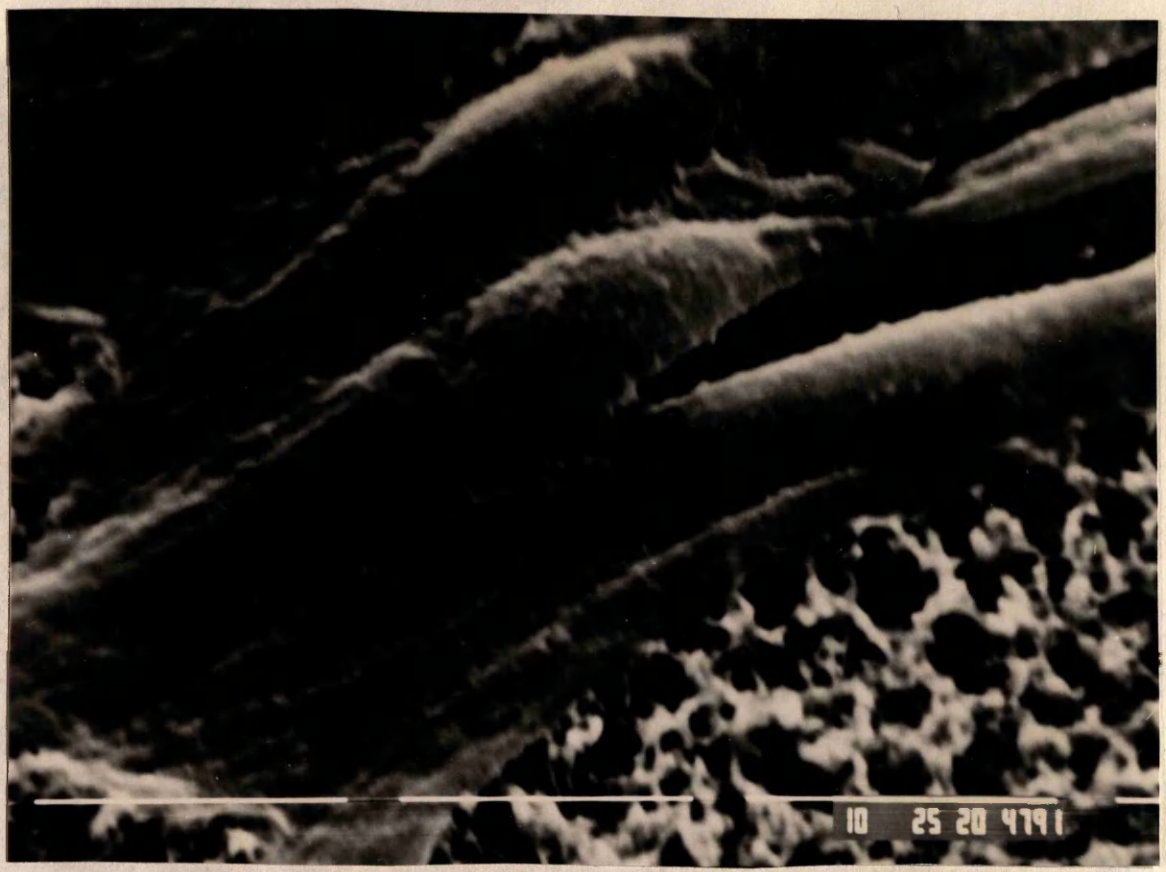


Fig. 60_ Columnar cells with the nucleus at one end of the cells. (posterior view) (Mag. X3500)

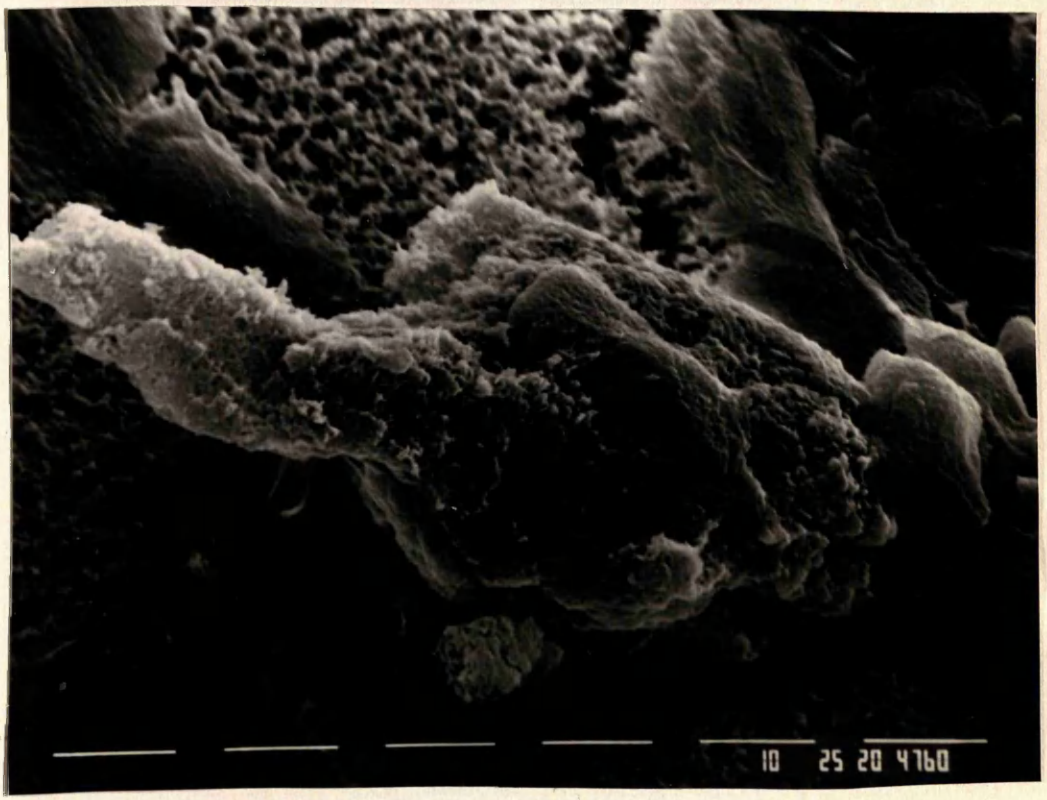


Fig. 61 _ Mucus from a Goblet cell in Sjogren's syndrome patient. (posterior view) (Mag. X1500)

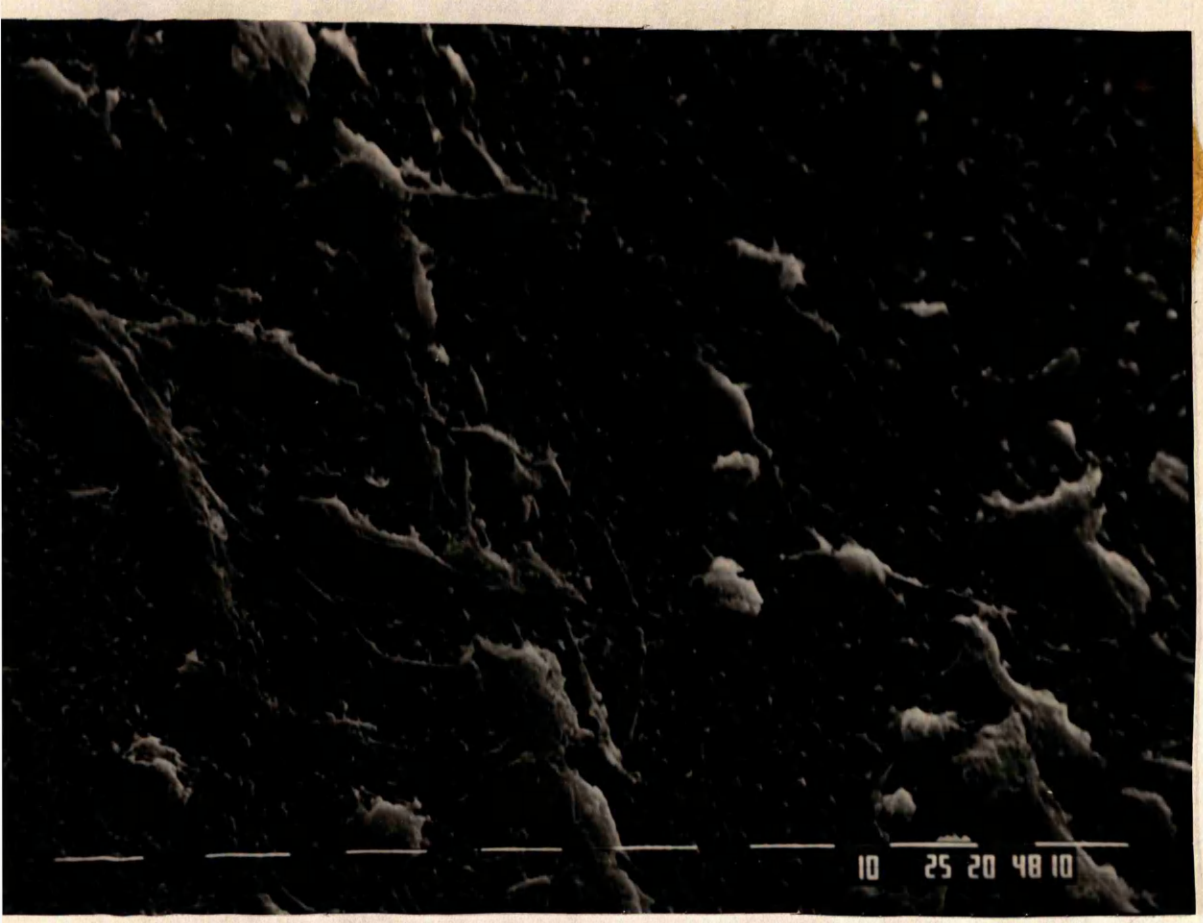


Fig. 62- Debris is seen on a filter paper from patient with Sjogren's syndrome. (Mag. X1000).

the shape of the epithelial cell borders in the different layers of the epithelium, have not been reported before. In the seventh layer, the columnar cells are generally rounded with their nucleus at one end while at the other end there is a small flat border (Fig. 41). The flat borders increase in length gradually as the cells approach the surface (Fig. 40). At the surface, they are completely flat apart from the central bulge of the nucleus (Fig. 36).

The epithelial cells in Sjögren syndrome patients, show flat polygonal as well as cuboidal and longitudinal cells. This indicates a rapid loss of the superficial epithelial cells which was not compensated by an equally rapid turnover of mature flat cells. Pfister and Renner (1977) found in experimental dry rabbit cornea that clumps of cells of two layer thickness are lost within 24 hours. They also found a significant reduction of microprojections in the epithelial cells.

Wanko, Lloyd and Mathews (1964) found that the gross cell structure of human tissue samples of various ages did not differ substantially.

The different degrees of brightness between light and dark types of cells have been observed only in scanning electron micrographs. The degree of brightness may be due partly to the angle of the reflecting beam but it does not explain the structural difference between light and dark cells. Pfister (1973) found that dark cells have double the number of microvilli per square micron than light cells and the lenghth of the microvilli is one half those of the light cells. Nevertheless there is no known functional difference between the two types. Greiner, Covington and Allansmith

(1977) classified the epithelial cells to dark, medium and light cells. The dark and medium cells were similar in strucure in contrast to the light cells. They also suggested that the dark cells are probably older than the light cells. The presence of dark cells in the seventh and eighth layer contradict this suggestion.

The openings found in the first layer, are of two types, the intercellular orifices and the central holes. Dark et al (1974) described the intercellular orifices as openings of the underlying mucous crypts. Kessing (1986) examined the different types of crypts which are found in normal conjunctiva. The saccular type crypts are normally present at the fornix and the orbital areas although they are found near the limbus too. The central holes are the openings of empty goblet cells. Marsh and Swift (1969) examined the small intestinal mucosa under SEM. They showed empty goblet cells with fenestrated membranes at its opening which are adherent to the interior of the goblet cell, as seen in figure 12.

The globular cells at the eighth and fourth layers are probably immature goblet cells or lymphocytes. The cell at the eighth layer has a smoother surface and is slightly smaller in size than the cell at the fourth layer. The argument regarding the origin of the goblet cells has not been settled. One assumption that the conjunctival goblet cells are formed from ordinary epithelial cells, of an intermediate or superficial situation, due to external irritation. However Parsons found goblet cells at all depths of the epithelium and Binder assumed that the goblet cells form from the basal epithelial cells.

The clear advantage of this technique is the demonstration

of the extension and the distribution of dendrites and nerve fibres in the different layers of the conjunctiva which can not be seen in histological sections. However it is difficult to distinguish between dendrites of young melanocytes and Langerhan cells and the nerve fibres.

The choice of the bulbar conjunctiva with its multiple layers and wider structural differences gives a better understanding of the morphology of the epithelial cells throughout the different layers. The superficial epithelial layer of the two volunteers shows a cellular uniformity of shape and size which is absent in the conjunctival specimens of dry eye patients where there is a tendency toward longitudinal shaped cells.

The use of the impression cytology technique in scanning electron microscopy is a new concept. It provides a panoramic view of the deeper layers of the conjunctiva, showing in detail the size, shape and the inter - relationship of the epithelial cells, goblet cells and inflammatory cells of normal and pathological conditions. Furthermore, SEM gives a third dimensional view to the cellular structures which is a valuable help in following the changes that occur in the epithelial cells as they reach the surface. It may give us a better understanding of the structural changes that affect the epithelium directly or indirectly from the surrounding tissues.

There are two main drawbacks to this technique. First, the cellular strucure of the epithelial cells is unavoidably disturbed during the impression on the conjunctiva. This happen regardless of how much care is taken in reducing the eye globe movement or maintaining the steady handedness of the operator. Secondly, the

conjunctival cells are viewed by their posterior surface since the anterior surface is attached to the filter paper.

CHAPTER NINE

Conclusions and Recommendations

Dry eye disorders are complex conditions with varied manifestations and causes. The management of these conditions is a challenge to clinicians as well as a wide range of researchers. For the patient, there is no cure for most of these disorders. However, the important factor is that the clinician and the patient should work together for the relief of the symptoms.

The work described in this thesis is divided into four parts. The first part is the standarisation of the optimum filter paper surface. The second part is the clinical results of topical retinol acetate on Sjögren syndrome patients with dry eyes. The third part comprises the cell culture trials and the last part deals with examining conjunctival epithelial cells under scanning electron microscopy.

All the clinical and laboratory studies in this thesis has included the use of "Millipore filter paper". The filter paper has proved its efficiency in providing one or two layers of the conjunctival epithelium. The filter paper is very fragile and difficult to handle. The use of plastic in the

preparation of the Applicator, made it easy to put a uniform pressure on the conjunctiva without any damage to the filter paper. It also made the staining procedure described in chapters five and six easy to handle and more efficient, by staining a number of specimens at one time. Another important feature of the "Millipore filter paper" which was described in the thesis is its ability to grow cells on its surface. It also has a resistance to any damage or change to its surface in the face of different chemical and physical preparatory procedures. The main disadvantage of the "Millipore filter paper" is its thickness, which makes it difficult to cover with a glass cover slip after mounting on a glass slide for light microscopy. Therefore I would recommend a thinner filter paper which would be easier to prepare and also easier to examine the cellular details.

"Millipore filter paper" has uniform pore size, however, the company produces different pore sizes. Several researchers chose different pore sizes for impression cytology. The choice of pore size is empirical. Therefore I would recommend a trial for the optimum pore size in obtaining the maximum amount of epithelial cells and for obtaining them in single layers.

The result of standaisation of filter paper for impression cytology showed clearly that the dull

surface yields more epithelial cells than the shiny surface. The presence or absence of surfactant has no effect on the yield of epithelial cells.

Impression cytology as a non-invasive technique has proved to be of immense value in the diagnosis of epithelial cell lesions. The period of the staining procedure takes about one hour, therefore the results can be available in a short period of time.

The results of the treatment of dry eyes in Sjögren syndrome patients with retinol acetate ointment were not satisfactory. These results are in accordance with other researchers who used vitamin A preparations for dry eye conditions with the exception of cases of malnutrition and specifically in vitamin A deficiency where the results are very encouraging. It may be that vitamin A preparations have no significant effect in the management of dry eyes in general and Sjögren syndrome patients in particular.

In the cell culture trials, the filter paper was used first to obtain epithelial cells from the conjunctiva and then using the filter paper for epithelial growth. After pipetting the epithelial cells on the filter paper surface, the number of cells were few. The use of more than one filter paper would increase the initial number of cells. The experimental work which would merit pursuit as a sequel to this thesis could be as follow. My first recommendation is

to find the optimum number of filter papers in a dish that will give the highest initial number of epithelial cells for cell culture. The second recommendation is to find out the optimum time the epithelial cells should be left on the filter paper before pipetting them from the filter paper surface. The third recommendation is to examine the effect of different growth factors on the epithelial cell culture. The fourth recommendation is to study the effects of wide range of substances including drugs on the epithelial cell culture. Also comparative studies of cell cultures between the superficial and deeper conjunctival epithelial layers could be carried out with an emphasis on the morphological difference between the relatively new epithelial cells taken from the deeper layers of the conjunctiva and the older cells of the superficial epithelial layers. Further studies on these cells by SEM and TEM could also be carried out.

The study of the conjuctival epithelial cells by scanning electron microscopy using impression cytology is a new concept. It showed the changes in morphology of the epithelial cells at different depths. It also showed nerve fibres and dendrites spreading between the epithelial cells. The technique revealed the posterior surface of the epithelial cells. There may be minimal alteration in the position

of epithelial cells with each other caused by the pressure on the conjunctiva. This technique opens, for the future, a wide range of morphological studies of normal and abnormal ocular surfaces in human and animals. It also provides the opportunity for further studies of the normal morphological changes of the deeper layers of the ocular surface. Moreover, further detailed structural studies of cells of indefinite origin, can be performed using transmission electron microscopy. For example, in chapter eight, there were spherical cells of indefinite origin which could be examined further, by taking a tiny part of the filter paper which included these cells and then processing and examining them under TEM. This may provide information concerning the type and origin of such cells.

In conclusion there is a wide range of further research required by different relevant disciplines. This work covers a very small part of ongoing research in dry eye disorders. The development of the methods and techniques described in this thesis will hopefully provide strategies for further investigations.

APPENDIX 1

LIST OF EQUIPMENT AND REAGENTS

The equipment and reagents employed in all the experiments in this thesis are listed. The suppliers and manufacturers' addresses are listed at the end of the appendix.

	Product	Source
1.	Centrifuge Machine	Rotamixer DeLuxe
2.	Chemical Solutios and Powders	
	Aluminium sulphate	Sigma
	Basic Fuchsin	George T. Gurr
	Activated Charcoal	B.D.H.
	Dodeca tungstophosphoric acid	B.D.H.
	Ethyl alcohol	B.D.H.
	Ethylene glycol	B.D.H.
	Formaldehyde 36%	B.D.H.
	Glacial acetic acid	B.D.H.
	Haematoxylin anhydrous (C1 No 75290)	Sigma
	Hydrochloric acid (conc.)	B.D.H.
	Magnesium Sulphate	B.D.H.
	Orange Green	Sigma
	Periodic acid	Sigma
	Potassium metabisulphite	B.D.H.
	Sodium hydrogen bicarbonate	Koch-Light Lab. Ltd.
	Sodium iodate	B.D.H.
	Sodium metabisulphite	B.D.H.
	Xylene	B.D.H.
3.	Cell culture dishes	
	24 well multidishes (Model FB-16-24-TC)	(Nunclon ®) Flow Laboratories Ltd
	Tissue culture Petri dishes with airvent	(Nunclon ®) Flow Laboratories Ltd
4.	Critical Point Drier (CPD 750)	"Emscope"
5.	Eye Drops	
	Benoxinate HCL 0.4% Minims	Smith & Nephew
	Rose Bengal Minims	Smith & Nephew
	Saline Minims	Smith & Nephew
6.	Flourets	Smith & Nephew

7. Films

Ektachrome 400 colour slides	Kodak
Ilford FP4 ISO 125/22°	ILFORD Ltd.

8. Filter Papers

Millipore Filter Paper	"Millipore" Ltd.
Whatman Filter Paper No.1	"Whatman" Ltd

9. Glassware

Glass slide	Chance Propper
Cover slide	Chance Propper
Staining trough	Griffin & George
GIBCO bottle	GIBCO

10. Laminar Air Flow Hood

"Microflow" type

11. Microscope

Scanning Electron Microscope (JSM - T200) — Jeol Ltd.

Leitz contrast-phase inverted microscope

12. Miscellaneous

Schirmer paper	Clement Clarke Ltd
Swing Handle Slide rack	Raymond A. Lamb

13. Mounting Medium

DePex	George T. Gurr
Permount	Raymond A. Lamb

14. Plastic PVC sheet (Product No.7332163) — D.I.Y. Plastic Ltd.

15. Scanning Electron Microscopy Preparation

Cylinder Stubs	Agar Scientific Ltd.
Glutardehyde 3.5%	
Cacodylate Buffer 0.2M	

16. S.E.M. Coating

Unit E 5000 — Polaron Equipment Ltd.

17. Tissue Culture Preparation

Dulbecco's growth medium	GIBCO
Tryptose Phosphate Broth	Flow Labs Ltd.
NaHCO3 7.5%	
L-glutamine	GIBCO
Antibiotic-Antimycotic	Flow Labs Ltd.
NaOH	
Foetal calf serum	GIBCO
Sterile water	
1ml plastic pasteur pipette	

Source	Address
Agar Scientific Ltd.	66A Cambridge Road Stanstead, CM24 8DA
B.D.H.	Burnfield Avenue, Thorniebank, Unit 7 Altens Trade Centre Glasgow G46 7TB
Chance Propper	Spon Lane, Smithwick Worley.
Clement Clarke Int. Ltd.	15 Wigmore St. London W1H 9LA
D.I.Y. Plastic Ltd.	Lynton Road, Cheney Manor, Swindon, Wilts. SN2 2PN
Emscope (CPD 750)	Emscope Laboratories Ltd. Kingsnorth Technology Park Watton Road, Ashford, Kent TN23 2LN
Flow Laboratories Ltd.	P.O. Box 17 Second Avenue Industrial Estate Irvine, Ayrshire Scotland, KA12 8NB
George T. Gurr	London NW9, England.
Griffin & George	Bishop Meadow Road, Loughborough, Leices. LE11 ORG
ILFORD Ltd.	Mobberley, Cheshire
Jeol Ltd.	1418 Nakagami, Akishima, Tokyo 196
Koch-Light Lab Ltd	Colnbrook, Berks.
Kodak	Eastman Kodak Co. Rochester, N.Y. 14650
"Millipore" Ltd. UK	Millipore House The Boulevard, Ascot Road, Coxley Green, Watford, Herts.,

Nunclon	
Polaron Equipment Ltd.	21 Greenhill Crescent Hollywell Industrial Estate Watford, Herefordshire WD1 8XG
Raymond A. Lamb	Raymond Lamb 6 Sunbeam Road London NW10 6JL
Rotamixer DeLuxe	Hook & Tucker Insruments Ltd.
SIGMA	SIGMA Chemical Co. Ltd Fancy Road, Poole, Dorset, BH17 7NH
Smith & Nephew	S&N Pharmaceuticals Ltd. Bampton Road, Harold Hill, Romford, Essex RM3 8SL
"Whatman" Ltd.	Unit 1, Coldred Road, Industrial Estate, Parkwood, Maidstone, Kent.

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