TEAR PROTEIN COMPOSITION AND CONTACT LENS WEAR

by

Murchison George Elton Callender

A thesis submitted for the degree of

Master of Philosophy

in the

University of Aston in Birmingham

October 1985

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Summary

Tear Protein composition has been extensively investigated since lysozyme, one of its major components, was discovered by Fleming in 1922. Much of the current interest lies with the analysis of its components and their interaction with the contact lens during wear.

Because of the limit volume of tear fluids, some of its components are not readily accessible for analysis. Recent developments in micro-electrophoresis and immunochemistry have enabled the identification, characterization and quantification of proteins found in tears.

The use of hydrogels in contact lens wear have been plagued with problems associated with proteinaceous deposits and chemical preservatives which appear to provoke an adverse eye reaction. The aetiology of which may be immunologic and/or toxic. Preservatives such as Thimerosal and Chlorhexidine appear to bind or complex with the denatured protein on the lens. These preservatives concentrate and are later released onto the eye to precipitate a toxic reaction. In addition the preservatives may inhibit the four principal proteins - Lysozyme, Lactoferrin, Specific tear prealbumin and IgA, that play a protective role against infection.

A 24 week group comparative study was initiated to investigate changes in tear protein composition with hydrogel lens wear and its associated care regimen. Acrylamide gel electrophoresis was used to look at the distribution of the total protein profile while immunoelectrophoresis was employed to quantify lysozyme and IgA levels among four groups of subjects.

In general, it was found that there was not significant difference among the groups in the total protein, lysozyme and IgA (secretory) levels over the period of 24 weeks ($p=0.0001$). Also there was no significant difference in protein profiles among the Group with respect to time ($p=0.0001$). However, there was a gradual increase in protein content with time which may be due to seasonal changes.

Key Words

Tears - Protein - Electrophoresis - Contact Lens - Hydrogels

INTRODUCTION

Much of the current interest in human tears lies with the analysis of its components and their interaction with the contact lens during wear. Understanding the composition of the tears can lead to a more meaningful analysis of subjects with normal tears or, with various ocular and systemic diseases or those wearing contact lenses.

Because of the limited volume of tear fluids some of its components are not readily accessible for analysis. Recently, sensitive analytical techniques have been developed for the identification, characterization and quantification of proteins found in tears. Such a development is not only important for understanding the physiologic role of tear proteins but is also a valuable diagnostic tool.

Considerable progress has been made in the last decade in the development of hydrogel contact lenses for the correction of refractive anomalies of the eye. Yet numerous questions concerning the interaction of hydrogel material with the tear fluid components remain unanswered.

It is the purpose of this dissertation to review the literature on the lacrimal system with special reference to the tear protein composition and to attempt to correlate clinically observable changes in the eye's response to hydrogel contact lens wear with changes in the tear protein composition.

ACKNOWLEDGEMENTS

I would like to extend my sincere thanks to the following people:

Professor Graham F.A.Harding for his encouragement and guidance during the course of the work presented in this thesis.

Dr. John L. Larke who initiated my involvement in this work while I was on sabbatical at his Soft Lens Research Laboratory,The University of Aston in Birmingham. His support and advice are greatly appreciated.

The members of staff and technicians of the Department of Ophthalmic Optics for their assistance.

Professors Emerson Woodruff and Jacob Sivak for their encouragement and the opportunity to complete this work during their respective terms of office as Director,School of Optometry, University of Waterloo Ontario, Canada.

Professor Paul Morrison, Department of Biology, University of Waterloo, for his advice,continual support and use of his laboratory facilities.

The faculty and staff of the School of Optometry, University of Waterloo for their advice and assistance.

The subjects who gave up their time to participitate in this study.

Mr Chris Young and Dr. Dan D. Sheni for their assistance with the statistical analysis of the data.

I would like to acknowledge the following organizations for their financial support to this study: Ontario Ministry of Health Fellowship, Nuffield Foundation Fellowship, Bausch & Lomb Soflens International, Ciba Vision Care Inc. and Burton Parsons-Alcon Inc.

I am grateful to Joan MacLean, Judi Carter, Louise Walchuk, Diane Marleau and Cynthia Baptiste for typing and processing the thesis through the computer services, University of Waterloo.

ABBREVIATIONS AND SYMBOLS

Angstrom	A
centimeter	cm
degree Celsius	oC
dioptre	D
gram	g
hour	hr
international unit	IU
litre	l
mean	x
mercury	Hg
microgram	ug
microlitre	ul
milliampere	mA
milliequivalent	mEq
milligram	mg
millilitre	ml
millimetre	mm
millimole	mmol
milliosmole	mOsm
minute	min
molar	M
mole	mol
nanometre	nm
parts per million	ppm
poly-hydroxyethyl methacrylate	PHEMA
poly-methyl methylacrylate	PMMA
second	sec
standard deviation	SD
standard error of the mean	SEM
statistical analysis system	SAS
volume	vol
week	wk

DEDICATION

This thesis is dedicated to my parents:

Elton

and

Dorothy (deceased 23/11/84)

My sincere thanks to my wife, Chloe, and daughters, Paula and Janielle for their love, understanding and support throughout the time spent on this thesis.

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Chapter 1

THE LACRIMAL SYSTEM

1.1 Introduction

The lacrimal system is of paramount importance for the maintenance of the optical integrity and normal eye function. An important contribution of this system is that it provides and maintains a continuous tear film covering the cornea and conjunctiva. The tears serve to protect, lubricate, cleanse and maintain an optically uniform corneal surface.

The system, as shown in Figure 1., consists of: (1) a secretory system, which provides the lipid, aqueous and mucin components: (2) a distributory system regulated by the eyelids and the blinking mechanism and (3) an excretory system which expels excess secretions and contaminants by way of the lacrimal duct (Jones, 1973; Holly and Lemp, 1977).

1.2 The Secretory System

Human tears are produced by a group of glands (Fig. 2) generally described as the lacrimal glands or the lacrimal secretory system (Botelho, 1964). Because of the complex neurogenic control of these glands they are classified as basic, reflex and psychogenic secretors (Botelho, 1964; Jones, 1966).

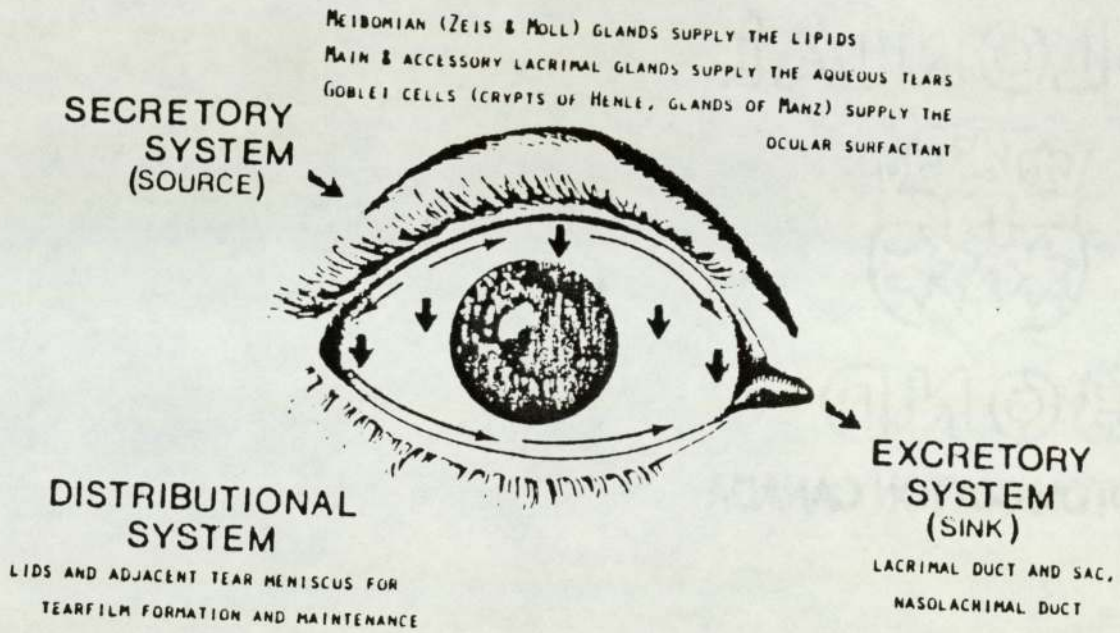


Figure 1: Schematic Diagram of the Lacrimal System (taken from Holly and Lemp, 1977)

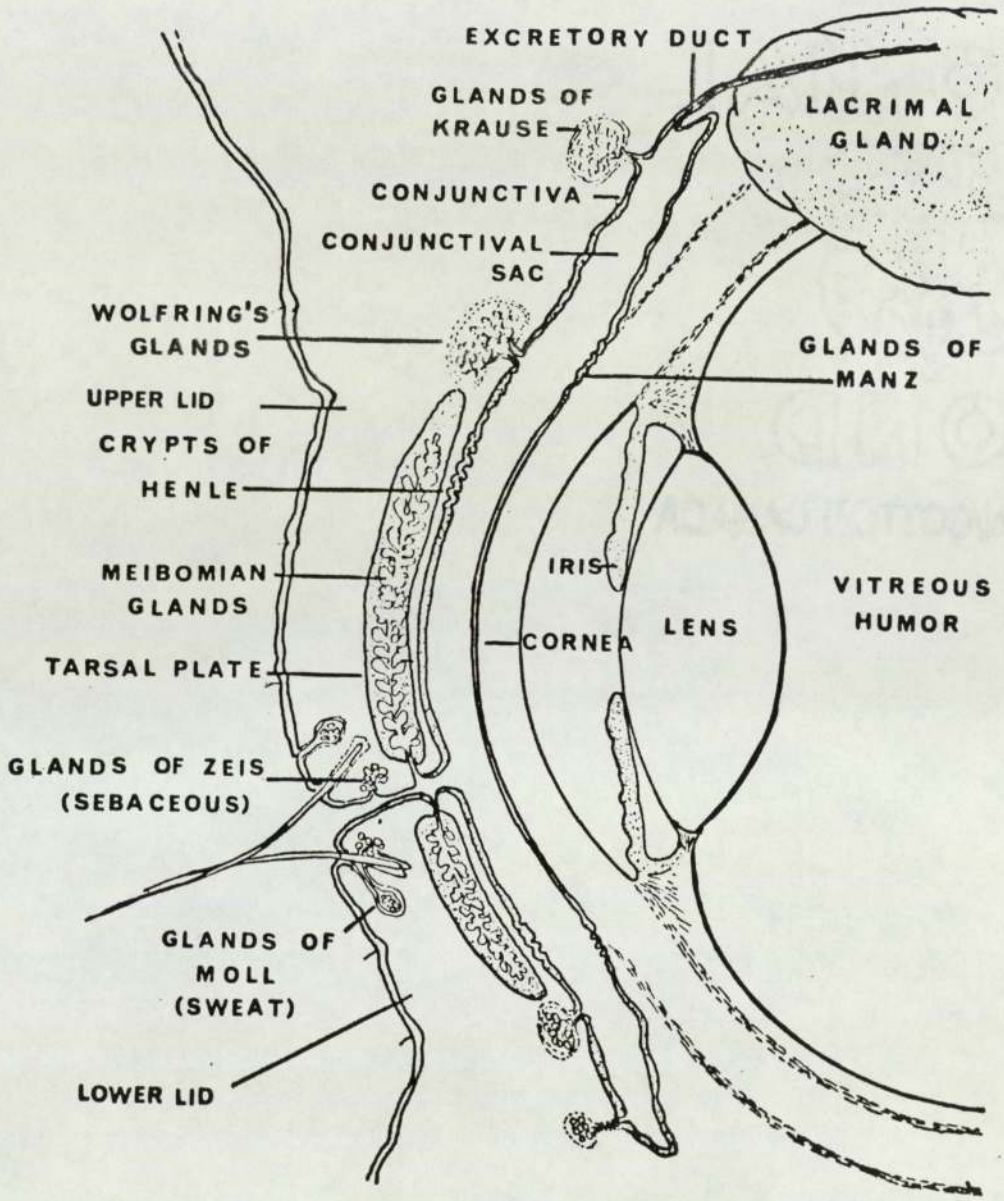


Figure 2: A Section of the Eye and Lacrimal Apparatus. Modified after Botelho (1964).

1.2.1 Basic Secretors

The basic secretors are exocrine glands with no known efferent nerve supply (Jones, 1966). These glands produce a continuous supply of secretions which are vital for the preservation of vision in some vertebrates. These include all vertebrates that spend all or some part of their life on land and two completely aquatic mammals, the whale and seal (Botelho, 1964).

Jones (1966) states that only the basic secretors are functional during the first few days or weeks of life in most new born. Normally during sleep or when the eyes are closed only the basic secretors are active.

The basic secretors consist of three types of glands which produce the triple-layered precorneal tear film. These glands are described by Wolff (1946) and more recently by Jones (1966) as the oily, aqueous (watery) and mucin secretors.

a. *The Oil Secretors*

The oil secretors consist of the Meibomian glands in the upper and lower tarsi, and the glands of Zeis in the palpebral margin of the upper and lower eyelids (Fig. 2). The major contributors to this oily secretion are the Meibomian glands. (Jones, 1966).

b. *The Aqueous Secretors*

These are the accessory lacrimal glands of Krause located near the upper and lower conjunctival fornices and the accessory lacrimal glands of Wolfring, three of which are in the upper margin of the upper tarsus and one in the lower tarsus (Fig. 2). There are also some minor glands in the plica and caruncle which together assist in the production of the middle or aqueous layer of the precorneal film (Jones, 1966).

c. *The Mucin Secretors*

The mucin secretors are the goblet cells of the tarsal conjunctiva and of the crypts of Henle along the upper and lower tarsi as shown in Figure 2 (Jones, 1966; McEwen, 1962).

There is also histochemical evidence of mucin-producing cells in the lacrimal glands (Allen *et al.* 1972; Jensen, *et al.* and Michelsen, 1969).

The amount of mucin secreted, by these glands per day has been estimated by Ehlers and co-workers (1972) to be 2 to 3 ul. Most of this mucin secreted forms the inner mucin layer of the precorneal film which is loosely attached to the microvilli of the corneal epithelium. The rest is dissolved in the aqueous layer.

1.2.2 Reflex Secretors

These are the principal lacrimal glands located superiorly and temporally to the globe, in a bony fossa, in the roof of the orbit (Wolff, 1946; Jones, 1966). They consist of a larger superior or orbital portion and a smaller inferior or palpebral portion (Fig. 2).

The reflex secretors are histologically similar to the accessory lacrimal glands of Krause and Wolfring but appear to differ in their neurosecretory control. Whereas the basic secretors appear to function independent of either afferent or efferent nerve supply, the reflex secretors are controlled by sensory fibers of the fifth cranial nerve (afferent pathway) and the seventh cranial nerve, which is the efferent pathway (Duke-Elder, 1968; Jones, 1966).

Recent studies by Gillette and co-workers (1980) confirmed that the lacrimal glands and accessory lacrimal glands are similar both histologically and in their secretory products. However, the presence of myoepithelial cells in the accessory gland tissue suggests autonomic innervation. This finding contradicts the classical differentiation of these two types of aqueous secretors.

The reflex secretors have only one efferent nerve supply but because of the many afferent pathways, the main lacrimal gland may be stimulated in four major ways according to Botelho (1964) and Jones (1966). These are peripheral, retinal, psychogenic and secretagogue stimuli.

a. *Peripheral Sensory Stimulation*

The sensory nerve endings of the conjunctiva, cornea, uvea, nasal mucosa and the skin in the immediate area of the eye may be stimulated by trauma, foreign bodies such as a contact lens, environmental pollutants etc., to activate reflex secretions. These secretions may also be activated when the basic secretions become inadequate.

b. *Retinal Sensory Stimulation*

Light entering the eye stimulates reflex tearing. Hence, under normal conditions retinal stimulation by light provides a reflex component of the basic secretions which is derived from the main lacrimal glands (Jones, 1966). The amount of this secretion is kept constant because of retinal adaptation to light. However, when the intensity of the light is suddenly increased or in pathological photophobia, this component is altered. Retinal reflex secretion is absent when the eyes are in the dark-adapted state and during sleep. (Jones, 1966).

c. *Psychogenic Stimulation*

This type of reflex stimulation is unique to man. It is never seen in lower animals (Botelho, 1964; Jones, 1966). Any strong emotional reaction, including joy and laughter, triggers tearing. This type of secretion is of no apparent physiological significance to the normal eye.

d. *Secretagogue Stimulation*

Stimulation of the secretory cells by "secretogogue" agents, such as mecholyl, pilocarpine and mustard gas derivatives, transported via the blood stream, cause both basic and reflex tearing (Botelho, 1964; Jones, 1966).

All of the aforementioned types of reflex secretions except peripheral sensory reflex, are unaffected by topical anesthesia of the cornea and conjunctiva. (Record, 1979).

1.3 The Distributory System

The tears secreted are distributed by the lids and the hydrostatic gradient in the tear meniscus along the edges of the upper and lower lids (Holly, 1980).

Jones (1973) believes that with each blink, there are changes in the pressure beneath the upper lid which help to distribute the tears. After each blink the pre-corneal tear film is reformed with the resurfacing of the mucin layer and the redistribution of the aqueous and lipid layers.

When the eyes are open, tear distribution to the exposed parts is made primarily by the physical forces of the surface, interfacial tension and gravity (Jones, 1973; Holly 1980). This is demonstrated by the flow of tears along the upper and lower marginal tear strip or meniscus. These join laterally, to form a tear pool. Medially the tear strips pass into the lacus lacrimalis which in turn carries the tears by capillary attraction and gravity into the lacrimal excretory system (Jones, 1973 and Maurice, 1973).

1.4 The Excretory System

The excretory system consists of the lacrimal puncta, canaliculi, sac and naso lacrimal duct. Tear drainage through this system is closely involved with the interaction of the lids, orbicularis muscles and preseptal muscles. The act of blinking propels tear secretions from the lacus lacrimalis through the lacrimal passage and out the inferior meatus into the nasal passage.

It is estimated that the excretory system removes 90 percent of the tear secretions; the rest is lost through evaporation (Jones, 1973).

Chapter 2

PHYSICAL PROPERTIES AND CHEMICAL COMPOSITION OF THE TEAR FILM

2.1 Introduction

The physical properties and chemical composition of human tear film have been extensively investigated over the last 200 years. However, because of the scantiness of the tear secretions and variability in composition, due to such factors as evaporation, inadequate mixing of the secretions of the various glands and the disproportionate contributions of the basic and reflex secretors, the published data, summarized in Table 1, are variable. (Altman, 1961; Lambert, 1983)

2.2 Physical Properties

The first documented chemical analysis on tears was done by Fourcrory and Vanquelin (1791) who described the tear fluid as a clear watery alkaline liquid which when heated, evaporates leaving a little oil and many salty substances. Duke-Elder's (1968) review of some of the early investigations on the physiochemical properties of this watery fluid indicates that it has a specific gravity slightly above unity; a conductivity ($\lambda \times 10^{-5}$) 1,950 to 2,272; viscosity (η) 1.050 to 1.405; surface tension (γ) 0.694 to 0.749 and refractive index 1.336 to 1.3369.

Other physical properties of tears, such as rate of secretions, osmotic pressure, pH and temperature, which are of a more recent era of investigation, require a more detailed review in light of their clinical importance in the formulation of ophthalmic solutions, including contact lens solutions and in the fitting characteristics of contact lenses.

Table 1. The Properties and Composition of Human Tears (Modified after Lambert, 1983)

Characteristic Physical Properties	Value
pH	6.5-7.6
Osmolarity	302 ± 6.3 mOsm/L
Volume	6.5 ± 0.3 µL
Evaporation rate	10.1×10^{-7} gm/cm^{-2}/sec^{-1}
Flow rate	1.2 µl/min
Refractive index	1.336
Surface tension	40.1 ± 1.5 dyne/cm

Composition	Concentration
Water	98.2%
Sodium	145 mEq/L
Potassium	20 mEq/L
Chloride	128 mEq/L
Bicarbonate	26 mEq/L
Calcium	2.11 mg/dL
Magnesium	Trace
Zinc	Trace
Glucose	3 mg/100 ml
Amino acids	8 mg/100 ml
Urea	7-20 mg urea N/100 ml
Oxygen	155 mg Hg (eyes open)
Total protein	0.9 ± 0.1%
Lysozyme	1.3 ± 0.6 mg/ml
Complement	Present
Mucus secretory substance	Present
Lysosomal hydrolases	Present
Lysosomal enzymes	Present
Lactate and Pyruvate	Present

2.2.1 Secretion Rate

The rate of tear secretion was first investigated by Schirmer (1903). He measured the rate of moistening of filter paper strips inserted into the lower conjunctival sac of patients with extirpated lacrimal sacs. The flow rate was found to be 0.67 gm per 16 waking hours which is equivalent to 0.6 to 0.8 ul per minute. The Schirmer technique, although widely used as a diagnostic test, is questionable because the irritation caused by the filter paper induces varying amounts of reflex secretions (Norn, 1965).

Recent studies based on the dilution rate of dyes and radioactive tracers applied to the marginal tear strip, provide a more reliable measure of the basic rate of tear secretions (Maurice, 1963; Mishima, *et al.* 1966; Norn, 1965; Sorensen, 1975). Mishima and co-workers (1966) measured the tear turnover rate as 16 percent per minute with the aid of fluorescein and a fluorophotometer. From this information they computed the average tear volume to be 7.0 ul and the average tear flow rate as 1.2 ul per minute. Sorensen (1975) found the rate of tear flow was 0.6 ul per minute by measuring the decay rate of radioactivity of a radioisotope placed in the tears. The results from these two recent techniques do not vary markedly from the less sophisticated Schirmer filter paper technique.

There is also general agreement among investigators that the tear flow rate decreases with increase in age and that there is minimal difference between the sexes except in young females who have a higher secretion rate than that of males (deRoetth, 1953; Furukawa and Polse, 1978; Henderson and Prough, 1950; Wright and Meger, 1962). In some ocular diseases such as kerato-conjunctivitis sicca (KCS) there is a significant reduction in tear secretion (Wright and Merger, 1962).

There are many drugs that affect the tear secretions. Antimuscarinics such as atropine and scopolamine are known to decrease tear secretions (Crandall and Leopold, 1979). Antimuscarinic compounds are also found in several over-the-counter preparations such as sedatives, nasal decongestants, antitussives, internal analgesics and antidiarrheals which decrease tear secretion and may result in symptoms of discomfort in contact lens wearers (Chang, 1977).

Garner and Rahi (1976) reported a reduction in tear secretions in 14 of 22 patients on Practolol, a beta - adrenergic receptor blocking agent, used in the management of cardiac dysrhythmia and ischaemic heart disease.

There are also many reports of drugs that increase tear secretions. Crandall and Leopold (1970) classified these as muscarinics, sympathominetic, antihypertensive and miscellaneous drugs, such as marijuana and heroin in chronic users.

2.2.2 Osmotic Pressure

The osmotic pressure of the tear fluid is of prime importance in the maintenance of the optical integrity of the cornea. Electrolytes such as sodium, potassium and chloride ions, are the main contributors to osmotic balance. Proteins, because of their high molecular weight and low concentration in tears, contribute an insignificant part of the total osmotic pressure (Mastman *et at*, 1961; Van Haeringen, 1981).

There is general agreement among investigators that the normal osmotic pressure of tears is similar to that of plasma which is equivalent to 0.90 percent sodium chloride solution (Krogh, *et al.* 1945; Mishima, 1965; Schaeffer, 1950). However, the tear tonicity is subject to dynamic change because of such factors as, tear evaporation between blinks, rate of tear flow and the amount of metabolites excreted into the tears (Mishima, 1965; Van Haeringen, 1981).

Terry and Hill (1978) using a dewpoint depression technique, found that tear samples collected from normal subjects during the waking day ranged from 0.90 to 1.02 percent sodium chloride equivalents. The mean value was about 0.97 percent sodium chloride equivalents or 310 mOsm/kg. These findings are in close agreement with those reported by Mastman and co-workers (1961) and more recently with Gilbard and co-workers (1978). It is interesting to note that with prolonged lid closure, the osmotic pressure of tears decreases. This decrease is attributed to a reduction in the evaporation of the basal tear secretion (Terry and Hill, 1978).

Since tear osmolarity is a function of tear secretion and evaporation, any abnormal decrease or increase in tear secretion will alter the state of hydration of

the cornea. In keratoconjunctivitis sicca (KCS) there is a decrease in tear production, as indicated by the Schirmer test (Mackie and Seal, 1981) which results in an increased tear osmotic pressure and dehydration of the cornea.

Gilbard and co-workers (1978) found an average tear osmolarity of 343 mOsm/L in a group of patients afflicted with KCS. On the other hand, normal patients fitted with contact lenses show a marked increase in lacrimation during the contact lens adaptation period. This reflex tearing dilutes the electrolytes and proteins, thus decreasing the tear osmolarity. This resulting relative hypotonicity reflects the corneal edema often seen in the early stages of contact lens adaptation (Callender and Morrison, 1974; Hill, 1978; Schmidt *et al.*, 1974; Uniacke and Hill, 1970).

2.2.3 pH

The tear pH closely approximates that of the blood plasma, pH 7.4 (Abelson *et al*,1981; Carney and Hill, 1976; McEwen, 1962). Wide variations have been found by Carney and Hill (1976) in the normal population and in a given individual at different times of the day. They found the pH ranged from 7.14 to 7.82. It appears to be lowest under conditions of prolonged lid closure as during sleep, because of the acid by-products associated with the relative anaerobic state, and increases as the eyes are opened because of the loss of carbon dioxide (Carney and Hill, 1976; Norm, 1977).

The tears are more alkaline with increased lacrimation (McEwen, 1962) and in certain external inflammatory diseases (Tapaszto , 1973). Tear samples collected directly from the lacrimal glands also showed a more alkaline pH (Rexed, 1958). The greater alkalinity of the tears in these reports appears to reflect the type of tear composition which McEwen (1962) attributes to the presence of the basic protein, lysozyme.

In spite of the variations in tear pH reported, it is maintained within a relatively constant range, because of the buffering system which consists of bicarbonate and protein species present in the tears (Carney and Hill, 1979; Iwata, 1973).

2.2.4 Temperature

The temperature of the tears is rather difficult to measure because of the scantiness of the fluid. However, it can be inferred that it is not too different from that of the corneal surface.

The temperature of the corneal surface has been measured Markovitch (1951) as 30°C, by Amano (1954) as 35°C and by Matthauis (1961) as ranging between 30 to 35°C. Ehlers (1965) cites the work of Braendstrup (1952) which indicated that the epibulbar temperature was higher in children than in adults and decreased with increasing age. The temperature was slightly higher nasally than temporally. Prolonged lid closure resulted in 0.5°C increase in temperature while a decrease of 1°C resulted if blinking was prevented for 5 minutes.

Hill and Leighton (1965) made the first attempts to measure the corneal temperature under a contact lens. They noted that the corneal temperature was altered by wearing contact lenses. Hamano (1978) reported that wearing a polymethylmetharcrylate (PMMA) rigid contact lens resulted in an initial decrease of 1.5°C in corneal temperature followed by a gradual increase. This initial decrease in temperature is due to the heat of evaporization of the tear film on the lens surface. As the lens surface becomes dry, the cornea is warmed up by thermal conduction. In contrast, wearing the polyhydroxyethylmetharcylate (PHEMA) soft contact lens, results in a decrease in corneal temperature with no subsequent increase. When the soft contact lens attains the corneal temperature, its water content decreases and its optical characteristics are altered. These changes in the soft contact lens are more pronounced in high water content soft lenses (Fatt, Chaston, 1980a & 1980b).

It is apparent from all these reports that the temperature of the tear film depends on such factors, such as the ambient temperature, the frequency of blinking, the rate of evaporation from the cornea and the type of contact lens material being worn.

2.3 Chemical Composition

Wolff (1946) was the first to describe the tear film as consisting of three layers: a superficial lipid layer; a middle aqueous layer; and a layer of mucin adjoining the corneal epithelium (Fig. 3). Since this description, numerous reports have appeared regarding the composition and function of each layer of the tear film herein reviewed.

2.3.1 Lipid Layer

The outer lipid layer of the tear film is approximately 0.1 um thick (Fig. 3). Its functions are to reduce the rate of evaporation of the underlying aqueous layer and to form a barrier along the lid margins to prevent the overflow of tears onto the lids (Mishima, 1965).

There is a lack of agreement among investigators on the nature and relative amounts of the different lipid classes. The three principal classes in tears are wax esters, cholestryl esters and triglycerides (Tiffany, 1978). Pes (1897) reported that the meibomian secretions contained fat, fatty acids and cholesterol. Linton, and co-workers (1961) identified neutral lipds, an unidentified lipid and some pospholipids. They could not detect the presence of cholesterol and free fatty acids. Ehler (1965), using a more reliable analytical procedure, found both cholesterol and cholesteryl esters, fatty acids, phospholipids and traces of trglycerides. Nicolaides and co-workers (1981) identified both wax esters and cholesteryl esters among the neutral fats as well as other hydrocarbons, free fatty acids and mono, di and triglycerides. This lack of agreement among investigators on the tear lipids composition is due not only to inadequate analytical procedures but also to sample collection techniques. In many studies the analysis was done on pooled samples, either from one individual or a group of individuals. Tiffany (1978) found that no two meibomian samples taken from the same individual are alike and that there are significant differences in lipid class composition between individuals with normal tear film functions.

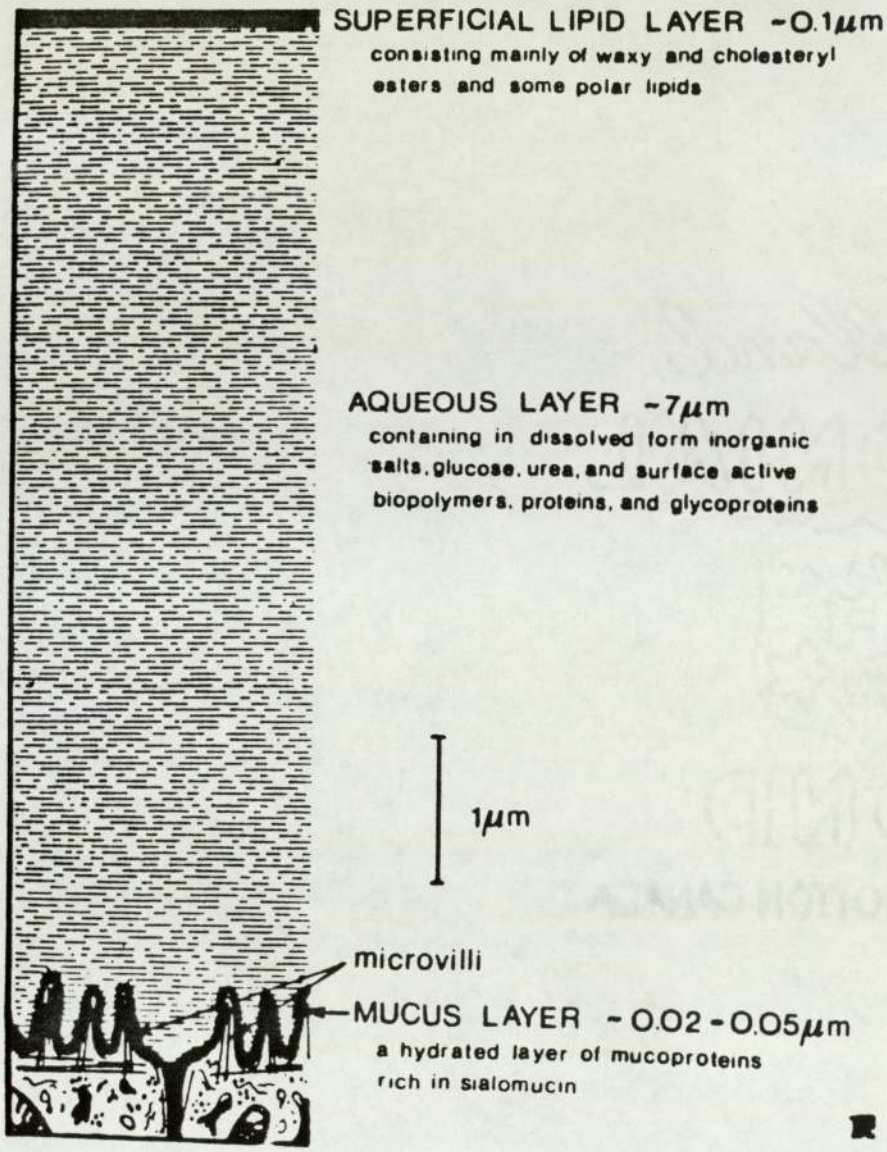


Figure 3: The Structure and Composition of the Tear Film (Taken from Holly and Lemp, 1977)

In spite of their high molecular weight, lipids melt at or near the corneal temperature (Brown and Dervichian, 1969). Andrew (1973) attributes the fluidity of these lipids to the number of branched chains and unsaturated hydrocarbons in the long-chain alcohols and fatty acid groups making up the lipid molecules. Recently, Nicolaides and Ruth (1982) found an unusual group of highly polar fatty acids in steer and human meibomian lipids which they believe aid in the spreading of the hydrophobic lipid layer over the aqueous layer.

2.3.2 Aqueous Layer

The aqueous layer makes up the bulk of the tear film; the thickness of which is about 7 μm (Fig. 3). It provides lubrication for the eyelid movement over the cornea and is the medium for transporting nutrients and antibacterial components necessary for the protection of the eye against pathogenic organisms. It also flushes away metabolic waste and foreign particulate matter which may be harmful to the cornea and conjunctiva (Holly and Lemp, 1977).

This layer is composed of 98.2 percent water and 1.2 percent solids (Ridley and Sorsby, 1940). The solids consist of both small and large molecular weight components. The small molecules make up the electrolytes, glucose, urea and free amino acids, while proteins, lipids and mucopolysaccharides constitute the macromolecules (Iwata, 1973).

The small molecules play an intrinsic part in the regulation of the osmotic pressure of the cornea and in supplying its metabolic needs (Van Haeringen, 1981). The principal inorganic molecules are the electrolytes sodium, potassium, chloride and bicarbonate ions. The concentrations of these have been compared with those in plasma or serum by Botelho (1964), Botelho, and co-workers (1973), Giardini and Roberts (1950) and Thaysen and Thorn (1954).

Sodium, the predominant cation among these electrolytes, was found by the Thaysen and Thorn (1954) to be equivalent to that of plasma and independent of the rate of tear secretion. The concentration varies between 142.5 and 147.0 mEq/L. In contrast, the potassium concentration is 3 to 5 times greater in tears

than in plasma of the same healthy group of subjects. A mean tear potassium level of 16.2 + 4.8 mEq/L was measured by Thaysen and Thorn (1954). This evidence supports the theory that potassium is actively secreted by the lacrimal glands, while sodium is passively transported from the blood across the blood-tear barrier. In general, any changes in the sodium level initiates the opposite change in the potassium level in order to maintain the osmotic balance between the extracellular and intracellular spaces of the cornea (Iwata, 1973).

Tapaszto (1973) found no significant difference in the concentrations of sodium and potassium between tears of healthy and diseased eyes. In fact, his findings were similar to those reported by Thaysen and Thorn (1954).

Chloride ions, like sodium and potassium play an important role in the osmotic regulation of the cornea. The mean tear chloride concentration of 128 ± 5.2 mEq/L appears to be higher than that in plasma (105.6 mEq/L) and independent of the tear secretion (Balik, 1955; Botelho, 1964; Giardini and Roberts, 1950; Iwata, 1973; Thaysen and Thorn, 1954). This evidence suggests that chloride ions are actively secreted by the lacrimal glands.

The bicarbonate ions form a buffer system with carbonate ions which may be responsible for the regulation of the tear pH (Iwata, 1973). Botelho (1964) measured the tear bicarbonate concentration as 26 mEq/L which is not signficantly different from that of serum (21-30 mEq/L).

Other inorganic ions listed in Table 1, such as calcium, magnesium, zinc, manganese and phosphate are found in low concentrations in the aqueous layer (Avisar, *et al.*, 1977; Calderia, *et al.* 1982; Tapaszto , 1973; Uotila, *et al.* 1972). The role of calcium, magnesium and zinc in diseased eyes is not clear. However, Tapaszto (1973) found that their concentrations are increased about twofold in bacterial conjunctivitis and blepharoconjunctivitis; while in iritis and iridocyclitis their concentrations are decreased to one half the normal values. Iwata (1973) suggests that calcium and magnesium may be associated with cellular enzyme activity and the regulation of cell membrane permeability.

In addition to the electrolytes, the aqueous layer contains low molecular weight organic components such as glucose, urea and amino acids. Most investigators have found that the glucose level in tears is much lower than in blood. Values ranging from 3 to 10 mg percent have been reported. (Balik, 1961; Gasset, *et al.* 1968; Giardini and Roberts, 1950). Because of this low tear glucose concentration, it is doubtful that this source of glucose plays a significant role in corneal metabolism (Maurice, 1973).

Glucose in tears originates mainly from the blood; only a small amount appears to be produced by the conjunctival goblet cells (Iwata, 1973). Evidence to support its origin from the blood is presented by Tapaszto (1973) who reported that in diseased eyes the tear glucose concentration may increase 2 or 3 times the normal value depending on the glucose concentration in the serum. Gasset and coworkers (1968) and Sen and Sarin (1980) found that most diabetics show an elevated tear glucose level following the administration of the glucose tolerance test. Such evidence suggests that the elevated glucose load in the blood results in transudation of glucose through the blood-tear barrier in the lacrimal gland.

The urea concentration in tears is reported by Thaysen and Thorn (1954) to be equivalent to that in plasma. They found a mean tear to plasma ratio of 1.02 ± 0.07. The level of plasma urea ranged from 32.8 to 138.5 mg/100 mL. Balik (1959) found the tear urea concentration higher than that in plasma. However with increased lacrimation the urea level decreased. Since the urea concentration is independent of the rate of lacrimation, it would appear that it is not produced by the lacrimal gland.

Among the nitrogenous compounds, free amino acids are also present in the aqueous layer. Balik (1958) reported the amino acid concentration in tear to be 7.58 mg/100 ml, which is 3 to 4 times greater than in serum. Flachmeyer and Weichert (1963) isolated approximately 17 amino acids from human tears. These are listed in Table 2. It is not known whether all or some of the amino acids found are secreted by the lacrimal glands, filtered from the blood or are due to the deg-

Table 2. Amino Acid Content of Human Reflex Tears (Flachmeyer and Weichert, 1963)

Amino acid	Concentration Range (ug/ml)
Alanine	5.7 - 12.1
Arginine	2.9 - 4.3
Aspartic	4.0 - 7.0
Glutamic	3.1 - 7.3
Glycine	13.4 -26.2
Leucine & isoleucine	2.6 - 5.8
Lysine	1.7 - 4.6
Serine	3.8 -13.4
Taurine	0.8 - 3.2
Threonin	3.8 -10.8
Valine	1.7 - 4.6
α-aminobutyric	Trace
Histidine	Trace
Phenylalanine	Trace
Proline	Trace
Tyrosine	Trace
Histamine	Trace

radation of proteins and polypeptides. In addition, the role played by these amino acids in the synthesis of tear proteins or the maintenance of the integrity of the external ocular tissue is not clear. The aqueous layer also contains a variety of macromolecules. These consist of proteins, mucopolysaccharides, lipids and other large molecules (McEwen, 1962).

Proteins are a major tear component, they differ both quantitatively and qualitatively from those found in serum. The concentration ranges from 0.2 to 4.52 gm/100 ml depending on the technique of tear collection and the method of analysis employed (Krause, 1959). Table 3 summarizes the techniques employed and the mean tear protein concentrations reported by various investigators.

There is general agreement among investigators that these tear proteins can be electrophoretically separated into three principal groups of protein fractions. There is a fast anodal migrating group, the cathodal migrating group (lysozyme) and an intermediate group with a mobility similar to serum globulins (Brunish, 1957; Liotet and Reveilleau, 1965; Liotet, Warnet and Arrata, 1982).

Recently, as many as 60 protein fractions have been separated by Gachon and co-workers (1979), many of which have been identified and will be reviewed in greater depth in a subsequent chapter.

Table 3. Reported Levels of Human Tear Proteins (From Callender, 1973)

Investigator	Method of Stimulating Tear Flow	Method of Protein Determination	Tear Protein Value gm/100 ml.
Magaard (1882)	Unknown	Unknown	1.4638
v. Rotth (1922)	Ammonia	Refractometry	0.25 - 0.60
Ridley (1930)	Lemon Juice	Salting-out	0.699
Junnola (1952)	Methyl-mustard Oil	Nephelometry	0.136 - 0.592
Balki et al (1953)	Unknown	Colorimetry	4.52
Brunish (1957)	Onion Vapours and air pollutants	Colorimetry	0.40 - 0.60
Erickson (1958)	Unknown	Colorimetry	0.39 - 2.90
Krause (1959)	Bromacetone	Colorimetry	0.356
Callender (1973)	None	Colorimetry	1.02 $\pm$ 0.05

2.3.3 Mucin Layer

The innermost layer of the tear film, the mucus layer, is approximately 0.02 to 0.05 um thick (Fig. 3). Mucus plays a critical role as a wetting agent by reducing the interfacial tension between the corneal epithelium and the aqueous layer and by stabilizing the extremely thin precorneal tear film between blinks (Holly and Lemp, 1971; Lemp, Holly, Iwata and Dohlman, 1970). In addition to lowering the interfacial tension, mucus maintains this low tension by masking and removing hydrophobic contaminants, such as meibomian lipids and epithelial cell debris rich in phospholipids (Holly, 1981).

The mucus layer is composed of mucopolysaccharides and glycoproteins, which are carbohydrate-protein complexes (Iwata, 1973). Histochemical studies by Norn (1969) showed that mucus threads collected from the lower fornix contain carbohydrates. Since then, numerous studies have shown, in addition to a number of tear-derived proteins and lipids, three principal mucus-type glycoproteins (GP1, GP2 and GP3M) of high molecular weight, detectable by electrophoretic methods (Iwata and Kabasawa, 1971; Moore and Tiffany, 1979, 1981). The two largest of these GP1 and GP2, appear to be aggregates consisting of the third (GP3M) held together by disulphide and other types of bonding (Moore and Tiffany, 1979). Further analysis of these indicates the presence of a high proportion of the amino acids, serine and threonine and the sugars, fructose, mannose, galactose, glucose, galactosamine, glucosamine and sialic acid (Moore and Tiffany, 1981; van Haeringen, 1981).

The site of origin of these glycoproteins has been demonstrated by Moore and Tiffany (1979) to be exclusively in the conjunctival goblet cells and not the lacrimal tissue as indicated by Allen, *et al.* (1972).

The tear film instability may be used as a clinical tool in the diagnosis of tear film deficiencies. This is a measure of the time elapsed between the last blink and the appearance of the first dry spot or break in the tear film. A tear film breakup time (BUT) shorter than 10 seconds is indicative of an abnormally unsta-

ble tear film (Lemp, 1973). Observations of a simultaneous occurence of tear film instability and a decreased goblet cell population in patients afflicted with Sjogren's syndrome by Ralph (1975) have lead to the speculation that mucus deficiency is the cause of tear film instability in these dry eye conditions. However, Dohlman and his co-workers (1976) did not find a marked deficiency in mucus concentrations in these conditions but suggested that a qualitative difference in the glycoprotein composition may be responsible for the tear film instability.

Chapter 3

HUMAN TEAR PROTEINS

3.1 Introduction

The proteins in human tears have been the object of intensive investigation since lysozyme one of the major tear proteins, was discovered by Fleming in 1922.

In early studies paper electrophoresis was the method of tear protein separation. This method did not provide optimum conditions for maximizing the separation of the many protein fractions. Consequently, only a maximum of six fractions have been detected by this technique (Brunish, 1957; McEwen and Kimura, 1955).

More recently, other types of supporting media have been used and more tear protein fractions have been separated and identified (Sapse, Bonavida, Stone and Sercarz, 1969). In general, up to 14 protein components can be detected from a single tear sample by acrylamide gel electrophoresis (Callender, 1973). However, Gachon and co-workers (1979) have detected at least 60 protein components from pooled human tears separated by two-dimensional acrylamide gel electrohoresis.

Three principal groups of tear proteins are herein described according to their relative electrophoretic mobilities, these are: the anodal albumins, cathodal lysozyme and the components of the intermediate group.

3.2 Anodal Proteins

Tear albumins make up 25-35 percent of the total proteins in normal human tears (Liotet, *et al.* 1982). They are the fastest migrating protein fractions located at the anodal end by acrylamide gel electrophoresis (Bonavida, *et al.*, 1969). These proteins are separated into two components: specific tear prealbmin and serum albumin.

3.2.1 Specific Tear Prealbumin

Specific tear prealbumin is a major tear protein fraction found in tears of most animal species but not found in other body fluids (Bonavida, *et al.* 1969).

This protein has the same electrophoretic mobility as serum prealbumin but differs in immunochemical and physical properties. Antiserum raised to serum prealbumin does not react with tear prealbumin (Bonavida, Sapse and Sercarz, 1969; Josephson and Weiner, (1968). Hence the name "Specific tear prealbumin" (STP) was proposed by Bonavida and co-workers (1969). In addition, its molecular weight, 15,000 - 20,000 daltons, is much lower than serum prealbumin 61,000 Bonavida *et al.*, 1969).

This anodal tear protein shows genetic polymorphism in acrylamide gel electrophoresis (Azen, 1976). It may be separated into 5 subfractions all of which are not always present at the same time. Band 1 is usually in combination with the slower migrating major band (2,3, or 4) or a faster major band (5). These genetic markers in tears may be of use in genetic investigations (Van Haeringen, 1981).

The absence of specific tear prealbumin in serum suggests it must be locally synthesized. Bonavida and co-workers (1969) identified sites of protein synthesis by culturing slices of the lacrimal tissue in a medium containing ^{14}C-labelled amino acids. These sites were assayed by radioimmuno-electrophoresis and gel electrophoresis to ascertain the presence of STP. Other evidence in support of STP production by the lacrimal glands is the absence of this protein in human tears after surgical removal of the lacrimal gland or in ocular diseases, such as Sjogren's syndrome, where the lacrimal glands are non-functional (McEwen *et al.* 1957, Erickson, 1955).

The role of specific tear prealbumin is still unclear. Josephson and Wald (1969) showed that STP interacts with lysozyme to enhance its bacteriocidal properties. They postulated that at pH 7.0 lysozyme, a positively charged protein, is unable to interact efficiently with bacteria which are also positively charged. Specific tear prealbumin, a negatively charged protein, neutralizes the bacteria thus increasing the activity of lysozyme.

Sapse and co-workers (1968) postulated that acidic STP and basic lysozyme, being oppositely charged proteins, provide a buffering system in the tears. Any alteration in the ratio of these two components results in eye irritation. Hence the ratio of STP to lysozyme may be used as a diagnostic index of the condition of the lacrimal gland.

3.2.2 Serum Albumin

Serum albumin, a relatively minor protein component of normal tears, migrates in an electrophoretic field at a slightly slower rate than specific tear prealbumin (Bonavida, Sapse and Sercarz, 1969). Its molecular weight is 69,000, daltons (which is greater than STP) and is immunologically similar to blood albumin (Josephson and Lockwood, 1964).

Serum albumin is not synthesized by the lacrimal gland. Its concentration increases after mechanical stimulation, and in certain disease states (Josephson and Lockwood, 1964). Such an increase can be considered to be an indicator of a physiological response at the blood-tear fluid barrier. The function of this protein in tears is not apparent.

3.3 Cathodal Protein Component

There is only one negative migrating tear protein component separated by electrophoresis. This has been identified by McEwen *et al.* (1955) as lysozyme.

Lysozyme makes up 20 to 40 percent of the total human tear protiens (Brunish, 1957; McEwen and Kiwura, 1955; Sapse, *et al.*, 1967; Liotet, *et al.*, 1982).

Since human tear lysozyme was first discovered by Fleming (1922) it has been subjected to extensive investigation. It is also found in other biological fluids and tissues in man, animals and plants. These include: saliva, serum, leukocytes, gastrointestinal mucous, spleen, kidney, liver, lungs, lymph glands, milk, eggs, turnips, cabbage and cauliflower (Selinger, Selinger and Reed, 1979).

The physicochemical properties of human lysozyme differ from other sources of this protein. When compared with hen egg lysozyme, its amino acid composi-

tion and antigenic properties are significantly different, but its molecular weight (14,000-15,000 daltons) and electrophoretic mobility in an alkaline medium are similar (Bonavida, *et al.*, 1967; Jolles and Jolles, 1967).

Tear lysozyme is produced by lysosomes, known celluar ultrastructures, found abundantly in monocyctes, polymorphonuclear leukocytes, eosinophils and basophils (Horwitz, *et al* 1978). The site of tear lysozyme production is not definitely known. However, there is evidence which suggests that it is produced in the lacrimal gland. Covey and co-workers (1971) found that there was no correlation of the lysozyme levels in paired serum and tear samples. The fact that the level in tears is significantly higher than in serum and independent of the rate of tear flow, provides added support for local synthesis. (de Koing and van Bijsterveld, 1984; Sapse, *et al*, 1968).

Recently, Gillette and co-workers (1981) identified sites of lysozyme in the acinar and ductular cells of the main and accessory lacrimal gland with the aid of an immunohistochemical technique. This evidence suggests that lysozyme is either produced in the lacrimal tissues or concentrated from serum.

Lysozyme (muramidase) is a mucolytic enzyme which catalyzes the depolymerization of sugars from peptidoglycan polymers which form the cell-walls of bacteria (Regan, 1950; Ronen, *et al.* 1975). This activity results in cell lysis, especially, of gram-positive organisms because their cell-walls are not protected by a lipopolysaccharide layer as is the case for gram-negative organisms (Selinger, *et al.* 1979).

Although the mechanism of action of human tear lysozyme is similar to that of hen egg lysozyme, its activity is 3 to 3.5 times greater at an equivalent concentration (Jolles and Jolles, 1967). This antibacterial activity is influenced by pH, temperature and the level of pyruvic acid in tears (Khan and Erdec, 1972; van Haeringen and Glasius, 1974).

In addititon to the bacteriolytic activity, several other functions of tear lysozyme have been cited by Gillette and co-workers (1981). These include: bacterios-

tatic action, bactericidal action without lysis, facilitating secretory IgA bacteriolysis in the presence of complement, determining the rate of lysis in an IgM antibody-complement system and promoting contact inhibition of cells. Lysozyme is also believed to be effective against viruses (Ferrari, *et al* 1959).

Lysozyme level in normal tears differs from that in various ocular diseases (Regan, 1950; Tapaszto , 1973). In normal tears the level does not vary with changes in tear flow rate (Sapse, *et al*, 1968; van Haeringen and Glasius, 1974). However, Ridley (1928) noted a decrease in lysozyme concentration with prolonged tearing.

Most investigators agree that there is no significant variation between sexes, race or diurnal pattern (Pietsch and Pearlman, 1973; Regan, 1950). However, Regan (1950) reported that repeated measurements done on the same individual showed a high degree of variability in lysozyme levels over periods ranging from one week to one year.

Many investigators have found that age has no effect on the tear lysozyme level (Avisar, *et al*, 1979; Regan, 1950; Sen and Sarin, 1980), while others noted a gradual decline with advancing age (Mackie and Seal, 1976; Pietsch and Pearlman, 1973). The decrease in lysozyme activity seems probable since the tear production decreases with age (Henderson and Prough, 1950). Decreased tear volume however, does not necessarily indicate a low concentration of lysozyme as Pietsch and Pearlman (1973) have observed many subjects with low tear production but normal lysozyme levels. This lower lysozyme level observed with advancing age may be a reflection of a reduction in the number of acinar and ductular cells in the lacrimal glands.

Lysozyme has been reported to be reduced or absent in some ocular diseases such as conjunctivitis, corneal ulcers, herpes keratitis, trachoma, and systemic lupus erythematosus with paralimbal keratitis (Milton, 1965; Regan, 1950; Sen and Sarin, 1980; Tapaszto , 1973). In most cases the levels are not consistently reduced to be of diagnostic value. However, in keratoconjunctivitis sicca (KCS)

and Sjogren's syndrome, the lysozyme levels are consistently and significantly lower than in normal tears. Therefore the tear lysozyme assay can be used to compliment the Schirmer tear test in the diagnosis of these cases (van Bijsterveld, 1969).

Other health problems which reflect a reduction in tear lysozyme levels are, severe protein-caloric malnutrition in children (Watson, *et al*, 1978) and smog irritation (Sapse, *et al*, 1968.

The use of some therapeutic drugs appear to alter the level of lysozyme. Erickson (1960) studied the effect of several ophthalmic drugs on the tear lysozyme level in normal subjects. She found that scopalamine was the only drug to decrease the tear lysozyme concentration significantly. Johnsson and co-workers (1978) investigated some antimicrobial agents, such as chlorhexidine and thimerosal, which are used as preservatives in some ophthalmic solutions, including contact lens solutions. They found, by *in vitro* studies, that only chlorhexidine decreased the lysozyme activity.

The effect of several β-adrenergic blocking agents on tear lysozyme were investigated by Mackie and co-workers (1977). They found that patients taking practolol showed a marked reduction in tear lysozyme level. However, Strasser and Grabner (1982) did not find a reduction in tear lysozyme level in patients treated with timolol, a beta-blocking drug for glaucoma treatment.

It appears that the measurement of tear lysozyme concentration may be a useful indicator for the early detection of adverse effects of some drugs and disinfectants.

3.4 Intermediate Protein Components

The intermediate components consist of variety of proteins that originate from either the lacrimal glands or the serum. These proteins may be classified as anibacterial agents, some of which are metal complexing proteins, immunoglobulins and complements of the immune systems and enzymes.

3.4.1 Non-Lysozyme Antibacterial Factor.

Thompson and Gallardo (1941) reported that lysozyme was not the only antibacterial factor in tears and that there was a heat-sensitive factor primarily responsible for anti-staphylococcal activity. This observation was later confirmed by Friedland and co-workers (1972) who isolated and studied the physicochemical characteristics of this tear protein which they called a non-lysozyme antibacterial factor (NLAF).

Non-lysozyme antibacterial factor is a low molecular weight protein (5,000-7,500 daltons) with an anodal electrophoretic mobility similar to the intermediate tear protein fractions (Friedland, *et al.* 1972). Such a difference in electrophoretic mobility enables its separation from lysozyme and quantification of its specific activity. Unlike lysozyme, it lacks the ability to lyse the cell wall of *Micrococcus lysodiekticus* as demonstrated by spectrophotometric assay. Nevertheless, this factor shows a stronger antibacterial effect against many potential pathogens, few of which are affected by lysozyme even at high concentrations (Friedland, *et al*, 1972).

Ford and co-workers (1976) found this non-lysozyme antibacterial factor to be similar or identical in action to beta lysin, an antibacterial factor previously identified in platelets, serum and other body fluids. They found that tears contained more beta lysin-like activity than beta lysin in serum and aqueous humor.

The site of production of tear beta lysin is not known. Since platelets, the primary source of beta lysin, are not found in tears, it is suggested that it may be actively secreted or selectively filtered from the plasma and concentrated in the lacrimal gland (Ford, *et al.*, 1976).

Recently, Selsted and Martiniz (1982) and Jansen and co-workers (1984) reinvestigated the presence of NLAF and/or beta lysin. These investigators were unable to detect tear fluid fractions with structural and antibacterial properties reported for NLAF and beta lysin.

3.4.2 Metal-Complexing Proteins

There are three metal-complexing proteins which are almost always present in human tears. These proteins, identified as lactoferrin, transferrin and ceruloplasmin, appear to have a protective role in the defense mechanism of human tears and are discussed below.

3.4.2.1 Lactoferrin

Lactoferrin is an iron-complexing protein first identified in bovine milk, human tears, and a number of external secretions (Masson, *et al*, 1966). Its distribution parallels that of lysozyme which Masson and co-workers (1969) suggest are complimentary or synergistic in their functions.

Because normal plasma lactoferrin concentration is 1.5 mg percent (Bennett and Mohla, 1976) and normal tear lactoferrin is 145 mg percent (Broekhuyse, 1974), it appears that tear lactoferrin is locally produced. Immunohistological studies by Gillette and Allansmith (1980) confirmed that lactoferrin is localized in the acinar epithelial cells of both main and accessory lacrimal glands. More recently, Stuchell, and co-workers (1981) showed that the concentration of lactoferrin increased with reflex tearing. Both studies therefore lend support for the lacrimal secretory system as the primary source of lactoferrin in human tears.

Purified lactoferrin is a thermolabile protein with a molecular weight, 82,0000 daltons, which, like transferrin, binds reversibly to iron ion but differs from transferrin both in chemical and immunological properties (Broekhuyse, 1974). It is an anodal migrating protein located among the globulin (intermediate) factions.

Lactoferrin has both bacteriostatic and bacteriocidal properties (Gillette and Allansmith, 1980). Oram and Reiter (1968) demonstrated its activity against *Bacillus subtilis, Bacillus stearothermophilus, staphylococcus aureus, staphylococcus epidermidis* and *Pseudomonas aeruginosa.* They found that in the presence of iron the virulence of some gram-negative bacteria increased. Thus it would appear that lactoferrin complexes with iron making essential iron ions unavailable for microbial metabolism. Lactoferrin also binds to proteins such as albumin, IgA,

IgG and ovalbumin. The complex formed has an electrophoretic mobility between the original proteins (Hekman, 1971). This interaction with specific antibodies IgA and IgG may produce a more powerful antimicrobial system than either lactoferrin or the specific antibodies alone.

3.4.2.2 Transferrin

Transferrin is an iron-complexing protein reported to be present in tears (Sapse, *et al*, 1969). However in some reports it can only be detected in tears, along with serum albumin and IgG, after mild trauma to the conjunctival mucosa (Josephson and Lockwood, 1964). It is probable that transferrin is not locally produced but rather a transudate from serum.

Although it has the same electrophoretic mobility and molecular weight as lactoferrin, it is immunochemically different (Broekhuyse, 1974).

Transferrin has not been associated with any ocular diseases but the similarity of its physiochemical properties with lactoferrin, particularly its binding with iron, suggests a probable bacteriostatic-related activity in depriving microbes of this essential metabolic element. Schade (1963) found that human serum transferrin inhibited several species of bacteria. However, Oram and Reiter (1968) found no inhibition of *Bacillus stearothermophilus* even at high concentrations of transferrin. They postulated that the iron-acceptor molecules in *B. stearothermophilius* possess a higher affinity for iron than serum transferrin and therefore survives in this medium. However, in the presence of lactoferrin the organism is unable to compete for the free iron. Lactoferrin has 300 times more affinity for iron than transferrin (Broekhuyse, 1974).

3.4.2.3 Ceruloplasmin

Ceruloplasmin is a copper-complexing, α-2-glycoprotein which is sporadically found in human tears (Sapse, *et al.* 1969; Liotet, *et al.* 1982). It is an anodal migrating protein with a molecular weight of 151,000 daltons (Josephson and

Lockwood, 1964). Although it is immunologically identical to plasma ceruloplasmin, its electrophoretic mobility in acrylamide gel differs somewhat (Sapse, *et al.* 1969). They attribute this difference to the sialic acid content of the glycoprotein or to genetic variations of the protein.

The site of tear ceruloplasmin synthesis is unknown. Its low concentration in tears suggests that it is not produced locally but may be transported from the blood. Josephson and Lockwood (1964) proposed an active transport mechanism since its high molecular weight prevents passive diffusion across the blood-tear barrier

The physiological role of tear ceruloplasmin is not apparent. Its serum concentration is decreased in Wilson's disease. This is a degenerative disease of the liver with ocular manifestations of copper deposition at the corneoscleral lumbus, known as Kayser-Fleisher's ring (Newell, 1965). Josephson and Lockwood (1964) suggest that this protein may be important in copper metabolism. In addition, it may play a role in certain detoxification activities, as it is a powerful oxidizing agent (Records, 1979).

3.4.3 Immunoglobulins

Immunoglobulins (Ig) are a complex group of heterogenous proteins that possess antibody activity. In humans, they are divided into five major classes: IgG, IgM, IgA, IgD and IgE, and subclasses within each group (Allansmith, 1982). The classes differ not only in antigenic specificity but also in physiochemical properties, distribution, turnover, quality and function (Harkness, 1970).

Human immunoglobulins are found in internal and external body secretions and in essentially every body component exposed to the lymphatic circulation (Hahn, 1982). Immunoglobulins found in the external secretions - tears, saliva, nasal and bronchial fluids, colostrum, breast milk, perspiration and secretions of the gastrointestinal tract are predominantly IgA but smaller amounts of IgG, IgM and IgI may be present (Allansmith, 1973; McClellan, *et al.* 1973).

3.4.3.1 Immunoglobulin A (IgA)

IgA in tears differs from serum IgA both chemically and immunologically. It consists of two molecules, each identical with serum IgA, linked by a glycoprotein chain called the secretory component or transport piece and a cysteine-rich polypeptide chain called the J-Chain (Allansmith, 1982; Harkness, 1970). Thus IgA in tears exists as secretory IgA (S-IgA).

Secretory IgA is a unique immunoglobulin in that it is the product of two types of cells - plasma cells and epithelial cells. Fluorescent antibody studies on the main and accessory lacrimal glands by Franklin and co-workers (1973) and more recently by Gillette and co-workers (1980) have identified IgA - containing plasma cells and also IgA and the secretory component. These have been localized in the epithelial cells, acinar lumina and intercellular spaces. It is unclear as to how and where the synthesis of S-IgA takes place. Allansmith (1982) suggests that the secretory component migrates to the epithelial cell surface where it combines with dimeric IgA. The combination is then secreted into the acinar lumen as secretory IgA. Secretory IgA adheres to mucus which then spreads as "immunologic paint" to protect the cornea and conjunctiva.

Secretory IgA appears to play a major role as a mucosal antibody paint with antibody specificity that is antiviral and antibacterial. Its viral neurtalizing activity in volunteers experimentally infected with rhinovirus, and its antibody activity to herpes simplex virus in normal subjects, have been documented (Bonavida, *et al.*, 1969). However, the mechanism by which it functions as an antibacterial agent is much more complex and not well understood. Dawson (1976) suggested that S-IgA may mediate compliment-dependent bacteriolysis or enhance phagocytosis. Williams and Gibbons (1972) suggested that it may act by inhibiting bacterial adherence to mucosal surfaces. This inhibitory action prevents the colonization by pathogenic organisms, thus allowing the unattached bacteria to be washed away by fluids bathing these external surfaces. This hypothesis has been confirmed by Reed and Cushing (1975) who showed that Shigella-induced kerato-

conjunctivitis in guinea pigs could be prevented by precoating the organism with S-IgA.

Most investigators found an average IgA level of 20-30 mg/100ml in normal tears (Little, *et al.* 1969; Sen, *et al.* 1976). Sen and co-workers (1978) found that the tear IgA in normal, healthy eyes appeared to increase with increase in age and it was higher in females. It does not follow a diurnal pattern but tends to fluctuate more widely than the serum levels (Allansmith, 1973; Horwitz, *et al.* 1978).

Secretory IgA levels may be altered in a variety of conditions thus the determination of its concentration in tears may be used as a diagnostic aid in many immunological disorders. Watson, and co-workers (1978) found in protein-calorie malnourished children, the concentration of tear S-IgA was significantly reduced in contrast to the elevated serum IgA level. These children showed a significant increase in susceptibility to infections of the mucosal surfaces which may be due to impaired production and/or binding of secretory component without a reduction in the synthesis rate of IgA.

Sen and Sarin (1979) found that the tear IgA level was significantly elevated in patients with certain type of acute external eye diseases such as bacterial conjunctivitis, blepharoconjunctivitis, corneal graft reaction and Keratomalacia. Some drugs also appear to alter the levels of secretory IgA in tears. Garner and Rahi (1976) reported a decrease or absence of S-IgA in tears from a group of patient who were taking practolol, a beta-adrenergic receptor-blocking agent. They found the level of tear IgG, transferrin albumin and lysozyme were unchanged in these patients. Johnsson and co-workers (1978) investigated the effects of contact lens disinfectants, chlorhexidine and thimerosal on the human eye. They found an absence of IgA and IgE in tears of patients after the use of thimerosal for one to two weeks. It was suggested that this disinfectant causes damage to the immunoglobulin - producing plasma cells.

3.4.3.2 Immunoglobulin G (IgG)

Tear IgG is identical to serum IgG both in its physicochemical and antigenic properties. This protein consists of two heavy chains held together by a pair of disulfide bonds and two light chains each of which is attached to a corresponding heavy chain, through a C-terminal cysteine, by a disulfide linkage (Harkness, 1970). The molecular weight of IgG is less than that of IgA (M.W. 150,000 vs 160,000 daltons) and its electrophoretic mobility is slower than IgA (Allansmith, 1982; Harkness, 1970).

IgG, the predominant immunoglobulin in serum, is present in low concentrations in human tears (Little, *et al.* 1969). In most investigations it is reported as being detectable but only occasionally quantifiable in normal tears (Bluestone, *et al.* 1975; Centifanto and Kaufman, 1975; Sen, Sarin, *et al.* 1978). However, McClellan and co-workers (1973) were the only investigators to quantify the tear IgG level. They found IgG levels of 14 mg/100 ml which were approximately equal to that of IgA (17 mg/100 ml).

IgG is produced by plasma cells at the rate of about 28 mg per day per kg of body weight (Records 1979). It is found in higher concentrations in circulating plasma and extravascular tissues than any other immunoglobulin. Allansmith (1979) reported that there is high correlation between serum and tear IgG levels. This suggests that serum IgG diffuses across the blood-tear barrier in the lacrimal gland where it is concentrated and released with the lacrimal secretions. Evidence to support the localization of IgG in the lacrimal tissues was first presented by Franklin and co-workers (1971) and confirmed by Gillette and co-workers (1980). In both studies, immunofluorescent staining was used to identify IgG in the interstitial and plasma cells of the main and accessory lacrimal glands.

IgG is the principal source of protective humoral immunity against infective organisms and their toxins in the extravascular tissue (Allansmith, 1982). Its mode of action is by opsonization, immobilization and fixation of complement.

McClellan and co-workers (1973) observed a rise in tear IgG levels in some eye diseases, such as herpes simplex keratitis, acute follicular conjunctivities and vernal conjunctivitis. They suggested the increase IgG levels was due to transudation of serum protein in tears. In contrast, lower levels of IgG were found in children with trachoma as compared to a control group. The role of IgG in the pathogenesis of trachoma is not clear.

3.4.3.3 Immunoglobulin E (IgE)

IgE is a unique immunoglobulin which has recently been isolated and shown to be capable of mediating an atopic type of hypersensitivity in man (Brauninger and Centifanto, 1971). This immunoglobulin consists of two light and two heavy chains, like IgG, but has a much higher carbohydrate content and consequently a higher molecular weight 200,000 daltons (Harkness, 1970).

The average levels of IgE in tears and serum have been measured by Allansmith (1972), as 61 ng/ml and 201 ng/ml respectively. Because of the sparsity of the protein, the site of production is not definitely known. Its anatomical association with mucous membranes suggests it may be locally synthesized (Brauninger and Centifanto, 1971). This hypothesis is supported by Allansmith and coworkers (1976), who identified IgE by an immunofluoresence technique in the plasma cells of the main and accessory lacrimal glands. They have also shown that the level of IgE in tears increases relative to that of serum IgE in allergic conjunctivitis which suggests transudation from serum.

The clinical importance of tear IgE has been demonstrated by Ballow and Mendelson (1980), who identified specific IgE antibodies to pollen by the radioallergosorbent test (RAST). IgE attaches to mast cells and basophils in the tissues through which it controls the release of blood components at the site of inflammation. The presence of an allergen causes the combination of allergen with the antibody IgE which is attached to the mast cell. This reaction alters the mast cell membrane, and causes the release of its histamine which produces the characteristic symptoms of allergic conjunctivitis (Allansmith, 1982).

The level of histamine in normal tears averages 10 ng/ml. This level is consistently higher only in vernal conjunctivitis (Allansmith, 1980).

3.4.4 Complement Components

The complement system is recognized as a higher order of the humoral immune system in the body's defense against infection (Yamanoto and Allansmith, 1979). It is composed of 18 or more plasma proteins which can be activated by certain antigen antibody reactions. These proteins are of extremely high molecular weights. C1 has a molecular weight between 600,000 and 1,000,000; C2, C6, C7 and C9 have 120,000 or less, and C4, C5 and C8 have between 200,000 and 300,000 (Allansmith, 1982).

The protein components may be activated by one of two pathways. The classic pathway and the alternate pathway (Allansmith, 1982; Yamamoto and Allansmith, 1979). The classic pathway is triggered when C1 binds to sites on IgG or IgM that complex with the antigen. This sets up a chain reaction in the sequence C4, C2, C3, C5, C6, C7, C8 and C9 to damage the membrane of the infective organism.

The alternate pathway involves the interaction of a group of at least four proteins, including Properdin and Factor B, which act directly on C3 bypassing the C1, C4 and C2 sequence of the classic pathway. In both pathways the C3 component is the pivot for activating the enzyme sequence C5 through C9.

Activation of the complement system in humans results in cell membrane lysis, polymorphonuclear leukocyte chemotaxis, release of histamine, opsonization and finally viral neutralization (Yamamoto and Allansmith, 1979).

The presence of these components in tears and their physiological role have been under investigation for the last decade. Chandler and co-workers (1974) detected C4 in normal tears using a hemolytic assay technique. Bluestone and coworkers (1979) using a sensitive electro-immunodiffusion technique, were only able to detect C3 in tears of the normal individual.

More recently, Yamamoto and Allansmith (1979) demonstrated the presence of hemolytic complement activity of each of the nine complement components of the

classic pathway in tears. The alternate pathway has also been shown to be present.

Kijlstra and Veerhus (1981) were unable to demonstrate the classic complement pathway in stimulated tears but found a factor which inhibited the hemolytic complement activity when added to normal human serum. They found that this heat-labile anticomplimentary factor has a molecular weight of approximately 150,000 daltons which distinguishes it from lysozyme and lactoferrin but does not distinguish it from the immunoglobulins.

The clinical significance of this anticomplementary factor is not fully understood. It may regulate complement activations on the external surface of the eye under inflammatory conditions.

The sparsity of these proteins (complement and anticomplement) in tears makes quantitative analysis difficult. Thus levels of these tear proteins have not been correlated with ocular diseases (Allansmith, 1982; Krjistra and Veerhuis, 1981).

3.5 Other Tear Proteins

A number of other protein components have been isolated from human tears. These include metabolic enzymes, lysosomal hydrolases and antiproteinases, among others (van Haeringen, 1981).

3.5.1 Metabolic Enzymes

Twelve metabolic enzymes have been identified in stimulated tears by van Haeringen and Glasius (1974a). They found the distribution of these enzymes indicated high activities of the glycolytic pathway enzymes (lactate dehydrogenase, pyruvate kinase and aldolase) and enzymes of the tricarboxylic acid cycle (malate dehydrogenase and isocitrate dehydrogenase). While those of the pentose - phosphate shunt (glucose - 6 - phosphate dehydrogenase), the sorbital pathway (sorbitol dehydrogenase) and the amino acid pathway (glutamate dehydrogenase, glutamate - oxalacetate transaminase and glutamate - pyruvate transaminase) were relatively low.

The possibility that these enzymes originated from the lacrimal gland is questionable since the corneal and conjunctival tissues, in contact with the tear fluids, may also produce these enzymes (van Haeringen and Glasius, 1974 a,b; Kahan and Ottovay, 1975).

Lactate dehydrogenase (LDH), the principal enzyme found in tears has been investigated more than the other metabolic enzymes (van Haeringen, 1981). Conflicting reports on its origin has been attributed to the differences in technique for sampling tears among investigators. van Haeringen and Glasius (1976a) did a comparative assay of LDH on samples collected by absorbent filter paper with those collected by microcapillary tubes. They found the LDH level was 10 to 20 times higher in samples from the filter paper than those from the microcapillary tubes. This suggests that the filter paper caused trauma to the corneal and conjunctival epithelia resulting in the release of LDH from desquamated epilthelial cells. MacKay *et al.* (1980) were unable to detect LDH in tear collected by capillary tubes, however elevated LDH levels were found in tear samples after gently rubbing the eyelids. Kahan and Ottovay (1975) found higher levels of LDH activity in tears and the corneal epithelium of rabbits compared with those of humans, while the activity in tear glands of both species was similar. In spite of the differences in species, it would appear that the source of LDH is from the corneal epithelium. The difference in the LDH-isoenzyme pattern between tears and serum found by van Haeringen and Glasius (1974 b) rules out the possibility of transudation of LDH from the blood into the lacrimal glands. Recently, Jacq *et al.* (1982) investigated the 5 isoenzymes of LDH. They concluded that tears, conjunctiva and lacrimal glands were poor in LDH-isoenzymes and that LDH in tears came mainly from other ocular tissues in contact with the tears; the cornea being the primary source.

Changes in the LDH content of tears and the distribution of the isoenzymes may be of diagnostic use in both ocular and systemic diseases. Kahan and Ottovay (1975) noted that various corneal diseases, mainly herpes cornea,have a tear LDH

content and isoenzyme distribution which are dissimilar from those of healthy individuals. Similar changes in tears of the diabetic reflect alterations in the metabolic processes in the cornea.

Fullard and Carney (1984) investigated the tear LDH levels relative to malate dehydrogenase (MDH) levels as an index of metabolic activity of the corneal epithelium under open and closed eye conditions. They found an elevated tear LDH/MDH ratio following overnight lid closure. This elevated LDH/MDH ratio was attributed to the hypoxic state which resulted in the unbinding of intracellular muscle type (M) LDH, increased cell membrane permeability and the efflux of LDH in the tears.

Other metabolic enzymes found in tears are amylase, hexokinase and glutamate - pyruvate transaminase (van Haeringen and Glasius, 1974C). Studies by these investigators suggest that all three enzymes are synthesized by the lacrimal gland.

The physiological role of amylase is not clear. Liotet (1969) suggests that it plays a role in glycogen metabolism by liberating glucose for the corneal epithelial cells. This enzyme shows a wide variation in activities between individuals, the level of which is dependent on the presence of calcium (Ca^{++}) in the tears (van Haeringen *et al.*, 1975). Frequent use of the ophthalmic medications containing EDTA, a calcium sequestering agent, would inhibit the amylase activity and may lead to an adverse eye reaction (Anderson and Leopold, 1979).

3.5.2 Lysosomal Hydrolases

The lysosomal enzyme activities of 10 acid hydrolases have been observed in the human lacrimal gland and tears (van Haeringen and Glasius, 1980). These have been idientified as: β-hexosaminidase, α-galactosidase, β-galactosidase, α-fucosidase, α-mannosidase, α-glucosidase, β-glucosidase, α-glucuronidase, acid phosphatase and sulfatase.

From studies on tear enzyme activities, comparing methods of tear collection, van Haeringen and Glasius (1976 a&b) concluded that these enzymes are produced

in the lacrimal gland. Singer *et al.* (1973) previously reported that hexosaminidase A levels in tears were 6 to 10 times higher than in serum. This fact makes it unlikely that these enzymes originate from the blood and are transported into the lacrimal gland. Although the high concentration of these enzymes in non-traumatized tear samples suggest they are relased from the lacrimal gland, there is no histochemical evidence in support of the conclusion by van Haeringen and Glasius (1976 a&b) that these enzymes are found in the lysosomes of the secretory cells in the lacrimal gland's acini.

Many of the inborn errors of metabolism can be identified by specific deficiency of the corresponding lysosomal enzymes in tears (Singer *el al.*, 1973; van Haeringen, 1981). The two commonly reported inherited diseases of metabolism are Tay-Sachs and Fabry.

In Tay-Sachs disease the inherited metabolic disorder is identified by the absence of β-hexosaminidase A activity in tears (Singer *et al.*, 1973). β-hexosaminidase consists of two fractions (A&B) of which fraction A is composed of 38 to 72% of the total activity; total activity reported by Singer *et al.* (1973) being 4576 ± 665 nmol per ml per hour.

Fabry's disease is another of these metabolic disorders in which the lysosomal enzyme, α-galactosidase, activity in tears can be used to diagnose the condition. This enzyme also exists in two forms (A&B) which are distinguishable by their relative thermostabilities. The major component, α-galactosidase A, is thermolabile and represents about 90% of the total activity; the remaining 10% activity is due to the thermostable component, α-galactosidase B (Johnson *et al.*, 1975). Thus the affected individual, carrier or normal can be identified by assaying tear samples for total α-galactosidase activity and again, after heating, for α-galactosidase B. The difference is the α-galactosidase A activity which is deficient in hemizygotes with Fabry disease and heterozygous carriers.

The use of tear samples for identifying patients with these inherited metabolic disorders from carriers and normals is a very practical and more economical

method than the use of serum. The collection of tear samples by absorbent filter paper and measurement of the relative amount of these enzymes is a sensitive diagnostic method for identifying these inherited disorders in the population. (Anderson and Leopold, 1979; Singer *et al.*, 1973).

3.5.3 Proteinase Inhibitors (Antiproteases)

Human tears have several inhibitors of proteolytic activity, the level of which is much lower than that in serum (Kueppers, 1971; Zirm *et al.*, 1976). These inhibitors have been identified as alpha 1 - antitrypsin, alpha 2 - macroglobulin, inter-α-antitrypsin, alpha 1 - antichymotrypsin and a thiol-dependent protease inhibitor, papain (Anderson and Leopold, 1981).

There is conflicting evidence on the origin of these protease inhibitors. van Haeringen (1981) reported that alpha 1 - antitrypsin was secreted by the lacrimal gland because its level in normal tear was not altered by mechanical irritation or hypersecretion of tears. He suggested the other antiproteases may originate in the corneal or conjunctival tissues. Immunologic studies both by Berman *et al.* (1973) and Zirm et al (1976) have shown that these tear protease inhibitors are similar to those in plasma. They also observed a concomitant increase in serum albumin with increasing antiprotease activity in tears of inflamed eye. From this they concluded that the presence of antiproteases in tears was due to a passive leakage of these proteins from blood vessels that supply the inflamed eye.

The main function of antiproteases in tears appears to be protection of the tissues of the cornea and conjunctiva. This is achieved by controlling the collagenolytic activity in the tissues. alpha 1 - antitrypsin and α_2 - macroglobulin appear to be responsible for most, if not all, of the collagenase inhibitory activity because of their broad specificity (Berman *et al* 1973). These two antiproteases show an increased activity in corneal ulcerations, bacterial and viral infections (Anderson and Leopold, 1981; Zirm *et al.*, 1976).

Anderson and Leopold (1981) investigated tear inhibition of the thiol-protease, papain, and the possible relationship of its inhibitory activity in patients with

external ocular diseases. They found low inhibitory activity with blepharitis and infectious conjunctivitis. While in allergic conjunctivitis the inhibitory activity was higher than in normal tears.

Bosmann *et al.* (1980) found the range of inhibitory activity in normal tears was from 0.49 ug to 1.14 ug. of papain inhibited per 10 ul tears. They indicated that this amount of antiprotease activity was more than enough to inhibit the residual papain activity on hydrogel contact lenses that were chemically disinfected after using the Soflens^{R} enzymatic (papain) cleaner.

Protease inhibitors in human tears may be of importance in controlling the course of ocular inflammation. The level of activity in tears may be of diagnostic and prognostic value for a number of ocular diseases. However, more sensitive methods of analysis need to be developed to differentiate the activities of the five proteolytic inhibitors identified.

Chapter 4

SYNTHETIC POLYMERS IN CONTACT LENS APPLICATION

4.1 Introduction

This historical development of contact lens materials warrants review in order to appreciate those unique characteristics in the designs of current synthetic polymers which make them compatible with the ocular tissues and tear fluids.

Fick (1888) described the first contact lens which was made of glass for the correction of refractive anomalies of the eye. In spite of the obvious shortcomings of glass (fragility and difficult to modify) it was used exclusively for many years until the introduction of synthetic plastics in the late 1930's (Bier and Lowther, 1977; Mandell, 1981).

Feinbloom (1936) developed the first scleral contact lens which had a plastic scleral portion fused to the glass corneal portion. Because of the toughness, optical properties, biocompatibility and relative ease of manufacturing, this plastic, polymethyl-methacrylate or PMMA, replaced glass.

The next period was devoted to the development of new lens designs in the PMMA material until the introduction of hydrogels by Wichterle and Lim (1960). Since then, research efforts have been concentrated on the development of contact lens material with qualities superior to those of PMMA, as it was realized that long term PMMA wear leads to corneal complications attributed to hypoxia (Mandell, 1981). This realization triggered the search for gas permeable materials.

4.2 Classification of Contact Lens Polymers

Polymers are long chain, high molecular weight molecules, made up of a number of repeating units linked together by covalent bonds, each derived from a starting unit called monomer (Refojo 1978). If the monomers are identical , the polymer is said to be a homopolymer. Polymers containing more than one type of monomer are called copolymers. These may be further described as random, alternating, block or grafted, depending on the arrangement of the monomers in the polymer chain (Bier and Lowther, 1977; Refojo, 1978).

Tighe (1979) classified these contact lens polymers as thermoplastic, synthetic elastomers and hydrogels, each having physico-chemical properties which reflects the uniqueness of the group; however, none having all the properties described by Pappas (1982) as the ideal contact lens material.

4.2.1 Thermoplastics

Thermoplastics are synthetic polymers which can be shaped or moulded under heat and pressure but are rigid at room temperature (Tighe 1982). Contact lenses made from these materials have good optical properties; however, they tend to be uncomfortable because of their rigidity and poor wettability. The principal material of this group is polymethylmethacrylate (PMMA).

4.2.1.1 Polymethylmethacrylate

Polymethylmethacrylate (PMMA) has been used extensively since Feinbloom (1936) reported on its use in a scleral contact lens design. The material can be synthesised by radiation or chemically induced polymerization of methylmethacrylate (MMA) in the presence or absence of solvents (Pappas 1982).

PMMA has most of the attributes of an ideal material, except it is rigid, hydrophobic and practically impermeable to oxygen, water and other metabolites necessary to support normal corneal metabolism (Refojo, 1973). Its oxygen diffusion coefficient (11×10^{-7} cm^2/hour) is approximately four times smaller than that of a hydrogel and five times lower than that of the synthetic elastomers

(Refojo, 1973). Because of its relative impermeability to atmospheric oxygen, PMMA lenses must be fitted in such a way that normal blinking facilitates a continuous exchange of oxygenated tears between the lens and cornea (Mandell, 1981).

To a great extent, because of the impermeability of PMMA, several other thermoplastics are currently being used, or suggested for use, as gas permeable rigid contact lenses.

4.2.1.2 Gas Permeable Rigid Materials

There are several gas permeable rigid materials many of which vary in their oxygen-diffusing characteristics due to differences in their polymeric formulation. Examples of these materials are cellulose acetate butyrate (CAB), silicone acrylates, t-butyl styrene and fluorocarbon copolymers (Greco, 1984).

Silicone acrylates are the most widely used of these gas permeable materials because of their enhanced physiological tolerance and potential for use as extended wear contact lenses (Benjamin & Simons, 1984; Levy, 1983; Zantos and Zantos, 1985). The formulation consists of varying proportions of siloxanyl alkylmethacrylate, methylmethacrylate, methacrylic acid or hydroxyethylmethacrylate (HEMA) and crosslinking agents (Andrasko & Bennett, 1985). The siloxanyl alkylmethacrylate provides the oxygen permeability characteristics, while the methylmethacrylate and methacrylic acid or HEMA provide the rigidity and the wettability characteristics respectively.

The properties of these polymers which allow increased gas permeability are the same ones that contribute to the problems of poorer wetting characteristics than PMMA. In addition, gas permeable materials have a greater affinity for protein and lipid deposits, which are a source of great discomfort to the contact lens wearer (Seidner and Sharp, 1984).

4.2.2 Elastomers

Synthetic elastomers are polymers made from crosslinked dimethylpolysiloxane with varying proportions of a silica filler added to provide the flexibility, pliability and resilience of the silicone lens (Bier and Lowther 1977; Phares, 1972). The entire mixture is polymerised at high temperature and pressure to form a moulded silicone rubber lens (Seger, 1980).

Tighe (1979) describes the properties of these elastomers as being intermediate between those of the thermoplastics and hydrogels. They possess a degree of toughness associated with the former and the softness or flexibility of the latter. The most outstanding feature of this material is its high oxygen permeability which greatly exceeds that of both thermoplastics and hydrogels. This is attributed to the polymer backbone of alternating silicone to oxygen atoms which has not only greater segmental mobility but much higher solubility for oxygen than polymers with all-carbon backbones (Tighe, 1981).

Comparative studies on the three classes of polymers have judged the silicone elastomers to have the best equivalent oxygen performance - 18% E.O.P. (Hill, 1972). This property makes them ideal candidates for contact lens usage.

An undesirable characteristic of the material is its high wetting angle (greater than 90^0) which makes it hydrophobic and uncomfortable to wear (Tighe, 1979). Surface modification by irradiation or chemical coating is necessary to improve the wettability of the finished contact lens (Refojo, 1973). However, such surface treatments are non-permanent and tend to alter the gas permeation and optical characteristics of the lens surface (Refojo 1973; Tighe 1979). In addition, silicone has a great affinity for lipid and protein deposits (Lipman, 1981). This accumulation of deposits compounds the problem of poor surface wettability.

4.2.3 Hydrogels

Hydrogels are three dimensional polymers which are characterized by their ability to take up and retain water. These polymers are held together by crosslinks which may be weak cohesive forces, hydrogen bonds, ionic bonds or covalent bonds (Bruck, 1974).

The classification of hydrogels by Erid *et al.* (1968) is based on three types of crosslinking or bonding. These are described as:

4.2.3.1 Covalently Crosslinked Hydrogels

These are three dimensional polymers such as polyhydroxyethyl methacrylate (PHEMA), a synthetic polymer developed for biomedical and contact lens applications (Wichterle and Lim, 1960, Wichterle, 1971).

4.2.3.2 Ionically Crosslinked Hydrogels

These are three dimensional, thermally reversible gels such as polyelectrolyte hydrogels which have been suggested by Michaels (1965) for use as membranes for ultrafiltration and dialysis, battery separators and surgical implants. Refojo (1967) suggested that these gels could be used for contact lenses because they are optically transparent and more permeable to water than non-ionic gels having similar water contents at equilibrium swelling.

4.2.3.3 Particle-particle Bonding Hyrogels

These are three dimensional microcrystal hydrogels formed by the interaction of discrete colloidal particles. These gels are derived from both natural and synthetic polymers; examples of which are the tobacco mosaic virus, collagens and nylon (Erdi *et al.* 1968). collagens have the most potential for contact applications (Refojo and Leong, 1980). However, there are no clinical studies to support this claim.

Of the three classes of hydrogels, covalently crosslinked hydrogels have stimulated most interest in their general applications as demonstrated by Wichterle and Lim (1960) who pioneered the work on HEMA. Since then there has been a prolif-

eration of a variety of hydrogels with improved properties for contact lens applications.

4.3 Hydrogel Contact Lenses

The introduction of hydrogel soft contact lenses has added a new dimension to the fitting of a contact lens. Techniques which apply to the fitting of a rigid thermoplastic lens cannot be used with hydrogels because of their unique physicochemical characteristics which depend both on the physical shape of the cornea and the chemical composition of the tears. This section will review the physicochemical characteristics of hydrogels, the manufacture of lenses, and their interaction with the tears and care regime.

4.3.1 Physicochemical Aspects of Hydrogel Lens Polymers

The designing of hydrogel polymers for contact lens applications requires consideration of the chemical, physical and physiological factors necessary to optimize the performance of the lens on the eye. If the contact lens is considered as an extension of the cornea, then the hydrogel material must meet the requirements of hydration, permeability, mechanical stability, surface wettability in addition to optical transparency (Peppas, 1982; Tighe, 1976).

4.3.1.1 Chemical Composition

The diversity of the chemical composition of polymers used in the manufacture of hydrogel soft contact lens is illustrated in Table 4. It is apparent, from this partial list of lenses available in Canada since 1970, that three major chemical components have been used to design hydrogel lens materials. Thus hydrogel contact lens can be classified into three groups based on chemical composition: (a) those consisting of poly (hydroxylethylmethacrylate) or poly HEMA (b) those consisting of poly HEMA copolymerized with one or more comonomers such as N-vinyl-2-pyrrolidone (VP) or methyl methacrylate (MMA) and (c) those containing principally poly (N-vinyl - 2 - pyrrolidone), usually copolymerized with at least one other non-HEMA polymer (Cordery, 1974; Refojo, 1978; Parker, 1983).

Group A lenses are based on HEMA as described in the original patents by Wichterle and Lim (1961, 1965). HEMA is crosslinked with varying amounts of ethyleneglycol dimethacrylate to produce hydrogels with water contents varying from 30 to 40 percent (Pedley *et al.*, 1980). Examples of these listed in Table 4 are Soflens^{R}, Hydron^{R} and Durasoft^{R}

Group B lenses are made from a modified HEMA, HEMA copolymers and HEMA terpolymers. Since homogenous poly HEMA can only be made to contain a maximum of 40 percent equilibrium water content, polymerization of HEMA with more hydrophilic monomers could produce hydrogels with water contents greater than 40 percent (Tighe, 1976). The principal monomer used for this purpose is N-vinyl pyrrolidone (VP) or its polymeric form, polyvinyl pyrrolidone (PVP), extends the range of water contents from 40 to over 90 percent, depending on the mixture of monomers (Pedley *et al.* 1980; Tighe 1976). Examples of lenses made from these copolymers are Softcon^{R} and Permalens^{R} with water contents 55 and 71 percent, respectively (Table 4).

Group C lenses are based on N-vinyl pyrrolidone or its polymeric form, polymerized with acrylic monomers (MMA) other than HEMA (Parker, 1983). Examples of these listed in Table 4 are Sauflon^{R} 70 and Sauflon PW^{R}, also marketed as B&L 70 and B&L CW. Other hydrogel lenses using methyl methacrylate and copolymers other than HEMA or PVP have been patented (Refojo 1978), one such example is the CSI^{R} lens which is made of a copolymer of glycerylmethacrylate and methylmethacrylate. The equilibrium water content of materials in Group C range from 38 to 79 percent.

Table 4. Composition and Water Content of Some Hydrogel Lenses. These are some of the lenses available in Canada since 1970.

COMPANY	TRADE NAME	POLYMER	HYDRATED REFRACTIVE INDEX	PERCENT WATER CONTENT
American Optical	Aosoft	HEMA /NVP /MMA	1.43	42.5
American Optical	SOFTCON	HEMA/PVP	1.40	55
Alden Optical	AL47	HEMA/MA/NVP	1.43	36.5
Bausch & Lomb	Soflens	HEMA	1.43	38.6
Bausch & Lomb	CW79	PVP/MMA	1.39	79.0
Bausch & Lomb	B&L 70	PVP/MMA	1.39	70.0
Calcon	Gelfex	HEMA/MMA	1.43	35.5
Canadian Contact Lens	Contaflex	HEMA/PVP	1.40	55
Central Canada C.L.	C-Flex 40	HEMA/MMA/NVP	1.43	40
" " "	C-Flex 50	HEMA/MMA/NVP	1.41	52
" " "	C-Flex 38	HEMA	1.43	38
CIBA Vision	CIBASOFT	HEMA	1.43	37.5
Cooper Vision	Permathin	HEMA/NVP/MMA	1.43	42.5
Cooper Vision	Permaflex	PVP/MMA	1.38	72.0
Cooper Vision	Permalens	HEMA/PVP	1.38	71
Cooper Vision	Duragel 75	PVP/MMA	1.37	73.5
Cooper Vision	Cooper 38	HEMA	1.43	38
Syntex Ophthal. Inc.	CSI	PGMA/MMA	1.44	38.5
Dominion C.L. Lab	Toyo 515	HEMA/VA	1.43	35.5
Freflex Canada Ltd.	Freflex	HEMA/MA	1.41	60
Frontier	Hydromac	HEMA/MA	1.40	52
Barnes-Hind	Hydrocurve II	HEMA/NVP	1.43	45
Barnes-Hind	Hydrocurve II	HEMA/Acrylamide	1.41	55
Ingram & Bell Canada	AMSOF	HEMA	1.43	43
Kelvin C.L. (Canada) Ltd.	TC50	HEMA/MA	1.44	50
" " "	TC75	HEMA/MA	1.39	75
Medical Optics	Sauflon 70	PVP/MMA	1.39	70
" "	Sauflon PW	PVP/MMA	1.39	79
Hydron Canada	Hydron	HEMA	1.43	38.6
N & N Optical	Toyo 515	HEMA/VA	1.445	35.6
" "	Toyo 1500	HEMA/VA	1.457	29
" "	M 79	HEMA/VA	1.43	37
" "	K 69	HEMA/VA	1.41	60
" "	N&N 70	PVP/MMA	1.39	70
Plastic Contact Lens Lab	Durasoft	HEMA	1.45	30
Plastic Contact Lens	PCL38	HEMA	1.43	38

CODE:

GMA	Glycerylmethacrylate
HEMA	Hydroxyethylmethacrylate
MA	Methacrylic Acid
MMA	Methylmethacrylate
NVP	N-Vinyl Pyrrolidone
PVP	Poly Vinyl Pyrrolidone
VA	Vinyl Acetate

4.3.2 Physical Properties.

The relevance of the various properties of a hydrogel lens material depends on whether the lens is intended for daily wear or extended wear or whether it will be heat disinfected or chemically disinfected. Such factors must be considered in terms of their relative importance to the lens application.

4.3.2.1 Hydration

Hydration is the most important and essential property of hydrogels for contact lens application. A hydrogel in the dry state (xerogel) is hard and glossy like PMMA and can be deformed under pressure or heat (Larke, 1978; Refojo, 1972). However, when hydrated it becomes soft and rubbery. Water acts as an internal plasticizer, allowing the polymer chains to move easily with respect to each other (Larke, 1978).

The amount of water absorbed by the hydrogel, expressed as the equilibrium water content, depends on the concentration and type of hydrophilic groups and the density of the crosslinks (Refojo, 1973). As the number of crosslinks are increased, the number of reactive sites decreases and the water content and pore diameter decrease (Refojo, 1972). In general, the amount of water absorbed has a profound effect on the permeability, mechanical strength and biocompatibility of the material. For example, increasing the water content of the material increases its permeability to water and water soluble molecules and ions; while, it reduces the mechanical strength and increases its sensitivity to changes in the ambient environment (Pedley *et al.*, 1980; Refojo, 1973).

The degree of swelling depends not only on the chemical composition previously discussed but also on such factors as pH, osmolarity, temperature, pressure and composition of the hydrating medium (Refojo, 1965, 1972; Yasuda *et al.*, 1966). Clinical evidence suggests that diurnal variations in tear osmolarity and pH may affect the dimensional stability of the lens fit and proneness of the hydrogel to deposit formation (Hill and Carney, 1970; Hill, 1978). Changes in hydration of the material brought about by variations in tear osmolarity and pH are insignificant when compared to those induced by heat sterilization.

The effect of temperature on the equilibrium water content can be quite complex but it is normally reversible. Tighe (1976) reports there is a small but significant linear variation over a wide range of temperatures with some hydrogels; while with others, an increase in temperature leads to an initial decrease in water content followed by a rapid increase in water content with the concurrent development of a translucence or opaque gel. Therefore, it is important to consider the water content of the hydrogel lens when it is on the eye at 35^0C and when heat is used as the procedure for lens disinfection.

The equilibrium water content also affects the optical transparency and refractive index of hydrogels. Tighe (1981) states that there is a linear relationship between refractive index and equilibrium water content. This is demonstrated in Table 4 which shows that the refractive index decreases with increasing water content.

4.3.2.2 Permeability

Hydrogels in the hyrated state have a certain porosity which selectively allows the penetration of water soluble molecular and ionic species in the water-filled polymer matrix. This porosity, commonly defined as the "average pore" radius of the network can be measured and calculated in various ways (Refojo, 1965). The average pore size increases with increasing water content as illustrated by Tighe (1982); water contents of 40, 60, 80 and 85 percent have average pore sizes of 4,6, 20 and $30A^0$ respectively.

Because of the pore sizes ($5-30A^0$), small water soluble substances such as fluorescein, oxygen, carbon dioxide, urea, glucose, electrolytes and other metabolites can diffuse in and out the hydrogel contact lens with relative ease (Refojo, 1972). Substances of larger dimensions, such as protein, bacteria and viruses cannot penetrate an intact hydrogel lens (Refojo, 1972).

At the equilibrium water content these hydrogels will have an equal concentration of solutes and ions between the gel matrix and hydrating medium. Some of these substances have a greater affinity for the polymer matrix than the aqueous

medium and as a result concentrate in the lens. Subsequent release of some substances, like ophthalmic preservatives (benzalkonium chloride, chlorobutanol and chlorhexidine), are known to be toxic to the ocular tissues of contact lens wearers (Refojo, 1972). This property of absorption and desorption of hydrogels is also a useful method of dispensing drugs into the eye in a continuous manner (Waltman and Kaufman, 1970).

The permeability of hydrogels to oxygen is an important property to be considered in selecting materials for contact lens application. Permeability of the material to oxygen depends on: (1) its resistance to the diffusion (D) of the dissolved gas and (2) the solubility (K) of the gas in the material (Refojo, 1979). The product of these two factors, DK, is the permeability.

Yasuda *et al.* (1966) found that the oxygen permeability of various hydrogel membranes was proportional to the equilibrium water content. This relationship was further investigated by Ng and Tighe (1976) with a variety of hydrogels used for or suggested for use as contact lens materials. These included HEMA, non-HEMA and polyelectrolyte complex gels with water contents ranging from 20 to 70 percent. They concluded that the oxygen permeabilities of hydrogels with water content 30 percent or less depended both on chemical composition and the proportions of "free" and "bound" water. While at higher water contents the oxygen permeability increased exponentially with increasing water content, irrespective of the chemical composition of the polymer. Furthermore, the permeability values at eye temperature 34^{o}C were almost double those values obtained at 25^{o}C

The presence of a hydrogel contact lens on the cornea acts as a limiting barrier to atmospheric oxygen. The amount of oxygen diffusing through the hydrogel contact lens occurs in accordance with Fick's Law of diffusion. This is expressed mathematically by Fatt and St. Helen (1971) as:

$$J = \frac{DK(Pa-Po)}{L} \qquad \text{.....(1)}$$

where J is the oxygen flux (ul $cm^{-2}hr^{-1}$); DK is the permeability of the material; (Pa-Po) is the difference in oxygen tension between the anterior and posterior surfaces of the contact lens (mm Hg) of a given thickness L (cm). It is apparent from this forumula that the amount of oxygen at the contact lens-cornea interface depends on the thickness of the lens and the permeability of the material to oxygen.

The compatibility of the hydrogel lens with the cornea will to a great extent, depend on the oxygen consumption rate of the corneal epithelium. The average oxygen consumption rate established by Hill and Fatt (1963) is 7.8 ul $cm^{-2}h^{-1}$. Ng and Tighe (1976) used existing data on human consumption rates to determine the lens design which would support the oxygen needs of the cornea. From the computed values, one can select the material with a known water content and corresponding DK value to design the lens which would provide the oxygen needs of a given cornea.

An alternative approach has been suggested by Decker *et al.* (1978) in which a relationship has been established between the oxygen transmissibility of the hydrogel lens on the eye and the amount of corneal swelling. It is apparent from these studies that the hydrogel lens *in situ* causes the oxygen tension at the contact lens-cornea interface to be lower than in the open eye without a contact lens. Therefore, the lens with the higher oxygen transmissibility will produce a lower corneal swelling.

4.3.3 Manufacture of Hydrogel Contact Lenses

Hydrogel contact lenses are made by either lathe-cutting or spin-casting methods. These methods require precise control of the temperature and humidity of environment to prevent partial hydration of the polymer during manufacture of the lens.

4.3.3.1 Lathe-cutting

This procedure is carried out with dehydrated (xerogel) semifinished blanks or blanks cut from the polymerized rods. The blanks are lathed much in the same way as a PMMA or other thermo-plastic materials. However, because of the hardness of the hydrogel material the lathe speed must be 6,000 rpm rather than the 3,000 rpm used with PMMA, to ensure a smooth surface finish (Skudder 1978). The surfaces are polished with aluminum oxide mixed with mineral oil, paraffin oil, oxylene or some other non-aqueous fluid (Bier and Lowther 1977).

Since the lens swells in an aqueous environment, the dimensions must be calculated for the dehydrate lens which when hydrated swells to the desired hydrated parameters. The finished lens is inspected for surface defects and the physical dimensions verified before hydration.

The lens is then hydrated to extract all residual leechables (unreacted monomers, catalyst and polishing compound) and cleaned with a nonionic detergent.

The lens is inspected, bottle and disinfected by heat or chemical methods.

4.3.3.2 Spin-casting

Spin-casting or centrigual casting is a unique technique developed by Wichterle (1961) and is the present manufacturing method used solely by Bausch and Lomb Soflens^{R} under licensing rights from National Patent Development Corporation.

The process starts with a mixture of two monomers: ethylene glycol monomethyl methacrylate. A predetermined volume of the mixture is injected into a concave spinning mould of known radius. Polymerization is initiated when the liquid spreads from the centre towards the edge of the mould and is completed while the mould is still spinning. This process takes place in an oxygen-free and climatically controlled environment.

The lens is then removed from the mould and placed in distilled water at temperature 190^{0}F to extract the unreacted monomers from the finished lens. After extraction, the lens is inspected for surface and structural defects and verified for power and other physical dimensions.

The lenses are then bottled in 0.9 percent saline and sterilized by autoclaving. Lenses moulded from the spin casting procedure depend on factors such as, the shape of the concave mould, volume of the mixture, speed of rotation, surface tension, gravity and centrigual force (Clements, 1978). Precise control of these variables is necessary to produce accurate and repeatable lens parameters.

4.4 Interaction of Hydrogel Lenses with Human Tears and Care Regimen

Hydrogel lenses are generally accepted by a significant proportion of the public. Furthermore, many wearers prefer hydrogel lenses to the rigid PMMA lenses because of the comfort experienced with the former. However, inspite of the comfort, there are several well-defined problems associated with hydrogel lenses that are not usually found with PMMA lenses. These problems include durability, contamination and disinfection. The focus of this section is on contamination and disinfection.

4.4.1 Contamination of Hydrogel Lenses

Hydrogel lenses, because of their hydrophilic properties, attract numerous contaminants to their surfaces. These contaminants include micro-organisms, tear proteins, mucus, lipids and other organic and inorganic compounds (Kleist,1979).

Bacteria, molds or fungi are capable of metabolizing hydrogel polymers. The degree of susceptibility to microbial attack is related to the composition of the polymer and the aqueous environment in which it is hydrated (Refojo, 1973). Some hydrogel lenses contain linkages that are more susceptable to attack than others (Refojo,1973). Furthermore, many of the surface contaminants from the tears serve as nutrients for microorganisms and, together with a well-regulated temperature and pH of the tears, ideal conditions exist for the proliferation of microbes. For these reasons, hydrogel lenses must be cleaned and disinfected daily after use.

4.4.2 Care Systems

Several commercially available care products have been developed for the cleaning and disinfection of hydrogel lenses. The efficacy and advantages of each of these methods have been exhaustively reviewed (Callender & Lutzi, 1979; Phillips, 1977; Lutzi & Callender, 1984).

The two principal methods of disinfection are heat and chemical.

4.4.2.1 Heat Disinfection

Heat disinfection was the first method approved by the U.S. Food and Drug Administration for use with the Bausch & Lomb Soflens contact lens. It is a widely used procedure for both medium and low water content lenses and is effective against all organisms except spores (Callender & Lutzi, 1979). However, this procedure has a number of shortcomings, notably lens discolouration, polymer degradation and surface spoilage from denatured protein and other surface contaminants (Koetting, 1975; Ruben et al, 1975).

4.4.2.2 Chemical Disinfection

Chemical disinfection, as an alternative to heat, is a simple and effective procedure for disinfecting hydrogel lenses. However, its safety has been questioned because of the ontowards adverse ocular reactions reported in the literature (Callender & Lutzi, 1979).

These disinfecting agents may be classified as:

1. Oxidative Agents

    a. Hydrogen Peroxide

    b. Iodophors

2. Antimicrobial Agents (preservatives)

These antimicrobial agents are used in the following combinations:

a. Chlorhexidine, Thimerosal and Disodium Edetate. eg. Flexcare^{R} (Burton Parsons-Alcon, Inc.)

b. Alkyl Triethanol Ammonium Chloride, Thimerosal and Disodium Edetate eg. Hydrocare^{R} Soaking Solution (Allergan Pharm., Inc.)

The principal oxidative agent which is compatible with all types of hydrogels is 3% hydrogen peroxide. This compound is an effective microbicide. However, because of its low pH 3.0 and strong oxidizing effect, this disinfectant must be neutralized before the lens could be placed on the eye. Neutralization is acheived by one of the following methods: platimum, sodium pyruvate or with a catalyst, catalase (Lutzi & Callender, 1984).

The combined antimicrobial formulations are equally effective in killing all organisms within 30 mins to 6 hours (Kaspar, 1976). However, because of the absorptive and adsorptive properties of hydrogels, preservatives can diffuse into the lens matrix and interact with the polar (-OH) charged regions of the hydrogel. This interaction results in preservative binding and an increased concentration in the lens (Sibley & Yung, 1973). Bound preservatives can be released from the lens into the patient's eye and cause ocular irritation.

Studies have shown that chlorhexidine binds weakly to HEMA lenses and is slowly released without adverse effects to the eye (Otten & Szabocisk, 1976; Refojo, 1976; Ruben, 1980). However, if proteins and other contaminants are on the lens, chlorhexidine binds to the contaminants in addition to the polymer, thereby increasing the concentration of the adsorbed preservative. Kaspar (1976) has shown that a dirty HEMA lens adsorbs twice as much chlorhexidine as a clean lens.

This increase capacity of soiled lenses to bind preservatives necessitates an effective cleaning procedure which would free lenses of deposits and ensure effective disinfection. Several commercially available methods for cleaning hydrogel lenses have been reviewed by Phillips (1977). These include surfactants, organic solvents, oxidizing agents and enzymes.

Chapter 5

STATEMENT OF THE PROBLEM

A common problem associated with hydrogel soft contact lenses is the accumulation of deposits after periods of wear varying from a few days to several months (Koetting, 1973). These deposits, which are mainly proteinaceous, become denatured on the lens surfaces with the use of the disinfection procedure (heat or chemical) required to keep the lens in an aseptic state when not worn (Allen *et al.*, 1978; Karagoezian, 1976; Wedler, 1977).

This denatured protein appears to provoke an adverse eye reaction, the aetiology of which may be immunologic and/or toxic (Allansmith et al, 1977, Cumming and Karagoezian, 1975; Spring 1977). In the latter case preservatives such as chlorhexidine and thimerosal may be solely responsible for the adverse reaction. These preservatives bind to protein deposits thereby increasing their concentrations above the level considered to be safe for use in the eye. Bound preservative may then be transferred from the lens to the eye and subsequently cause a toxic response or may act as an allergen which precipitates the allergic response (McMonnies, 1978).

Preservatives have also been implicated in interfering with the natural defense mechanism of the eye against infection. Johnsson *et al.* (1978), reported a disturbance in the microflora of the eye due to preservatives. In addition they demonstrated a decrease in lysozyme activity and the absence of tear IgA and IgG with the use of a chemical disinfection system containing 0.005% chlorhexidine.

Three types of cleaning agents have been developed for the removal and prevention of deposit formations. These are surfactants, oxidizing agents and enzymatic cleaners. The efficacy of these have been investigated by Hathaway and Lowther (1978). Of these three methods, a proteolytic enzyme (papain) has been

proven to be the most effective way of removing deposits and alleviating the adverse eye reaction (Allansmith *et al.*, 1977; Eriksen, 1980; Lowther, 1977). However, papain has been reported to produce asthma, rhinitis, urticara and angioedema in workers exposed to airborne papain in a meat tenderizing plant (Novey *et al.*, 1979). Thus the use of papain as a cleaner for hydrogel lenses may be another cause of the adverse reaction. Fichman *et al.* (1978) have demonstrated the presence of residual papain activity on hydrogel lenses after use. They suggested that the combination of the papain cleaner with the chemical disinfection system may lead to the red eye syndrome. To test this hypothesis a preliminary experiment was designed (see Appendix A). The results therein support the hypothesis that in the *in vitro* state papain binds to hydrogel lenses and forms a complex with mercury.

Recently there have been a number of case reports on hydrogel lens wearers manifesting an apparent sensitivity to the enzyme (Keller, 1983; Davis, 1983; Fichman *et al.*, 1978). In the absence of specific immunological studies to determine the specific allergen, the cause of the adverse response could be due to any number of chemical components interacting with the hydrogel lens and the eye.

The problems associated with the interaction of the tear components, preservatives and hydrogel lenses are very complex. Our current knowledge of the aetiology of these adverse ocular responses is largely due to "case histories". Such publications in themselves are useful but in the absence of sound scientific methodology the results may lead to unreliable interpretation. Thus, it is the purpose of this study to correlate clinically observable changes in the eye's response to hydrogel contact lens wear with changes in the tear protein composition.

There are four principal tear proteins that play a protective role in eye against microbial invasion. These are lysozyme, lactoferrin, specific tear albumin and the immunoglobulins IgA and IgE. All of these proteins are secreted by the lacrimal. The prolonged use of the hydrogel lens disinfections solutions containing antimicrobical chemical may inhibit the production of these antimicrobial proteins.

Thus making the eye more susceptible to the invasion of pathogenic organisms. The resulting inflammatory response will alter the tear protein concentrations. Liotet *et al.* (1982) found that inflammatory reactions induce an increase in serum albumin and immunoglobulin fractions with gel electrophoresis.

Therefore, in this study the tear protein composition will be monitored in the following manner:

1. The total protein concentration will be used as an index of overall change. An increase in concentration may be indicative of an inflammatory reaction.
2. The electrophoretic distribution of the tear protein profile may indicate changes in specific protein fractions relative to others.
3. Two of the principal antimicrobial proteins, Lysozyme and IgA will be studied. These may indicate specific changes in the protein composition. Tapaszto (1973) reported an increase in these tear proteins in supperative eye diseases. While in the presence of chemical disinfectants a decrease in lysozyme activity has been reported (Johnsson *et al.*, 1978).
4. The immune response to a hypersensitivity of the chemicals used in the hydrogel care systems may reflect a change in IgE. Therefore IgE will be studied.
5. The proteinase inhibitors in tears provide a protective function for the ocular tissues against proteolytic enzymes. Papain, a hydrogel lens cleaner, may alter the concentration of these proteinase inhibitors. Of the three proteinases present only alpha-1-antichymotrypsin will be monitored.

The information dervied from this analytical approach may be a major contribution to the understanding of the eye's response to hydrogel lenses and their care regimen.

Chapter 6

EXPERIMENTAL DESIGN AND METHODOLOGY

6.1 Experimental Design

In planning this study it was necessary to consider a clinical design which would yield maximum effect without bias.

There are three fundamental designs for clinical studies: the matched pair or paired organ type; the cross-over type; and the group comparative type (Maxwell, 1968). With a paired organ study, a smaller number of subjects could be used. However, this method was rejected because of the possible sympathetic effects resulting from the treatment of one eye on the contralateral eye. Callender (1973) noted that tear sampling of one eye caused an alteration in the tear protein concentration in the contralateral eye as a result of the sympathetic reflex.

The cross-over design involves subjecting the patient to one type of treatment and then to a second type of treatment: for example, heat disinfection of lenses for one period, followed by chemical disinfection for another period, or vice versa. The difference in response to the two treatments is assumed to be due to the difference in treatment. The results here may be affected by the patient's adaptation or sensitization to the first treatment. Thus it was felt that the cross-over design would be unsuitable for this study.

The most suitable design was a group comparative study wherein all treatment procedures were administered concurrently so that the interaction between groups and between periods of sampling could be compared without bias to adaptation or preconditioning.

6.2 Population Size

It was also necessary to determine the number of subjects that would give a precise estimate of the parameters and their differences to be measured. The principal parameter under investigation was the protein content with special reference to specific components in human tears. Obviously, the larger the population, the more precise would be the estimates of the parameters and their differences. However, financial resources and time imposed restraints.

In view of the data obtained from the sensitivity of the protein analysis techniques used in a previous study by Callender (1973), it was calculated that ten subjects per set of observations were required to measure a detectable difference between means of 0.35gm% total protein (p=0.05).

The statistical method for estimating sample size according to Davis (1967) was applied:

$$N = \frac{(U\alpha + U\beta)2}{D^2} \quad \text{.....(1)}$$

Where: $D = d/\sigma$ .....(2)

σ = Experimental error

d = Difference between Means, important to detect

α = Risk of ascertaining a difference when none exists

β = Risk of saying there is no difference when one exists

N = Number of observations required

From the previous study σ = 0.35 mg% and d = 0.35 mg% were obtained to compute N.

From the statistical tables:

$U = 1.96$; when $\alpha = \beta = 5\%$

$u\alpha = u\beta = 1.6444$

Therefore:

$$N = \frac{(1.6444 + 1.6444)^2}{(.35/.35)^2}$$

$N = 10.8$

To guard against the possibility of loss of data from subjects' non-attendance, for reasons unforseen at the start of the study, a 50% wastage rate over the period was estimated. Hence, 15 subjects per set of observations appeared to be ideal to avoid a reduction of confidence in the interpretation of the results.

6.3 Selection of Subjects

Subjects were obtained through posters (Appendix G) placed within the University of Aston in Birmingham, and the City of Birmingham Polytechinic Institute. Interested subjects were asked to make an appointment for a screening at the research clinic. This procedure was designed to eliminate those subjects with obvious pathologies, known allergies, subnormal tear production, low tear break-up time (BUT), and other health problems contraindicating contact lens wear. A detailed profile is given in Appendix G.

One hundred (100) caucasian subjects were screened and forty-two (42) were accepted for soft contact lens wear.

Of the volunteers rejected, because of very low spectacle prescriptions or emmetropia, fourteen (14) were asked to act as paid control subjects. It may be argued that a bias was introduced by accepting these subjects as controls. Nevertheless, since the primary aim of the study was to investigate changes in tear protein content in healthy eyes, it was reasonable to assume that there is no correlation between refractive status and tear protein composition. There is no information in the literature to refute this assumption.

A profile of the subjects selected is given in Table 5. The group consisted of 35 males and 21 females ranging in age from 16 to 45 (Mean: 24.8 years, S.D. $\pm$ 6.2 years).

Table 5. Subjects Profile

Group	N	SEX		AGE (YRS)		
		Male	Female	Range	Mean	S.D.
A.	14	9	5	19-37	23.8	±5.0
B.	14	7	7	19-35	24.2	±6.8
C.	14	10	4	16-32	22.4	±4.1
D.	14	9	5	19-45	27.6	±7.6
TOTAL	56	35	21	16-45	24.8	±6.2

6.4 Informed Consent

On completion of the screening, the selected subjects were given a demonstration of the method for tear collection and other clinical procedures required for data collection during the period of study (Appendix G). Subjects were informed that study was approved by the University Committee for Human Research. They were assured that none of the procedures to be utilized would be harmful to their vision. They were then asked to sign the consent form shown in (Appendix G).

6.5 Diagnostic Lens Fitting

Subjects selected for contact lens wear were fitted with the U3 and U4 Bausch and Lomb Soflens^{R} (polymacon) contact lenses according to standard clinical practice. This type of hydrogel lens was selected because of the ease of fitting, its reliable performance, comfort and good physiological response.

The lens powers ordered ranges from -0.50 D to -8.00 D (Mean. -2.7O D S.D $\pm$ 1.39). On completion of the diagnostic fitting , the subjects were randomly divided into three (3) groups of fourteen (14) contact lens wearers and the other fourteen (14) non-wearers acted as the control group (see Table 6).

TABLE 6. Treatment Code for Subjects in Group

Group ID	Subject Initials	Group ID	Subject Initials	Group ID	Subject Initials	Group ID	Subject Initials
A1	KJ	B1	CD	C1	CD	D1	DR
A2	LG	B2	LB	C2	PG	D2	AT
A3	SZ	B3	LD	C3	AB	D3	MR
A4	FH	B4	AW	C4	BO	D4	AH
A5	EC	B5	JS	C5	MN	D5	PM
A6	NL	B6	DM	C6	SC	D6	AE
A7	JR	B7	CT	C7	GD	D7	JB
A8	RA	B8	DD	C8	TC	D8	JH
A9	IP	B9	BA	C9	GT	D9	KT
A10	RC	B10	JL	C10	KH	D10	SF
A11	MW	B11	BH	C11	GT	D11	JL
A12	IM	B12	GB	C12	RS	D12	LU
A13	AR	B13	CW	C13	ML	D13	GM
A14	DB	B14	LS	C14	SC	D14	DR

Treatment Code

Group A = Heat: Salt Tablets + Enzyme Cleaner (papain)

Group B = Chemical: Flexcare + Preflex

Group C = Chemical: Flexcare + Preflex + Enzyme Cleaner (Papain)

Group D = Control

6.6 Experimental Procedures

The following procedures were carried out before data collection and analyses:

6.6.1 Treatment

The randomly divided groups were assigned the following treatment procedures:

Group A : Heat Disinfection
Non-preserved saline and Enzymatic (papain) cleaner

Group B : Chemical Disinfection
Flexcare + Preflex (surfactant) cleaner

Group C : Chemical Disinfection
Flexcare + Preflex + Enzymatic (papain) cleaner

Group D : Control group wearing spectacles if required.

6.6.2 Instructions on Care Regimen

Groups A, B and C were given verbal and written instructions on the care and hygiene procedures designed for their respective care regimes (Appendix G).

Group A was directed to clean their lenses twice per week with the Soflens^{R} enzymatic (papain) tablets, and to heat-disinfect their lenses daily, after use, with freshly made non-preserved saline in the Bausch & Lomb Aseptron^{R} unit.

Group B was directed to clean their lenses after daily wear with Preflex^{R}, a surfactant, then rinse and store overnight in Flexcare^{R}, a chemical disinfectant consisting of 0.001% Thimerosal and 0.005% Chlorhexidine.

Group C was given the same chemical system as Group B but in addition to the daily use of a surfactant, they were instructed to use the Soflens^{R} enzymatic (papain) tablets twice per week prior to overnight storage in the chemical disinfection solution. This procedure was designed to precipitate the mercuri-papain complex.

Group D was the control group of non-wearers. No treatment procedure was given.

6.6.3 Schedule of Visits

Prior to the start of the study, subjects in groups A, B and C were given instruction on lens insertion and removal and on the procedures for caring their lenses.

A baseline tear sample was collected from the right eye of each subject (including the controls) on day zero before the treatment procedure began.

In order to avoid variations in data due to diurnal rhythm, a Latin square appointment system was suggested by Hirji (1978). The simplicity of this system allows the construction of an appointment schedule for each subject within a group, and hence cancels out the diurnal variations in the parameters to be measured. Although this appointment system solves the problem of variability in data, it imposes limitations on the number of subjects the investigator could see within an hour. Hence, a modification of the Latin square model was adopted as shown in Table 7. This allowed each subject to be seen once at all possible times between 10.00 and 13.40 hours. In so doing, the mean data collection time for each group was similar. Also, note that each subject was always seen on a specific day of the week once every three weeks for a period of 24 weeks.

Unscheduled visits were available when necessary to attend to clinical problems or to replace lost or torn lenses. A spare pair of lenses for each subject was kept in stock at all times so that a replacement could be obtained immediately without loss of wearing time.

Table 7. Schedule for Data Collection

DAY / I.D.					WEEKS / TIME OF DAY								
M	T	W	TH	F	0	3	6	9	12	15	18	21	24
A1	A4	A7	A10	A13	10:00	10:20	10:40	11:00	11:20	11:40	12:00	12:20	12:40
B1	B4	B7	B10	B13	10:20	10:40	11:00	11:20	11:40	12:00	12:20	12:40	13:00
C1	C4	C7	C10	C13	10:40	11:00	11:20	11:40	12:00	12:20	12:40	13:00	13:20
D1	D4	D7	D10	D13	11:00	11:20	11:40	12:00	12:20	12:40	13:00	13:20	13:40
A2	A5	A8	A11	A14	11:20	11:40	12:00	12:20	12:40	13:00	13:20	13:40	10:00
B2	B5	B8	B11	B14	11:40	12:00	12:20	12:40	13:00	13:20	13:40	10:00	10:20
C2	C5	C8	C11	C14	12:00	12:20	12:40	13:00	13:20	13:40	10:00	10:20	10:40
D2	D5	D8	D11	D14	12:20	12:40	13:00	13:20	13:40	10:00	10:20	10:40	11:00
A3	A6	A9	A12		12:40	13:00	13:20	13:40	10:00	10:20	10:40	11:00	11:20
B3	B6	B9	B12		13:00	13:20	13:40	10:00	10:20	10:40	11:00	11:20	11:40
C3	C6	C9	C12		13:20	13:40	10:00	10:20	10:40	11:00	11:20	11:40	12:00
D3	D6	D9	D12		13:40	10:00	10:20	10:40	11:00	11:20	11:40	12:00	12:20

6.7 Methodology

The study employed both biochemical and clinical methods to monitor the ocular response to the treatment procedure of each group. However, the principal focus was on the biochemical analysis of tears collected at the predetermined intervals during the study.

6.7.1 Collection of Tear Samples

Over the last several decades a number of investigators have assayed the total protein and specific protein components in human tears. The values reported vary widely. van Haeringen (1981) attributed these discrepancies in tear protein values primarily to the methods of sampling. Tears may be collected by absorbent materials such as: Schirmer filter paper, cellulose sponges and cotton threads, or by glass capillary tubes.

6.7.1.1 Absorbent Materials

Collection of tears by an absorbent material requires that the pre-weighed material be placed within the conjunctival sac where it is soaked with tears. The tear-saturated material is then weighed to determine the sample volume. The tears are then eluted in an appropriate volume of saline or buffered solution. van Haeringen (1981) summarizes many of the flaws in this method: (1) it causes irritation to the conjunctiva and stimulates reflex secretion from the main lacrimal gland, (2) mucus and denuded cells adhere to the absorbent material, (3) it causes damage to the corneal and conjunctival epithelial cells, which results in the liberation of their contents; namely, metabolic enzymes, lysosomal enzymes and other proteins, and (4) there is poor recovery of the tear proteins when the tears are eluted in saline or the appropriate buffered solution.

Josephson and Lockwood (1964) found that the absorbent material caused trauma to the conjunctival sac. This resulted in increased levels of serum albumin, gamma globulin and transferrin not usually found in non-traumatized eyes.

6.7.1.2 Glass Capillary Tubes

Collection of tears by a glass capillary tube requires that it be placed in the lower tear strip at the margin of the external canthus (Fig. 4). Tears are drawn into the tube by capillary action with minimal stimulation to reflex secretion. Since the basal secretion rate of tears is approximately 1.2 ul per min. (Mishima *et al*, 1966) a 10 ul capillary tube could be filled in 7 to 10 minutes. In spite of the relative ease of sampling with this method, there is a potential risk of ocular injury if the researcher's hands are unsteady. Only fire-polished sterile tubes should be used with this method.

van Haeringen (1981) points out the essential differences between these two methods of sampling. In the capillary method, only freely floating tear fluid is collected; while with the absorbent material, not only fluid is collected, but also mucus and other cellular debris.

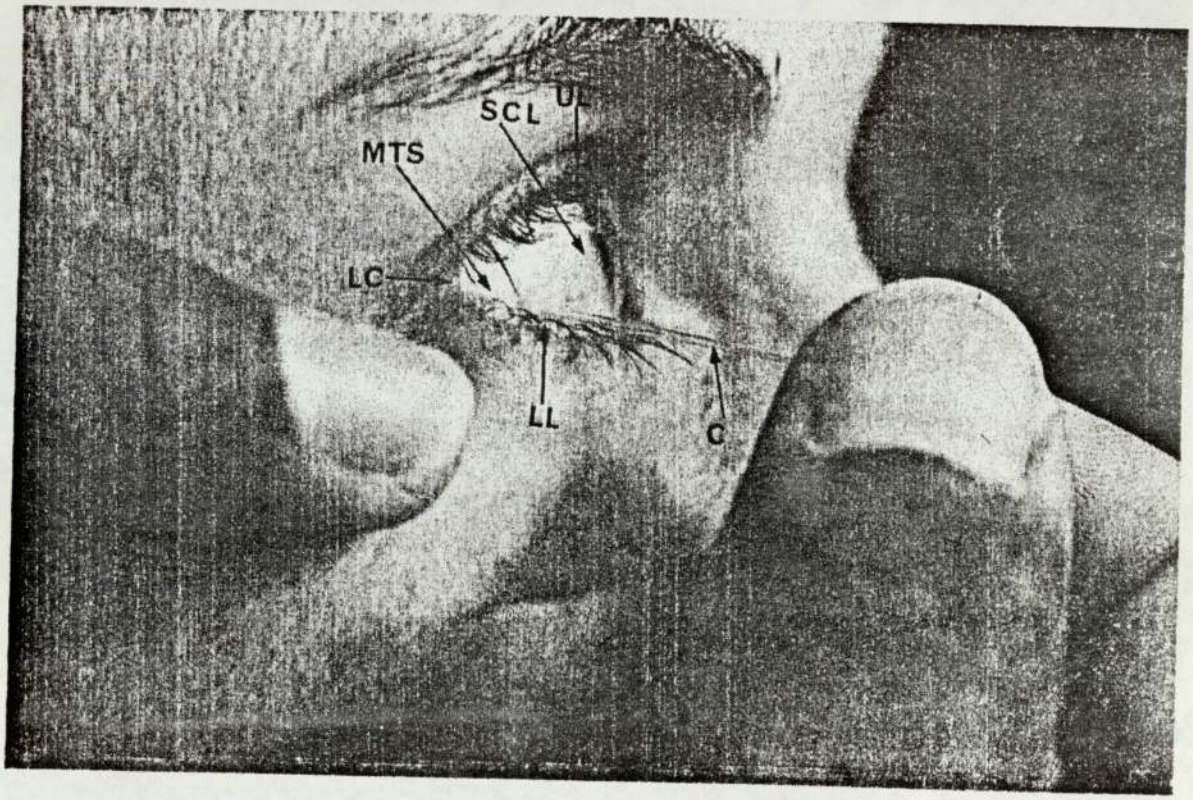
Stuchell and co-workers (1984) compared the quantitative effects of these two methods of sampling on several tear proteins. They found no signifiant difference in the concentration of lysozyme and lactoferrin in samples collected by either technique. In contrast, the concentration of serum proteins (albumin, IgE and transferrin) were significantly higher in tears collected with the absorbent filter paper.

In view of the numerous observations on the effects of the collection technique on the tear chemistry, the glass capillary tube method was chosen as the procedure for tear collection in this study. Previous studies by Callender and Morrison (1974) indicated that small sample volumes were more appropriate for tear protein micro-assays in that they minimized variations in protein values caused by evaporation, changes in the rate of secretion and reflex secretion. Experience has shown that the maximum time a subject could sit still during tear collection ranges from 5 to 10 minutes. Thus a maximum volume of basal tears one could collect in this time would be 6 to 12 ul.

Figure 4: Collection of Tear Sample.

A lateral view of a subjects right eye showing the collection of tear sample from the inferior marginal tear strip (MTS) with a 10 ul capillary tube.

1. C Capillary Tube.
2. LC Lateral Canthus.
3. LL Lower Lid.
4. MTS Marginal Tear Strip.
5. UL Upper Lid.



In this study tears were collected by placing a 10 ul sterile microcapillary pipette (Drummond Scientific) in the lower tear strip at the margin of the external canthus of the right eye of each subject (Fig. 4). Samples were collected while the contact lens was on the eye instead of after lens removal, as the trauma from lens removal would stimulate reflex tear secretion. Special care was taken to avoid any physical stimulation of the eye. If stimulation was suspected the sample was discarded.

Approximately 12 ul of non-stimulated tears were collected within 10 minutes. All samples were stored at -20 C in the sealed microcapillary tubes then brought to room temperature immediately before the tear protein assays were done. In this study all analyses were performed on a single tear sample to avoid multisampling error.

Because of the small sample volume,the tear protein assays had to be limited to total protein content, protein electrophoresis and two specific tear proteins - Lysozyme and Secretory IgA. IgE and Alpha-1- antichymotrypsin had to be deleted from the protocol because their concentrations were below the detection limit of the immunoelectrophoretic techniques being used for the tear protein analysis. To obtain measurable values much larger sample volumes would be needed.

6.7.2 Determination of Total Tear Protein Concentration

The Lowry *et al* (1951) method for total protein was modified for the determination of the protein concentration of 2 ul of tears. (Appendix B) A standard curve was prepared from a certified protein standard (Sigma Chem.). This calibration curve was checked by running 3 standards (5, 10, 20 ug per tube) whenever the tears were being analyzed. Samples were read in a Cecil DB Spectrophotometer at 750 nm.

6.7.3 Electrophoretic Distribution of Tear Proteins

Prior to the discussion of the electrophoretic techniques used in this study, a review of the theory of electrophoresis and its development are presented in Appendix C.

The technique of disc gel electrophoresis by Ornstein and Davis (1962) was modified for the separation of the low concentration of proteins in a 2 ul tear sample. This modification was originally demonstrated by Callender (1973), to resolve the tear proteins into 12 to 14 fractions without the use of 0.1% (w/v) sodium dodecyl sulphate and the large pore spacer gel (Appendix D).

Electrophoretic separation was conducted at pH 8.6 using 1.25 mA per gel for the negatively charged proteins. Lysozyme, a positively charged fraction, was not included, as this fraction was analysed independently.

After the proteins were separated, the gels were fixed and stained with Coomassie brilliant blue R in 10% acetic acid. The gels were then destained and the protein fractions (bands) were scanned at 570 nm with a Photovolt densitometer. The electrophoretic distribution of the protein fractions were quantified by measuring the area under the peaks with a digitizing table coupled to a PET commodore microcomputer Fig. 14 (Appendix D).

6.7.4 Determination of Tear Lysozyme Concentration

The Laurell *et al* (1966) technique of rocket immunoelectrophoresis described in Appendix E was used for the determination of the tear lysozyme concentration. A 1/2 ul tear sample for each subject and calibrated pooled tear lysozyme standards were applied to the appropriately labelled sample wells of the 1% Agarose plate containing the lysozyme antibody (DAKO Immunoglobulins Ltd).

The polarity of the Shandon electrophoretic chamber was reversed (samples at the positive electrode) and the current adjusted to 8 mA. Electrophoresis ran for a period of 16 hours. After this was completed the gel was washed for two days with several changes of 0.9% saline. This was followed by a one day wash in distilled water.

The gel was stained with Coomassie brilliant blue R in a methanol acetic acid mixture, destained and allowed to dry.

The rocket heights were measured and the lysozyme content of the unknown samples quantified from the linear calibration curve of the standards.

6.7.5 Determination of Tear IgA Concentration

The Laurell *et al* (1966) technique of rocket immunoelectrophoresis previously described, was modified for the determination of the IgA levels in the tears (Appendi E).

A 1 ul tear sample for each subject and three dilutions of a standard serum was applied to the sample wells of the 1% Agarose plate containing IgA (secretory) antibody (DAKO Immunoglobulins Ltd).

The polarity of the Shandon electrophoretic chamber was in the normal position (samples at the negative electrode) and the current adjusted to 8 mA. Electrophoresis ran for a period of 16 hours.

The gel was washed for two days with several changes of 0.9% saline, followed by a one day wash with distilled water.

The gel was stained with Coomassie brilliant blue R in a methanol acetic acid mixture, destained and allowed to dry.

The Rocket heights were measured and the tear IgA concentration of the unknown samples computed from the linear calibration curve of the standards.

6.7.6 Clinical Methods

Subsequent to the collection of the tear sample a modified contact lens aftercare assessment was done. The following items were recorded on the progress sheet (Appendix G):

1. History: special attention was paid to reports of red eyes, stinging, burning, irritation, itching, blurred vision, photophobia, excessive tearing and reduced wearing time, as these are symptoms of an adverse reaction to the care system and/or lens.

2. Visual Acuity: evaluated on an internally illuminated Snellen acuity chart with a constant external illumination.

3. Stability of visual acuity after the blink.

4. Over-refraction: when necessary to determine supplemental power required to improve the visual acuity.

5. Biomicroscopy: with lenses on, to assess lens performance and to check for deposits.

6. Biomicroscopy: with lenses removed, to assess corneal integrity with the use of fluorescein stain. Staining was classified as light, moderate or heavy; and the area of cornea stained was estimated as a percentage of the total corneal surface.

7. Upper lid eversion and examination: conducted on all subjects. The tarsal plate was classified as described by Allansmith *et al* (1977).

8. Pachometry: special attention was paid to the measurement of the central corneal thickness, since any form of corneal trauma results in swelling of the cornea. The pachometer used was a commercially available Haag-Streit corneal pachometer which was modified by Hirji (1978) to facilitate "blind" recording of the measurements. This apparatus was similar to that described by Mandell and Polse (1969). Prior to each session of data collection, the pachometer was calibrated with contact lenses of known thickness as advocated by Mandell and Polse (1969).

Central corneal thickness measurements were recorded as apparent thickness. These values were not converted to true corneal thickness as the investigator was only interested in the relative differences after the baseline measurements were obtained.

6.8 Data Recording and Analysis

The recording of the procedures described in Methodology generated a large volume of data. However, only the data pertaining to the biochemical analysis of tears collected and the corneal response to contact lens wear, as measured by pachometry, were analyzed.

The data was fed into the University of Waterloo IBM computer system where it was stored and retrieved for subsequent analysis. Statistical Analysis System (SAS) was used to compare the eye's response to hydrogel lens wear and care regimen among groups and between visits. Analysis of variance (ANOVA) and the Student Newman-Keuls test were the methods employed. A 5% level of significance was chosen for this study.

Chapter 7

RESULTS

7.1 Introduction

The data collected were stored in the computer and subsequently retrieved to ascertain the most appropriate method of analysis. The raw data presented in Appendix F, Tables 35 and 36 indicate that there are missing data and unequal group sizes at various visits.

7.2 Missing Data

Although fourteen (14) subjects were entered in each group, data were not collected from each subject for each scheduled visit because of withdrawals from the study or unkept appointments. The following subjects withdrew or were discontinued from the study:

1. Group A:

   Subject A12, age 20, failed to keep the baseline data collection appointment. She was ill and subsequently decided to withdraw from the study.

2. Group B:

   Two subjects were lost at the start of the study.

   a. Subject B11, age 21, did not keep the baseline data collection appointment. Numerous attempts to set up another appointment were futile. Subject withdrew from the study.

   b. Subject B13, age 26, attended the baseline data collection visit. Two hours after receiving his lenses he reported an adverse reaction (red eyes, itching, burning and general discomfort). The lenses were evaluated and found to be clinically acceptable. Purging the lenses

of the chemical disinfection solutions and prescribing heat for a week seemed to alleviate the problem. The subject was asked to resume the original treatment procedure prescribed so that he could continue in the study. However, the problem recurred. Subsequently he was diagnosed as being sensitive to thimerosal and was discontinued for the study.

3. Group C

Three subjects were discontinued due to adverse ocular reactions at different periods of the study.

a. C3, age 16, was discontinued because of occurance of red eyes and subepithelial infiltrates after the 18th week visit. Lens wear was discontinued and the subject monitored weekly for 3 weeks. During this period the red eyes cleared but the infiltrates were still present. After the infiltrates had cleared, lens wear was resumed and the adverse response recurred.

b. Subject C7, age 23, showed signs of an adverse ocular response (mild red eyes, discomfort and moderate staining of the cornea). Lens wear was discontinued to allow corneal repair and the lenses were purged. As a result no data was collected on 18th week visit. She resumed lens wear on the 21st week visit and the adverse ocular response was again observed. She was discontinued.

c. Subject C8, age 20, showed a similar type of adverse reaction as that of C3 at week 6. He was monitored weekly until the infiltrates had cleared by week 9. He resumed wear and the problem recurred. He was discontinued.

4. Group D:

There were no withdrawals and very few missing data.

The other reasons for missing data were loss of sample during the assay or subject unable to attend because of vacation. In these instances every attempt was

made to collect the data on the week prior to or the week after their scheduled appointment.

As a result of the missing data and unequal group sizes, the SAS linear models ANOVA and the student Newman-Keuls test were employed.

7.3 Biochemical Results

7.3.1 Total Tear Protein Determination

The total tear protein concentration was determined from the calibration curve derived from serial dilutions of a certified protein standard in Appendix B Fig. 13. These standards were freshly prepared for each set of determinations. The regression coefficient or slope and the coefficient of correlations (r) were used to calculate the protein concentration for each set of samples.

1. Baseline: Protein Concentration

   The tear protein concentrations for the baseline samples (visit = 0) for each group is presented in the raw data in Table 35 (Appendix F) and the mean protein concentration for each group is shown in Table 31 (Appendix F). These values show differences among the groups, however the differences are not statistically significant (df = 50, p = 0.05).

2. Treatment: Protein Concentrations

   The mean tear protein concentration for each treatment group per visit is given in Table 31 (Appendix F) and is graphically presented in Figures 5A and 5B. These graphs show an increase in protein concentration with time.

   The statistical analysis given in Appendix F. Tables 13 and 14 for protein as the dependent variable is summarized as follows:

   a. Group differences are lightly significant (p=0.04)

   b. Visit differences are significant (p = 0.0001)

   c. Group x visit interactions are not significant (p = 0.44)

d. There is some influence of the visit on the protein concentration. The estimated upward slope of the line is significant ($p = 0.0001$). Its rate of change is approximately 15 units per visit. However, the r-square value indicates that only 12.69% of the data was fitted by the model.

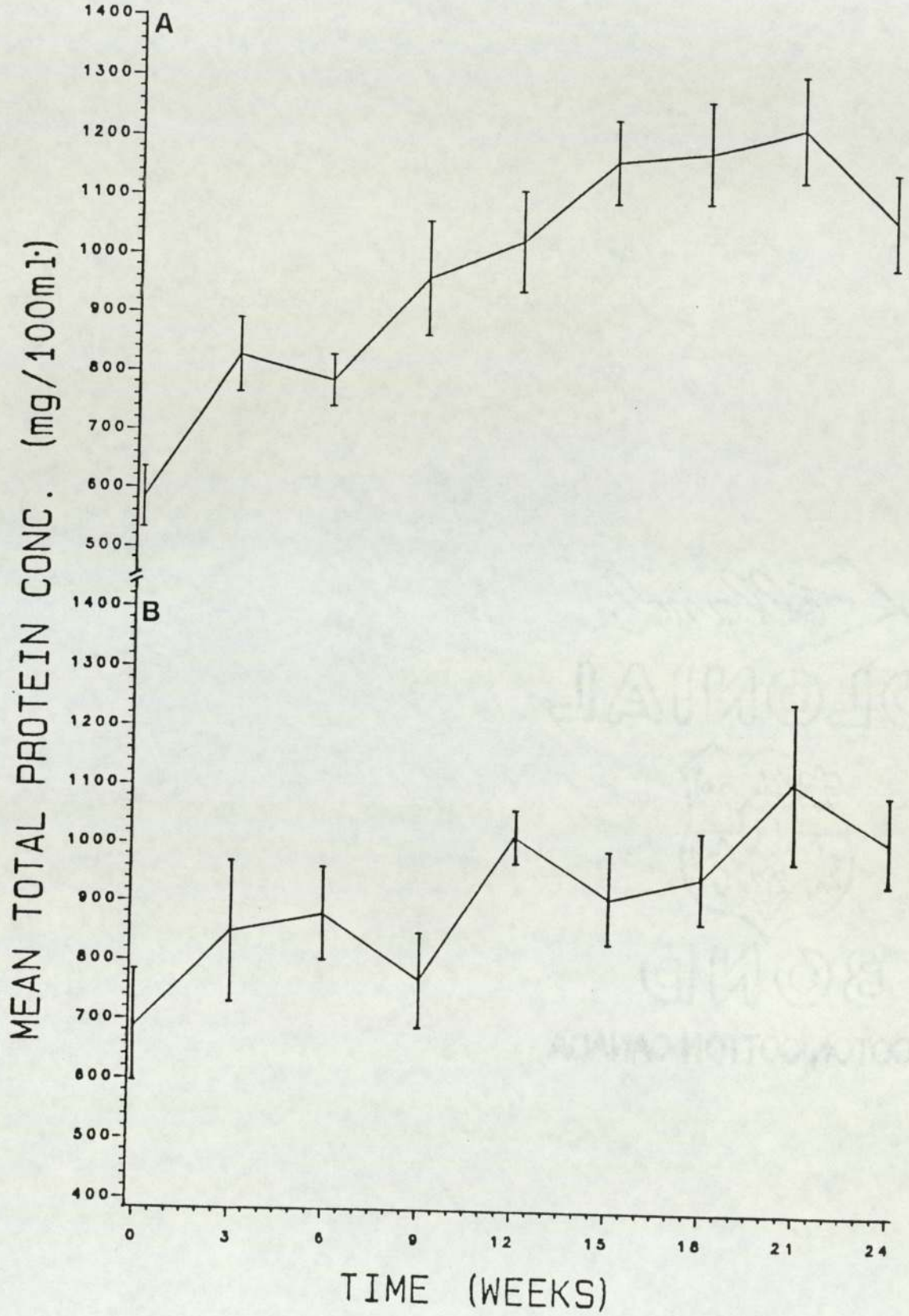


Figure 5a: Graph of the Mean Total Tear Protein During Study

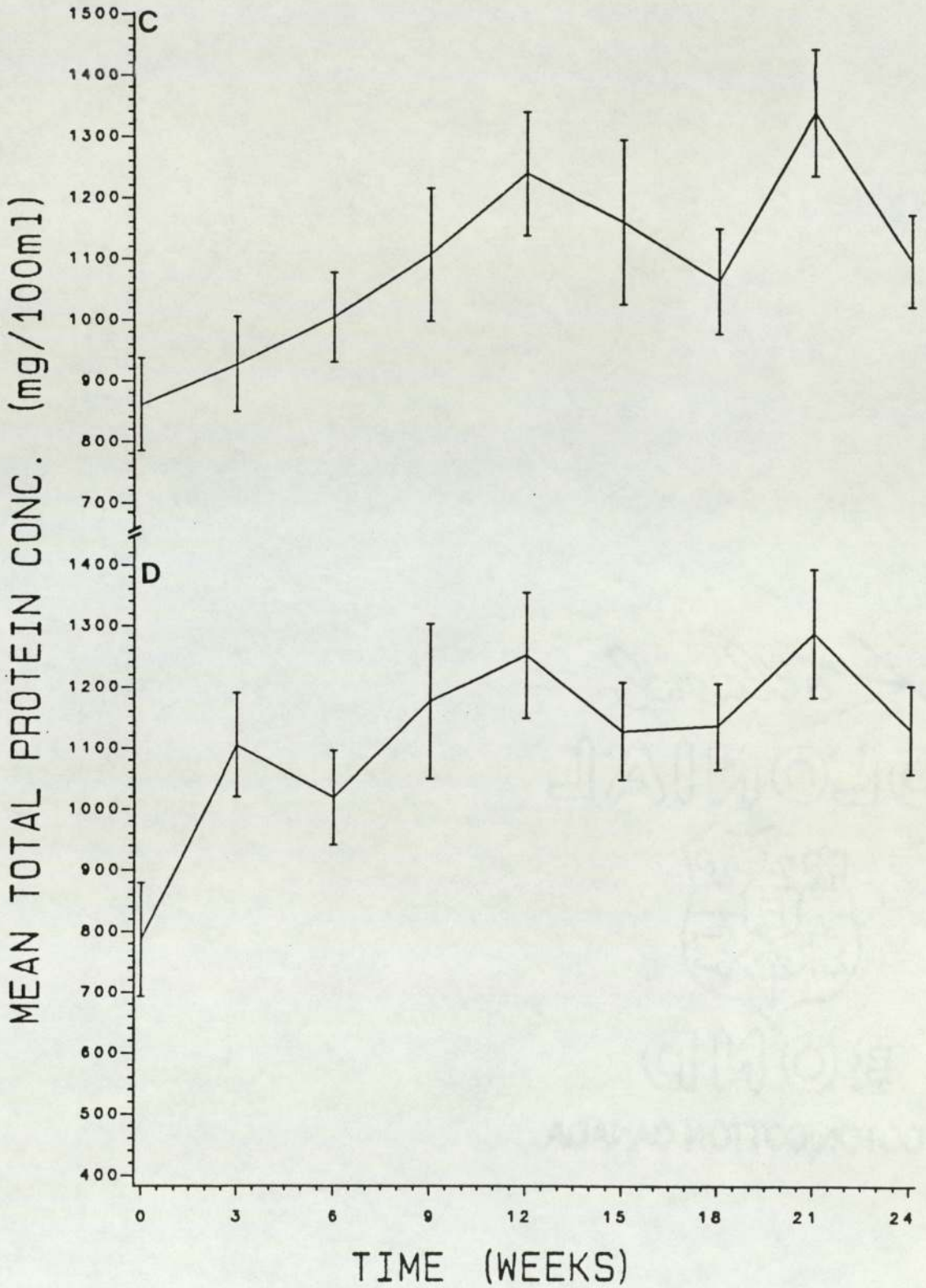


Figure 5b: Graph of Mean Total Tear Protein During Study

7.3.2 Electrophoretic Distribution of Tear Proteins

The technique of acrylamide disc gel electrophoresis by Orntein and Davis (1962) was modified to take a 2 ul tear sample per gel. The tear proteins were separated and their electrophoretic distribution quantified by measuring the area under the peaks with a digitizing table coupled to a microcomputer (Appendix D).

Figure 6 shows the distribution of the thirteen positive migrating fractions frequently observed as dense blue bands. These bands are graphically represented by peaks in Figure 6B which are labelled in decreasing order of their electrophoretic mobility in the acrylamide gel. Protein fractions migrating to the anode (+ ive) are denoted by Arabic numerals; the fastest being 1 and the slowest 13.

These protein fractions may be further differentiated into sets of protein I, II, III and IV and indicated in Tables 15-22 (Appendix F.) as PER 1, PER 2, PER 3 and PER 4 respectively. These sets of protein fractions are identified in the order of their relative mobilities. Lines of demarcation are taken as the deepest troughs between the sets of fractions.

1. Baseline: Distribution of Proteins

   The mean percentage distribution of the sets of fractions (PER 1, PER 2, PER 3 and PER 4) is presented in Table 34 (Appendix F). This distribution appears to be similar among subjects and among the four groups of subjects. The rank order is as follows:

   a. PER 3 50%
   b. PER 1 25%
   c. PER 2 13%
   d. PER 4 12%

2. Treatment: Distribution of Proteins

   The mean percentage distribution of the sets of fractions (PER 1, PER 2, PER 3, and PER 4) for each group per visit is presented in Table 34 (Appendix F) and graphically illustrated in Figures 7A, 7B, 7C and 7D.

Within each group of subjects, the graph for each set of fractions shows variability. Also among the four groups of subjects there is variability among the sets of fractions.

The differences among groups are statistically analyzed by the linear models in Appendix F, Tables 15-22, respectively for each set of fractions (PER 1, PER 2, PER 3 and PER 4) as the dependent variable. These may be summarized as follows:

a. PER 1:

For PER 1 as the dependent variable:

i. Group differences are not significant ($p = 0.43$)

ii. Visit differences are significant ($p = 0.0001$)

iii. Group x visit interactions are not significant ($p = 0.11$)

iv. Regression analysis shows no apparent linear trend ($p = 0.097$)

b. PER 2:

For PER 2 as the dependent variable:

i. Group differences are not significant ($p = 0.142$)

ii. Visit differences are significant ($p = 0.0001$)

iii. Group x visit interactions are not significant ($p = 0.99$)

iv. Regression analysis shows a significant positive trend over time ($p = 0.0001$)

c. PER 3:

For PER 3 as the dependent variable:

i. Group differences are not significant ($p = 0.20$)

ii. Visit differences are significant ($p = 0.0001$)

iii. Group x visit interactions are not significant ($p = 0.51$)

iv. Regression analysis shows a significant negative trend over time ($p = 0.0001$)

d. PER 4:

For PER 4 as the dependent variable:

i. Group differences are not significant ($p = 0.11$)

ii. Visit differences are significant ($p = 0.004$)

iii. Group x visit interactions are not significant ($p = 0.42$)

iv. Regression analysis shows no significant trend ($p = 0.1$)

There is an apparent increase in protein content in PER 2 with a concomitant decrease in PER 3. The net change appears to increase the overall protein content with time.

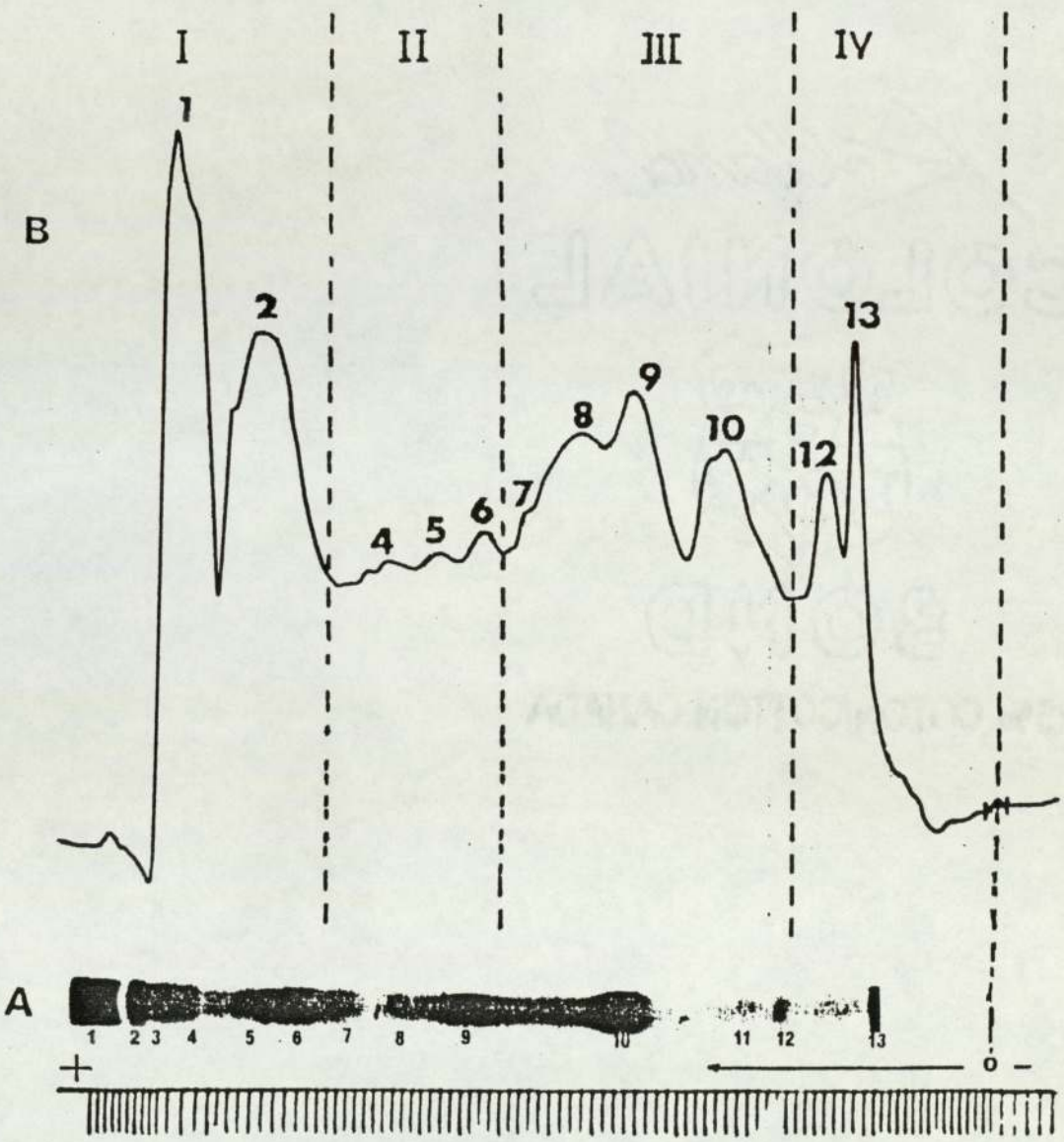


Figure 6: Electrophoretic Separation of Tear Proteins into 13 Fractions

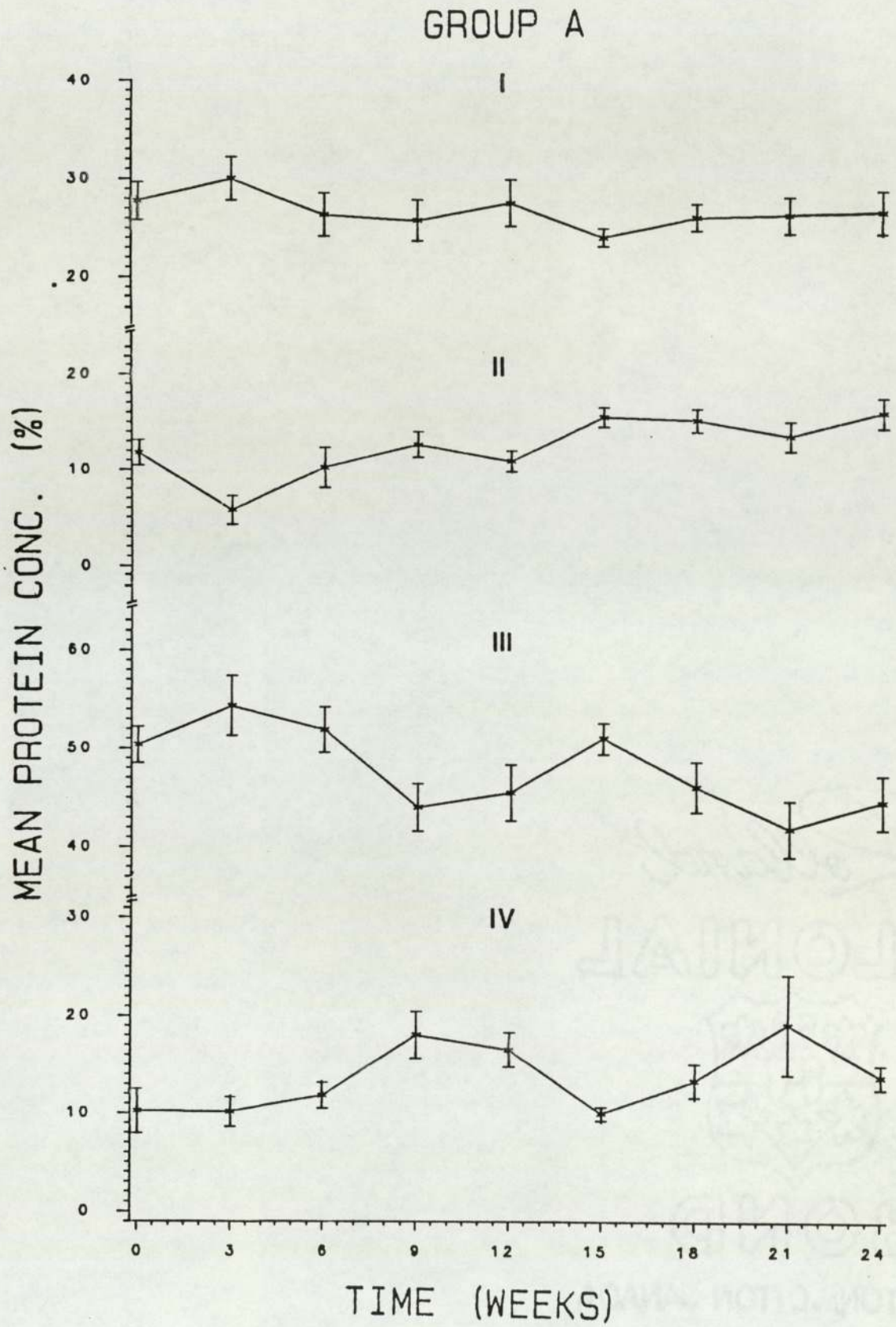


Figure 7a: Graphs of the Mean Percentage Distribution of Sets of Fractions (I, II, III & IV).

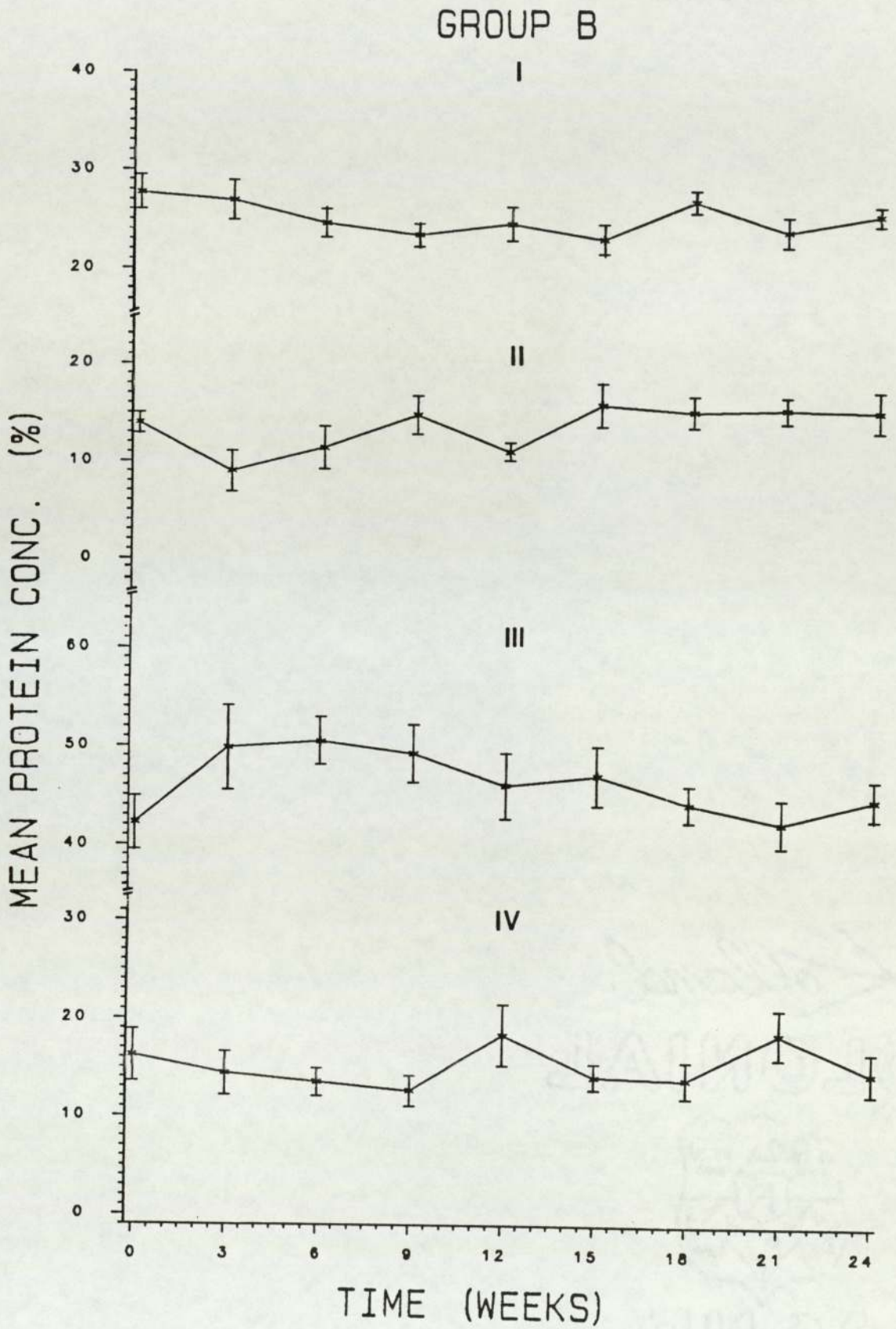


Figure 7b: Graph of the Mean Percentage Distribution of Sets of Fractions (I, II, III & IV).

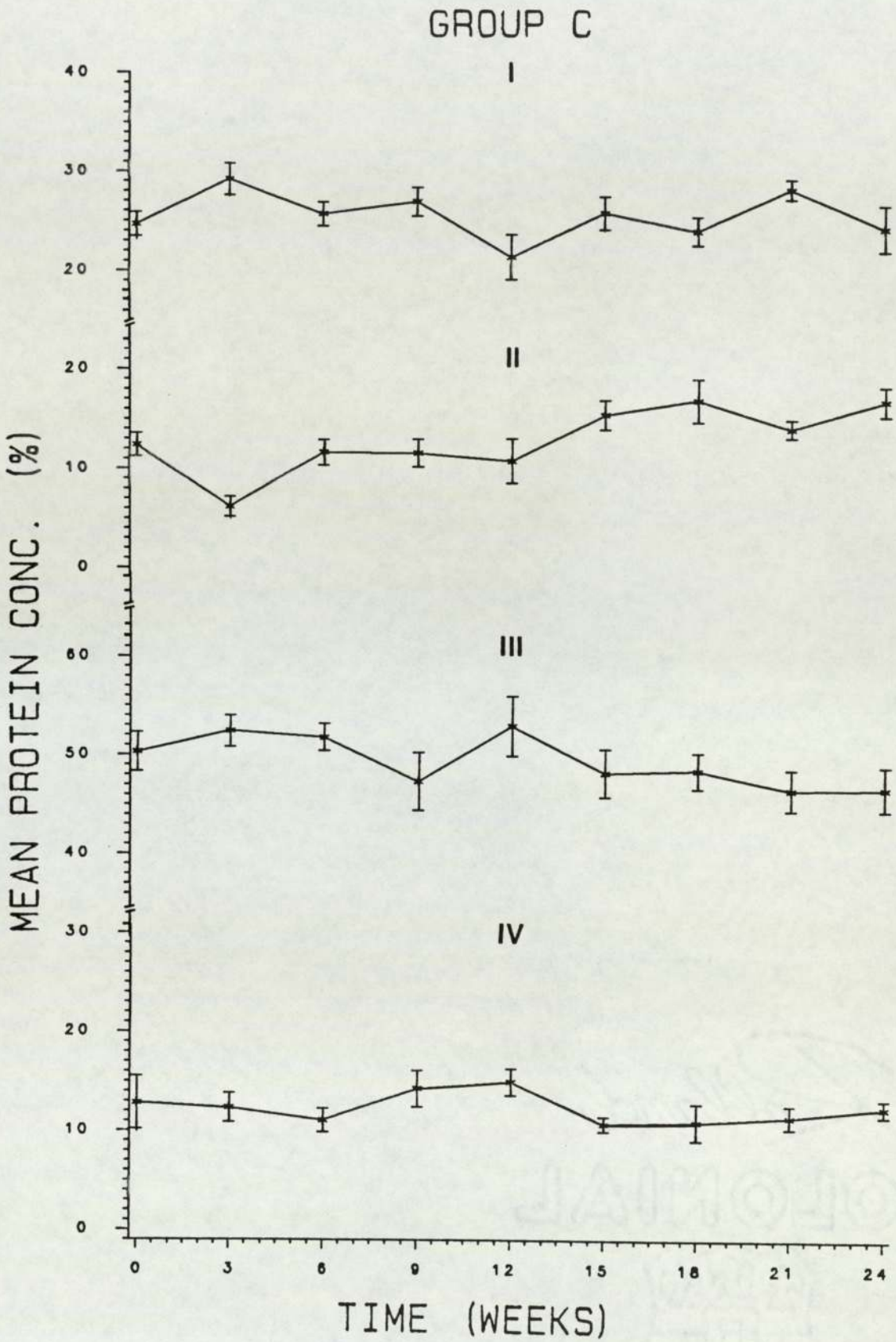


Figure 7c: Graph of the Mean Percentage Distribution of Sets of Fractions (I, II, III & IV).

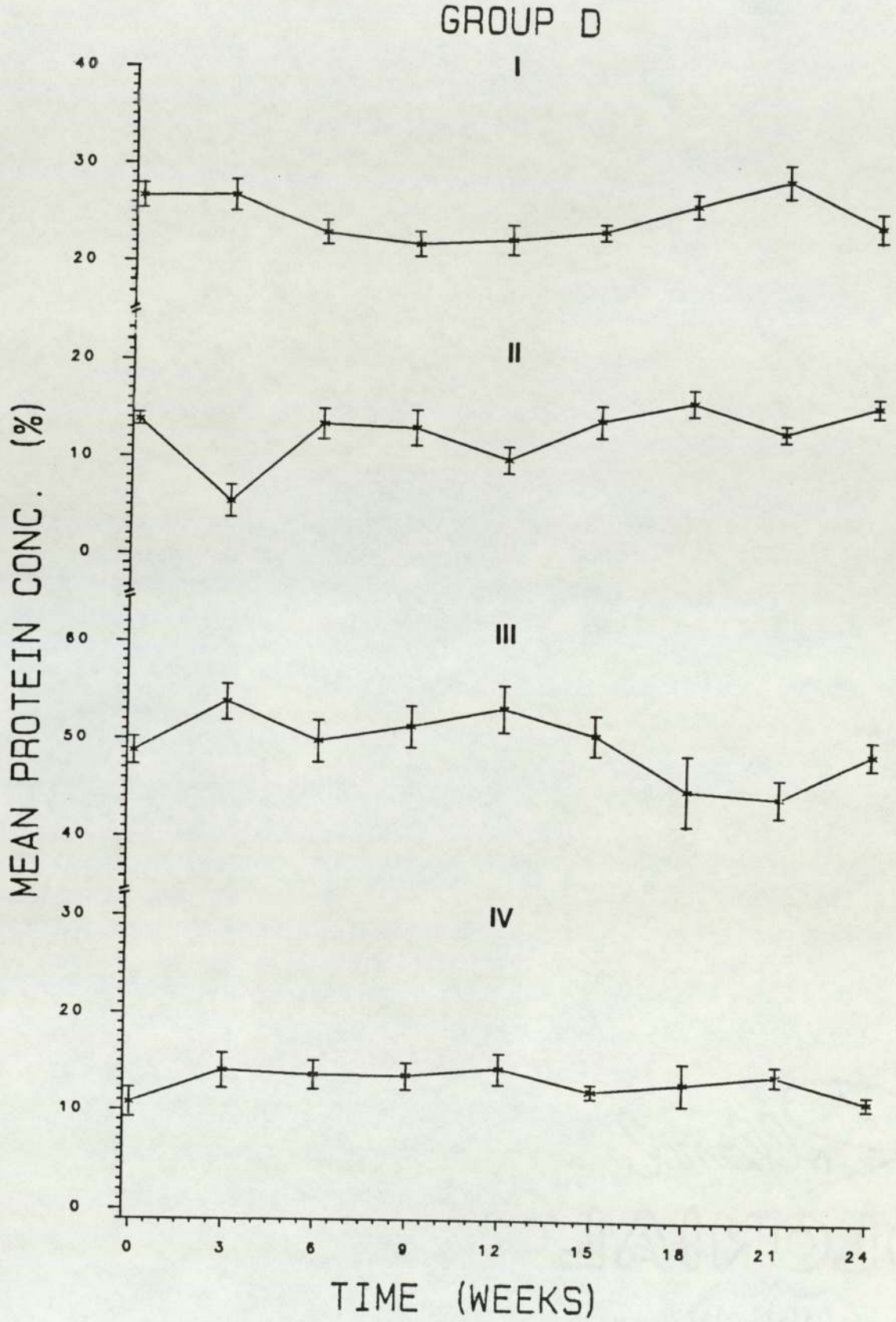


Figure 7d: Graph of the Mean Percentage Distribution of Sets of Fractions (I, II, III & IV).

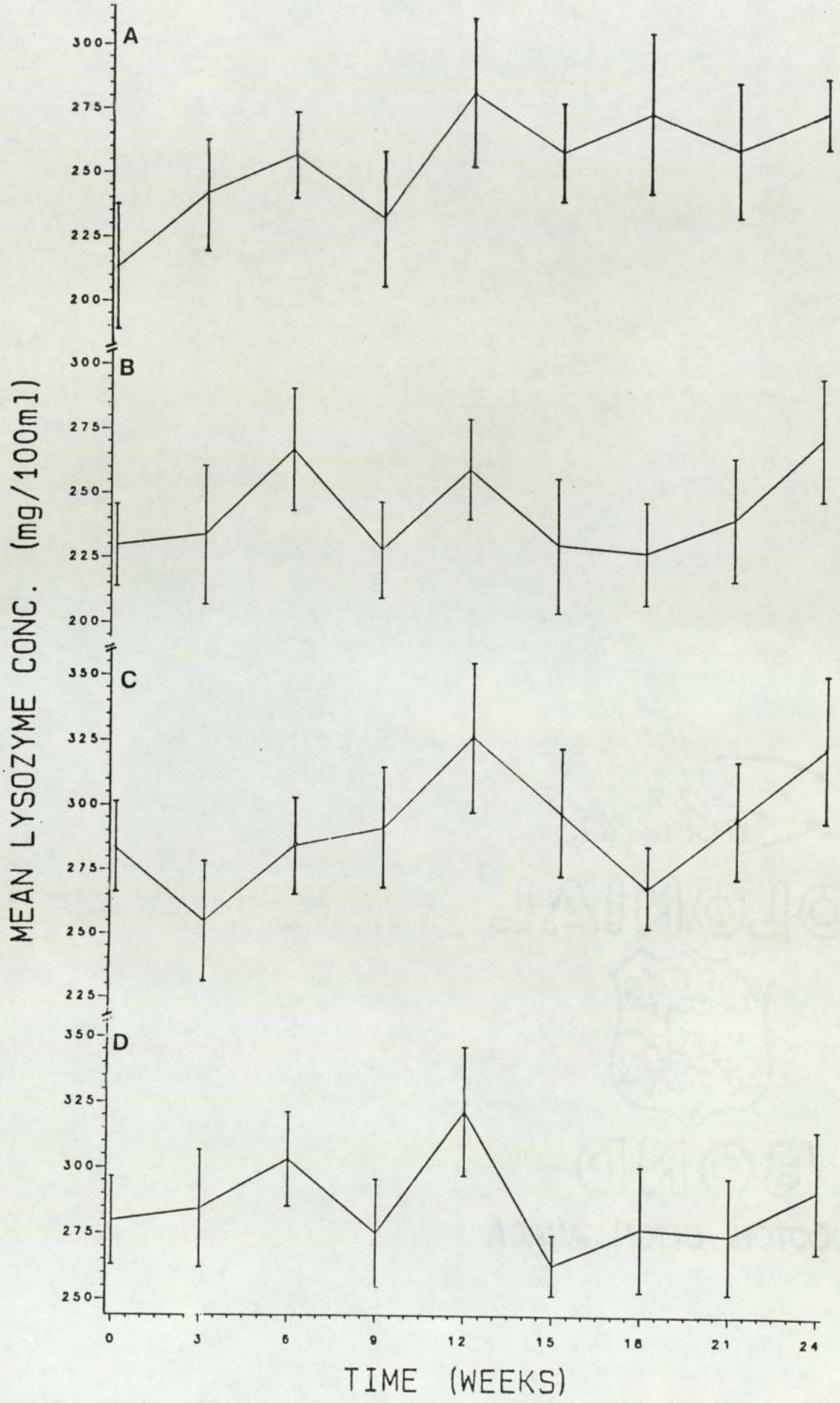


Figure 8: Graph of the Mean Lysozyme Concentration.

7.3.4 Percentage Lysozyme in Total Protein

There appears to be a linear tend towards a decrease in the percentage lysozyme in total protein which is graphically illustrated in Figures 9A and B from the data presented in Table 31, Appendix F.

The statistical analysis for the percentage lysozyme in total protein as the dependent variable is presented in Tables 25 and 26, Appendix F. The analysis may be summarized as follows:

1. Group differences are not significant ($p = 0.75$)
2. Visit differences are significant ($p = 0.0001$)
3. Group x visit interactions are not significant ($p = 0.96$)
4. Regression analysis shows a significant negative linear trend ($p = 0.0001$)

It would appear that changes in specific protein components, excluding lysozyme, affect the ratio of lysozyme to total protein. However, only 13.69% of the data fits this negative slope.

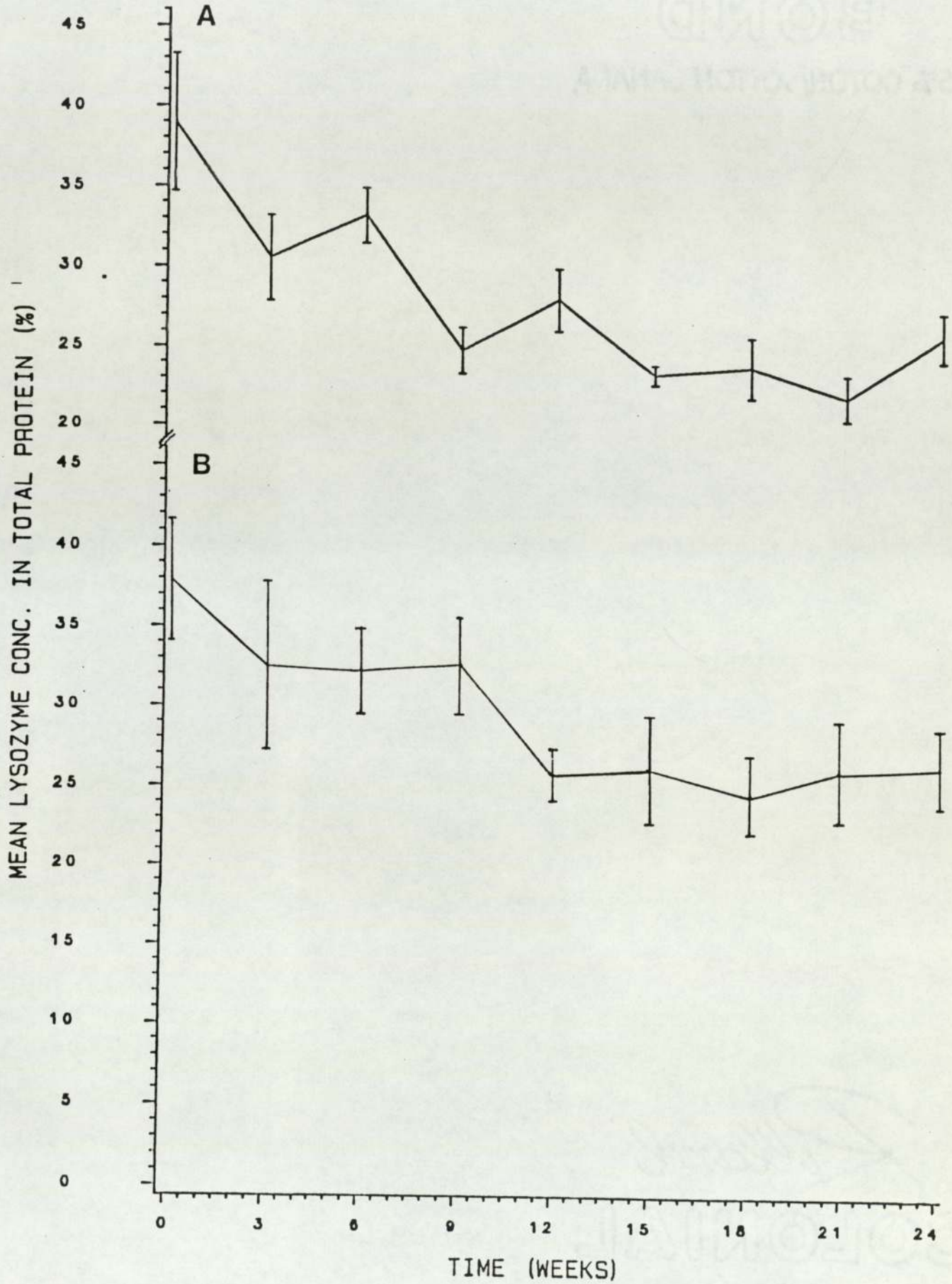


Figure 9a. Graph of the Mean Percent Lysozyme in Total Tear Protein

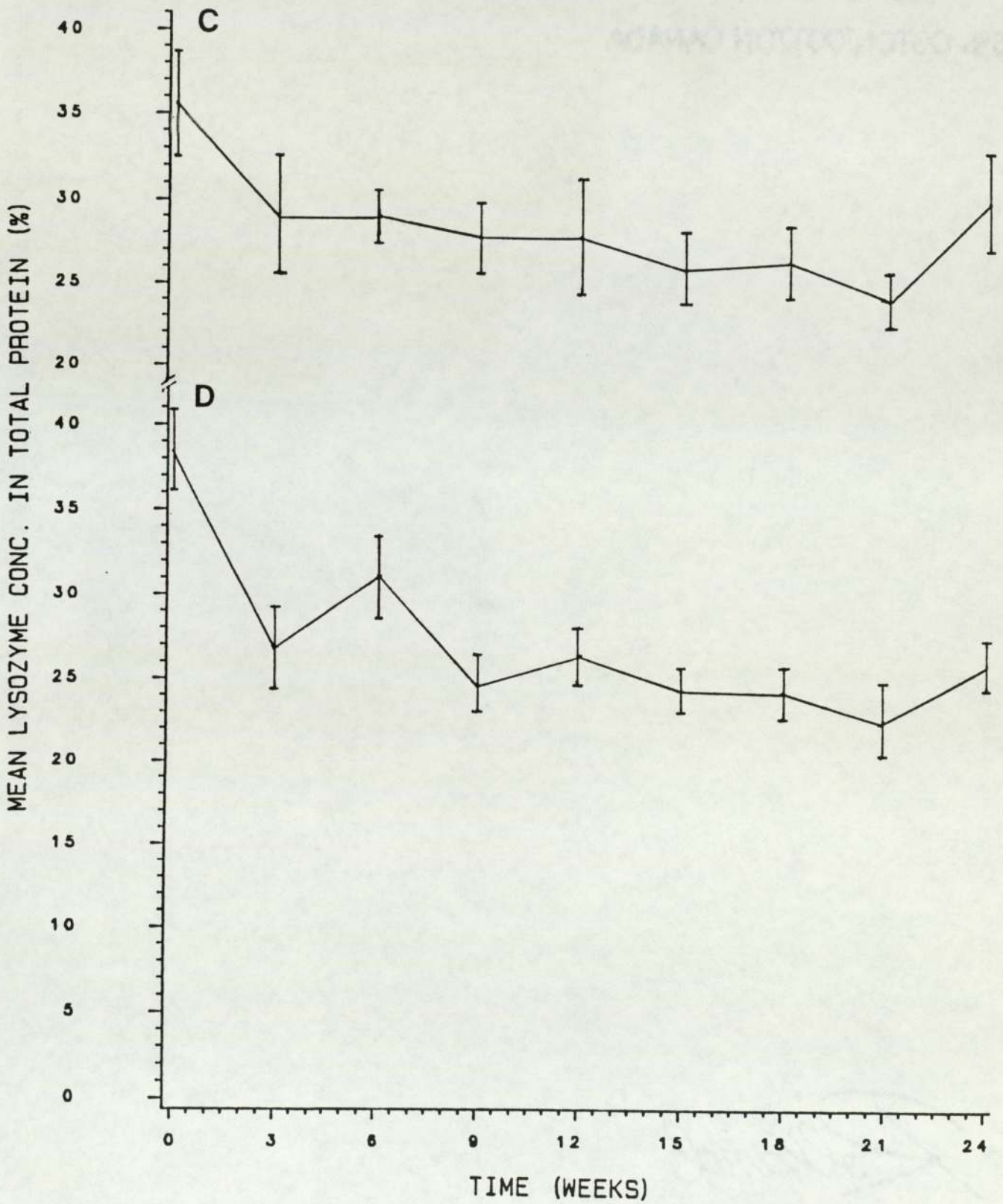


Figure 9b. Graph of the Mean Percent Lysozyme in total tear protein

7.3.5 Tear IgA (secretory) Concentration

Rocket immunoelectrophoresis was used to determine the tear secretory IgA concentration for each group. A calibration curve was prepared from a serum standard for each set of determinations (Appendix E.). The coefficient of correlation (r) obtained from the serial dilution of standards (peak height vs concentration) was used to calculate the concentrations of the measured peak heights of the unknown tear samples.

1. Baseline: IgA Concentration

   The tear IgA (secretory) concentration for the baseline samples (visit = 0) for each group is presented in the raw data in Table 35 (Appendix F). The mean lysozyme concentration for each group shows that there is very little variability in the baseline IgA levels among the groups. The differences are not statistically significant (df=49; p=0.05).

2. Treatment: IgA Concentrations

   The mean IgA (secretory) concentration for each group per visit is given in Table 31, Appendix F and graphically illustrated in Figure 10. Inspection of the error bars (SEM) suggests that there is no difference in the mean values between visits.

   The statistical analysis for IgA as the dependent variable is presented in Tables 27 and 28 (Appendix F). The analysis may be summarized as follows:

   a. Group differences are not significant ($p = 0.66$)

   b. Visit differences are not significant ($p = 0.06$)

   c. Group x visit interactions are not significant ($p = 0.43$)

   Thus it appears that the level of IgA is not influenced by the treatment procedures (heat or chemical disinfection) nor is it influenced by time.

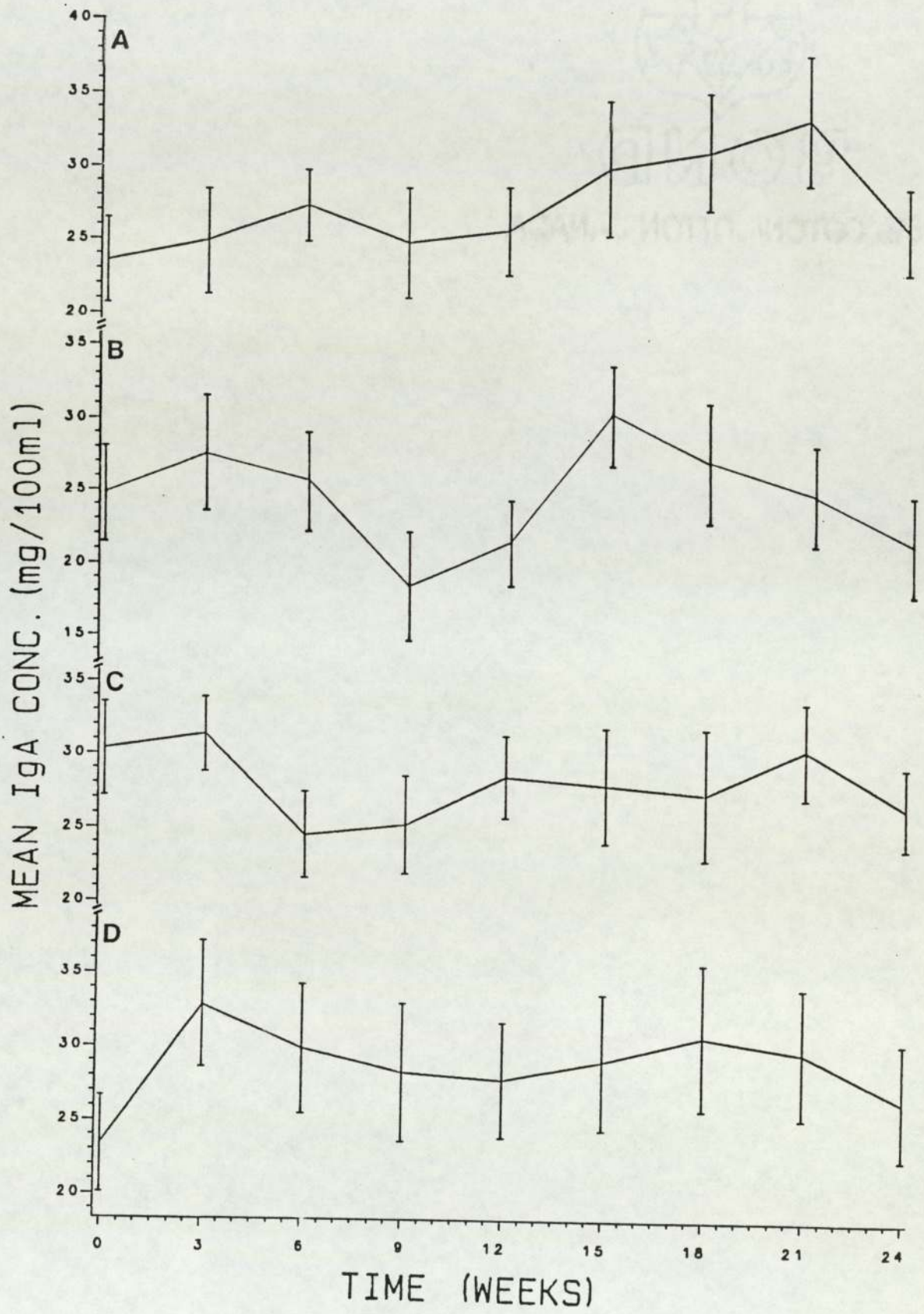


Figure 10. Graph of the Mean IgA Concentration

7.3.6 Percentage IgA in Total Protein

The percentage mean IgA in total tear protein for each group per visit is tabulated in Table 31, Appendix F and graphically illustrated in Figures 11A and B.

Statistical analysis shows 58.8 percent of the data fits the model presented in Tables 28 and 29 (Appendix F). The analysis for the percent IgA in total protein as the dependent variable may be summarized as follows:

1. Group differences are not significant ($p = 0.88$)
2. Visit differences are significant ($p = 0.0001$)
3. Group x visit interactions are significant ($p = 0.01$)

Comparing IgA to IgA in total protein it seems that the "visits" have no influence on IgA as the dependent variable but are of significant influence on the IgA in total protein model. Therefore, it would appear that changes in the total protein content affect the ratio of IgA to total protein.

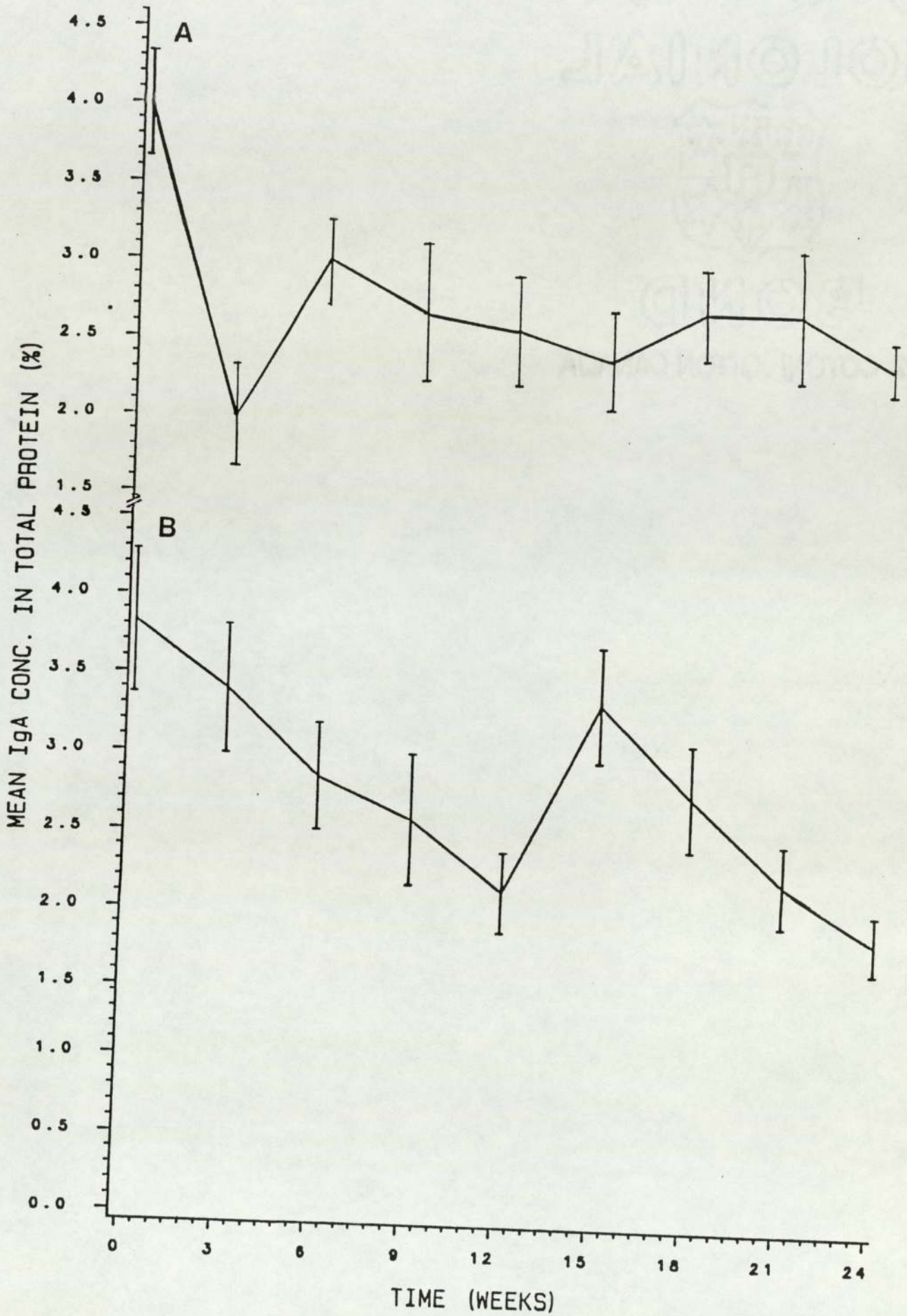


Figure 11a: Graph of the Mean Percent IgA in Total Tear Protein

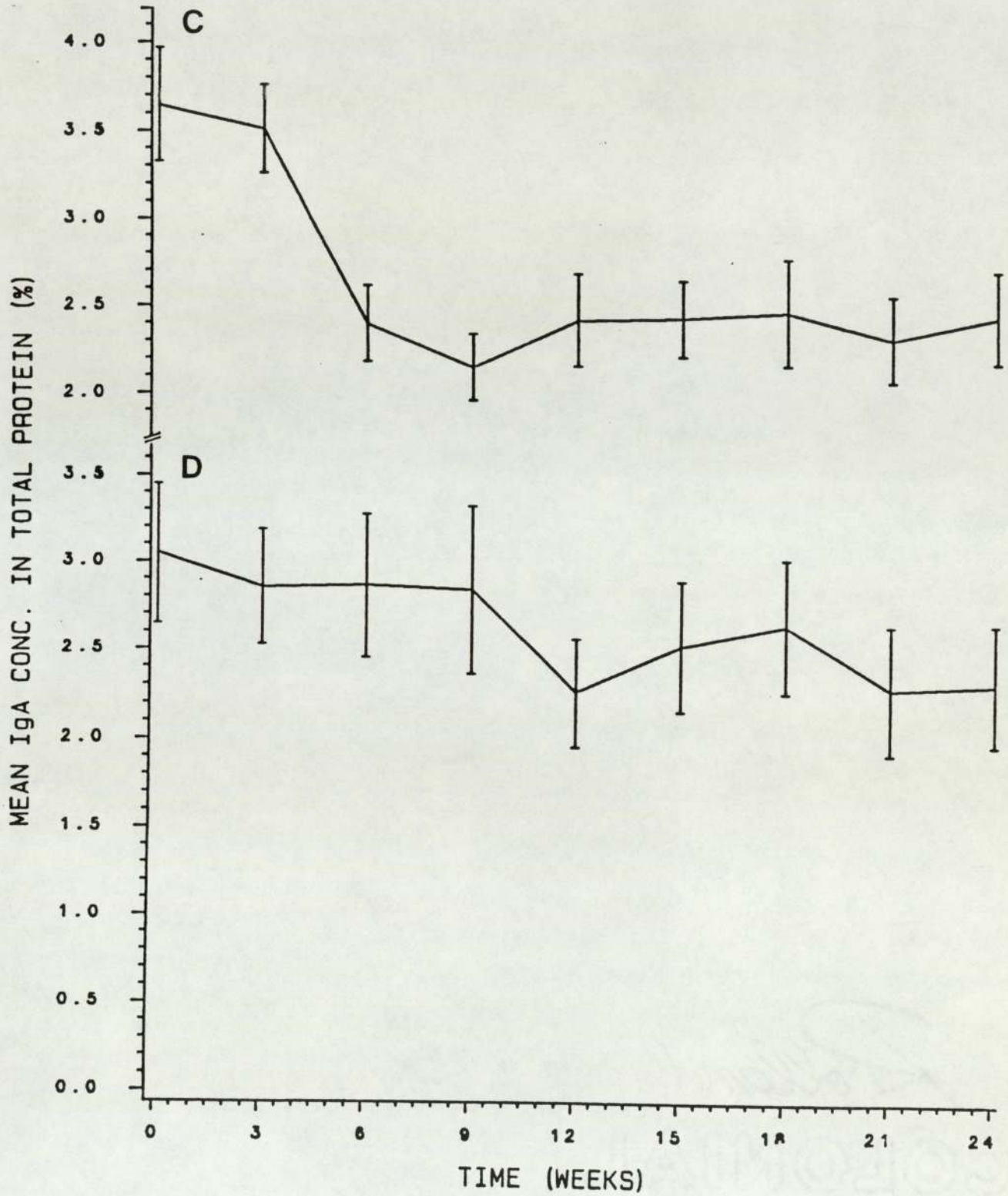


Figure 11b: Graph of the Mean Percent IgA in Total Tear Protein.

7.4 Clinical Results

General assessment of visual acuity, refractive status and corneal curvature were evaluated in accordance with standard optometric procedures. There were no apparent differences in these parameters among the group or between the groups over the period of 24 weeks.

The frequency of those clinical signs which are indicative of an adverse ocular response to the treatment procedures summarized in Table 8 are reported herein:

7.4.1 Pachometric Measurements

The apparent corneal thickness data obtained by the self-recording pachometer are presented in Table 31 (Appendix F) and are graphically illustrated in Figure 12.

The mean baseline thickness appears to be similar among the groups and compares with the normal values reported by Mandell and Polse (1969).

The mean differences among the groups during the period of treatment are statistically analyzed by the linear models presented in Tables 32 and 33 (Appendix F). The r-square value indicates 92.1% the data fits the model. The analysis of variance for the corneal thickness (cornea) as the dependent variable may be summarized as follows:

1. Group differences are not significant ($p = 0.77$)
2. Visit differences are significant ($p = 0.0001$)
3. Group x visit interactions are not significant ($p = 0.56$)
4. Linear regression analysis shows no significant trend over time ($p = 0.51$)

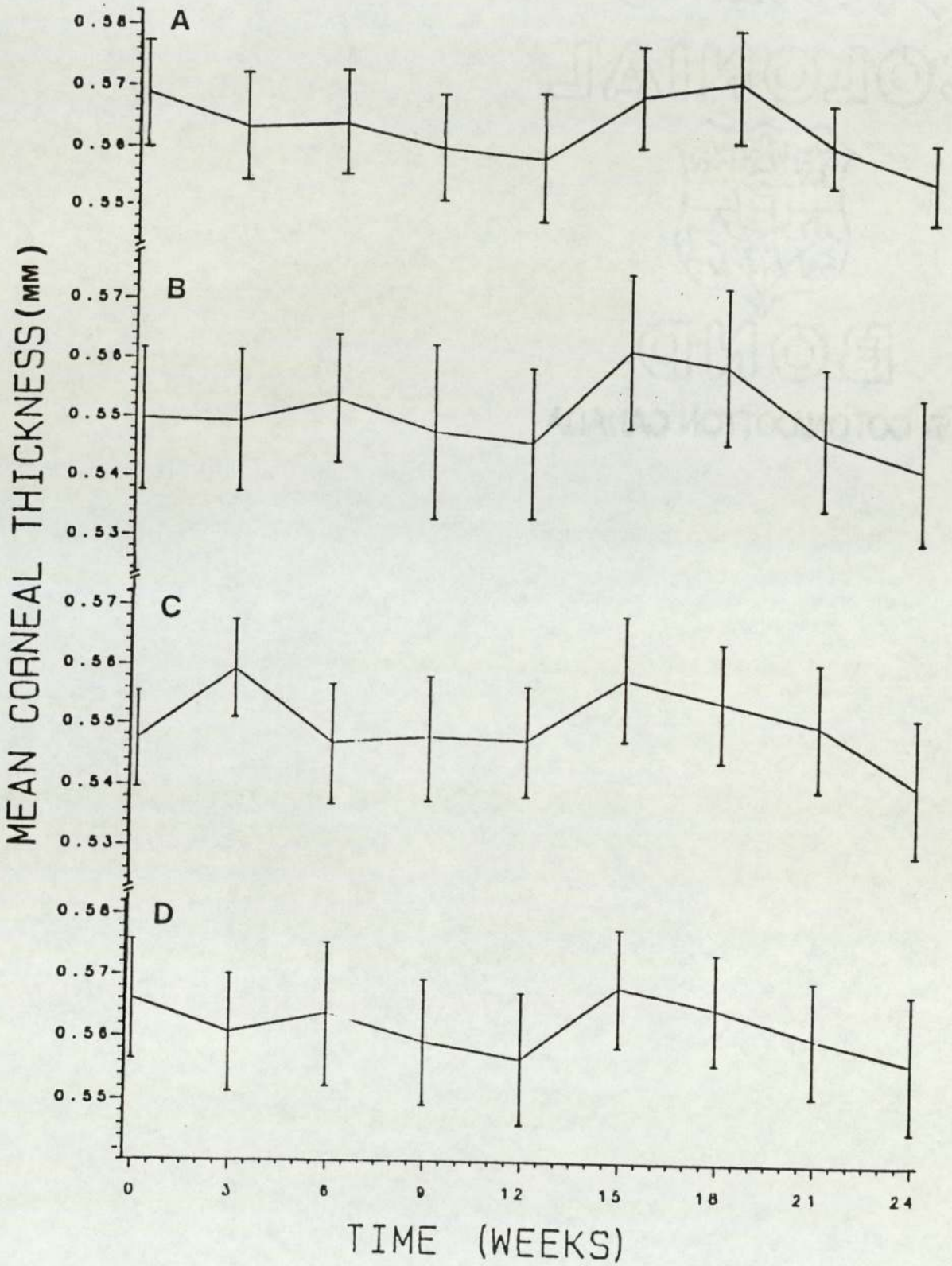


Figure 12. Graph of the Mean Apparent Corneal Thickness

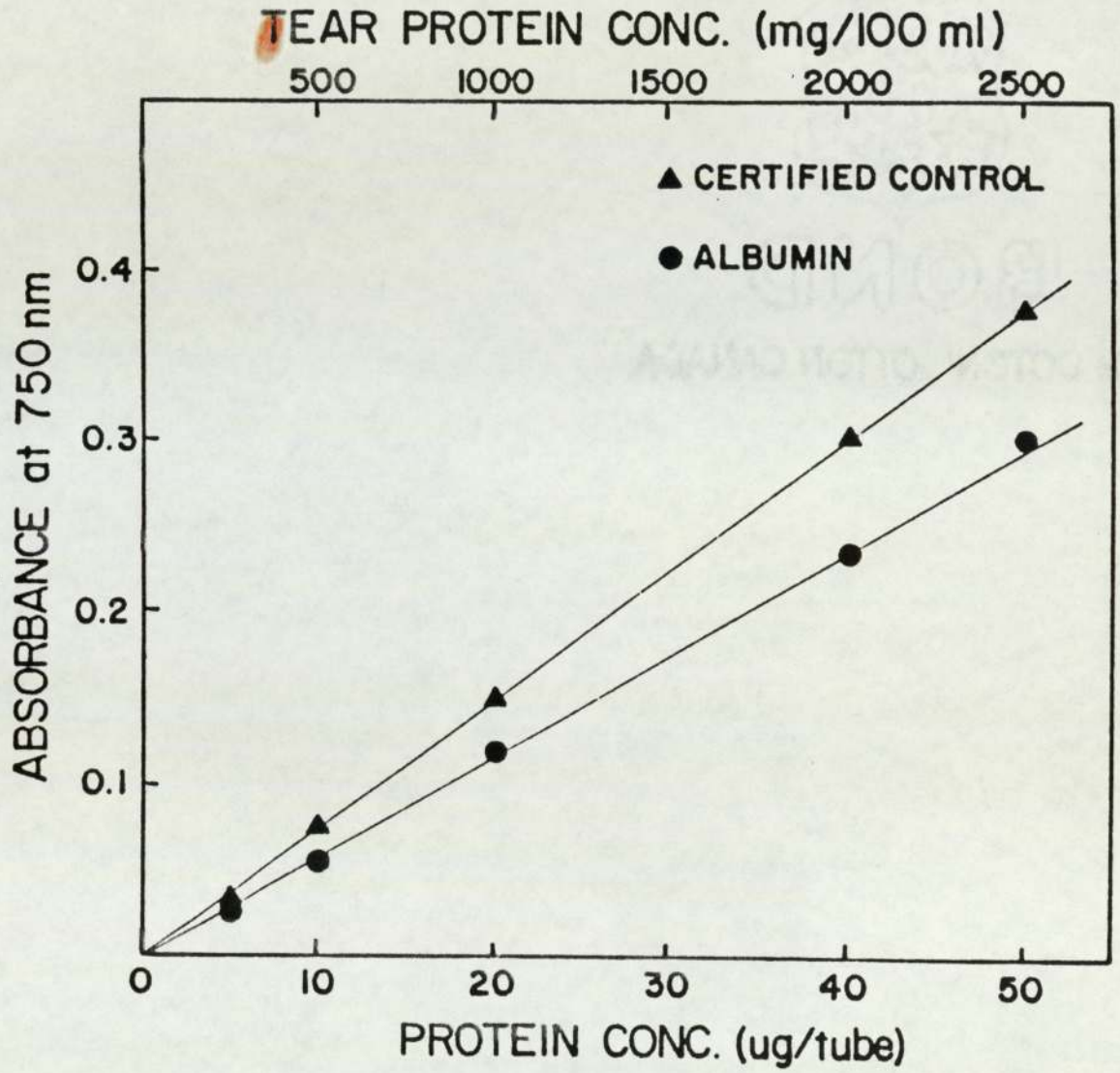


Figure 13: Comparison of Calibration Curve of Certified Protein Control vs Serum Albumin

7.4.2 Corneal Staining

Corneal staining was assessed before and after treatment with the aid of 1% sodium fluorescein and the biomicroscope. An eyepiece graticule was used to assess the area of staining observed during the examination.

Staining was almost always present in Groups B and C and only occasionally in Group A and D (Table 8). The incidence of staining greater than 1% of the corneal surface area was 38.5% for subjects in Group B, 36% in Group C and only in 7.7% of Group A. These differences were judged to be significant (df = 49; p = 0.05).

7.4.3 Complications

1. Red Eyes

   Four (4) subjects developed red eyes (one from Group B and 3 from Group C). The subject in Group B developed red eyes during the first week. Further investigation confirmed that he was sensitive to Thimerosal. The three subjects in Group C with red eyes appeared to be sensitive to the Chlorhexidine and/or possibly the residual papain-preservative complex on the lens.

2. Subepithelial Infiltrates

   Two of the three subjects with red eyes in Group C also had subepithelial infiltrates. The first case (C8) was observed during Week 7 and second case in Week 18. Lens wear was discontinued and the condition cleared in 3 weeks. The solution-papain treatment may be the causative agent.

3. Giant Papillary Conjunctivitis

   There was no apparent change in the appearance of the tarsal conjunctiva in any of the contact lens wearing groups. However, one control subject, (D11) showed grade 1 giant papillary conjunctivitis at Week 18. This was attributed to a spring allergy.

Table 8. Incidence of Adverse Eye Reactions

Adverse Reactions	Number (Percentage) in Group			
	A	B	C	D
Red Eyes	0.0	1 (7.7)	3 (21.4)	0.0
Corneal Infiltrates	0.0	0.0	2 (15.4)	0.0
Corneal Area Stained 1% or More	1 (7.7)	5 (38.5)	5 (36.0)	0.0
Conjunctival Changes	0.0	0.0	0.0	1 (7.0)
Total Number	13	13	14	14

Chapter 8

DISCUSSION AND CONCLUSIONS

8.1 Introduction

Human tears are a mixture of secretions from the lacrimal glands, conjunctival glands and other small glands which empty their contents into the conjunctival sac. Because of the complexity of its composition, the protein values obtained vary widely, indicating a number of variables operating; one of which is the method of sampling.

In this study care was taken to avoid stimulation. The values obtained will be interpreted as those of non-stimulated tears and will be the basis for the discussion and conclusions.

8.2 Discussion

The mean total tear protein concentration of 1.024 g/100 ml for the four groups studied compares favourably with current value of 0.9 g/100 ml by Lambert (1983). In earlier studies there were many discrepancies in the reported values which are attibuted to the method of sampling (van Haeringen, 1981). In most cases an irritant was used to stimulate tear flow (Table 3). Brunish (1957) reported a decrease in tear protein content in stimulated tears. This observation was supported by Callender and Morrison (1974) in a study wherein a decrease in tear protein concentration was observed during the initial stages of adaptation to PMMA contact lens wear. This change in tear composition was attributed to excessive tearing.

The method employed for assaying these tear proteins is another reason for the discrepancy. For example, colorimetric methods may be influenced by non-protein nitrogenous substances. The method used in this study is minimally affected by these substances (Lowry *et al*, 1951).

In this study there was no significant difference in protein levels among the groups, however, there was a linear upward trend with time. This trend may be attributed to seasonal variations in the tear protein, as the study progressed from Winter to Summer. It could be postulated that the increase in protein concentration was due to an increase in tear evaporation as the subjects tended to be more outdoors where the temperature and humidity could not be controlled as within the University environment.

This increase in protein concentration may also be due to an adaptive phenomenon. Although there was no apparent stimulation caused by the sampling techinque, reflex tears could have resulted from the awareness of the samples being taken in the earlier visits.

A third explanation may be that there is transudation of serum proteins caused by conjunctival irritation due to environmental conditions.

Electrophoretic separation of the tear proteins indicated that 12 to 13 bands or protein fractions were usually present with all groups. The number of fractions detected is in agreement with that reported by Sapse *et al* (1967). Gachon and co-workers (1979) detected at least 60 fractions. However, this higher number of fractions reported was not obtained with a single sample but with pooled tear samples, using a crossed immunoelectrophoresis method. Many of the fractions identified are not normally present in the tears.

In order to facilitate the analysis of these fractions it was necessary to divide them into four (4) distinct areas which correspond to their relative mobilities from the cathode to the anode. The data derived indicated that there was no significant difference between groups over time. However, there was a significant alteration in the two intermediate areas identified as II (PER 2) and III (PER 3). The linear regression analysis indicated a significant increase ($p=0.0001$) in PER 2 fractions with respect to time. Previous studies by Callender (1973) identified serum proteins as the major component of these fractions (PER 2). This observation, in addition to the increase in total protein lends support to the hypothesis

that the increase in protein concentration may be due to conjunctival irritation from sampling or environmental conditions.

There is no agreement in the literature on the way serum proteins enter the tear fluid. The incidence of high serum albumin levels in tear fluids of conjunctival hyperemia has lead Jannsen and van Bijsterveld (1983) to conclude that serum proteins enter tears by leakage from the conjunctival capillaries. From the clinical evaluation of subjects in this study, there was no redness of eyes except in those four cases, B13, C5, C7 and C8, in which there was an adverse response to the chemical disinfection procedure. Since all groups showed an increase concentration in fractions II (PER 2) and in total protein content, this increase must be attributed to seasonal changes or sampling technique.

Lysozyme and IgA are both important antibacterial agents in tears which have a protective function. These two proteins appear not to be affected by the modes of treatment. The mean lysozyme level for the overall study was 269 mg/100 ml. This value is much higher than those values compiled by Stanifer *et al* (1983) in Table 9. The higher value obtained is attributed to the modified immunoelectrophoretic technique employed and the fact that a human tear lysozyme standard was used instead of egg lysozyme. Human tear lysozyme is 3.5 times more active than egg lysozyme (Jolles and Jolles, 1967).

There was no difference in lysozyme levels among the groups although there was variablility within each group. Horwitz *et al* (1978) noted a diurnal variation in lysozyme levels in normal subjects. The level was lowest between midnight and 3:00 a.m. and peaked between 9:00 a.m. and 12:00. Since all samples collected during this study were between 10:00 a.m. and 1:40 p.m., then they are peak values and are quite comparable with the 200 mg/100 ml reported by Horwitz and co-workers (1978).

It is interesting to note that there was no significant linear trend in the lysozyme level with time. This is due to the fact that the lysozyme concentration is independent of the rate of secretion. This has been documented (van Haeringen and Glasius 1974c).

Table 9. Reported Levels of Lysozyme Assays in Tears of Normal Subjects (from Stanfier et al, 1983)

Assay	Method of Collection	Normal tear lysozyme Concentration	Reference Standard
Schirmer Lysoplate Assay	Schirmer Paper	[a]40-70 unit activity/ul	Hen Eggwhite Lysosyme
Schirmer Lysoplate Assay	Schirmer Paper	[b]≥22.5mm diameter lysis	Hen Eggwhite Lysozyme
Schirmer Lysoplate Assay	Schirmer Paper	1.7mg/ml	Human Tear Lysozyme
Spectrophotometric	Microcapillary tube	6.1mg/ml	Hen Eggwhite Lysozyme
Immunodiffusion	Micropipette	1.3mg/ml	Human Serum Lysozyme
Electrophoresis	Filter paper (basal) Schirmer Paper (reflex)	0.65mg/ml 1.60mg/ml	Human Leukemic Urinary Lysozyme

a. "Unit activity" is the amount of enzyme required to cause a decrease in the spectrophotometric absorption of M. lysodeikticus substrate of 0.001 in the first minute of reaction.

b. clear zone. under standard conditions.

This study also shows that the use of solutions preserved with chlorhexidine during hydrogel lens wear do not alter the lysozyme content in tears. This finding is not in agreement with that of Johnsson and co-workers (1978) who reported a decrease in lysozyme activity in *in vitro* studies on chlorhexidine. Both Groups B and C used Flexcare^{R} which contained 0.005% chlorhexidine. There are reports of drugs decreasing the activity of lysozymes; one example is Practolol which causes ocular toxicity with longer term use (Mackie and Seal, 1975). It is prudent therefore, that long term studies be done to determine the effect of chlorhexidine on the tear lysozyme level.

It is also interesting to note that the tear IgA level did not show a significant difference among the groups, however, there was some variability within each group. Similar results have been reported by Mannucci and co-workers (1984) from the tears of patients on daily wear contact lenses. However, they found a significant increase (approximately twice the level) in the tears of patients on extended wear lenses. In the absence of an immumopathological condition, it was suggested that the elevated IgA was due to the continuous mechanical stimulation of the conjunctival tissues in extended wear. Their elevated value of 23.8 + 14.8 mg/100 ml is within the range of normal values found in the present study. Horwitz and co-workers (1978) did not find a diurnal trend but noted the wide variation more in normal subjects. Similar observations were found by various investigators as shown in Table 10. McClellan and co-workers (1974) found values ranging from 8 to 60 mg/100 ml.

The present investigation shows as a mean value for all samples 27.2 mg/100 mls which is comparable with those of Sen *et al* (1976) and Sen and Sarin (1979).

The percentage lysozyme in total protein and the percentage IgA in total protein provide some interesting information. Although there was no linear trend with respect to time in the analysis of either lysozyme or IgA, there was a significant linear trend ($p=0.0001$) when each of these proteins is expressed as a percentage of the total protein. Since the levels of lysozyme and IgA are independent of

Table 10: Reported Levels of IgA in Tears of Normal Subjects.

Reference	No. of Subjects	IgA (mg/100ml)
Brauninger and Centifanto (1971)	24	9 - 50
Garner and Rabi (1976)	100	125 (IU/ml)
Little et al (1969)	10	21
Mannucci et al (1984)	17	11.3 ± 2.9
McClellan et al (1973)	74	17
McClellan et al (1974)	61	22 (8 - 60)
Sen et al (1976)	50	24.6
Sen and Sarin (1979)	90	26.0 ± 13.2
Present Study	53	25.6 ± 11.5

the rate of tear secretion, their percentages in total protein should be similar with respect to time. In this work the percentage lysozyme in total protein and that of IgA in total protein showed a decrease with time. It is evident that the decrease observed is influenced by the increase in a protein component not assayed. The protein profile obtained by acrylamide gel electrophoresis suggests it is a protein in Group II (PER 2).

The clinical results indicate that subjects in Group A (thermal disinfection) were trouble free throughout the 24 weeks of the study. There was no apparent difference between the ocular response of this group and control group D. However, the persistence of corneal staining, which was present in 38.5% and 36% of subjects for Groups B and C respectively, is indicative of the solution intolerance reported by Coward and co-workers (1984).

The incidence of infiltrates in 14.4% of Group C is of interest since infiltrates are due to an inflammatory response which may be viral, bacterial, toxic or other causes. Ophthalmological consultations indicated in both cases that the condition was neither bacterial nor viral in origin. This lead to a postulation of a toxic and/or allergic reation which may be excerbated by the Thimerosal-papain complex.

In spite of the adverse ocular response observed in the chemical disinfection Groups B and C, there was no significant difference in corneal thickness nor tear protein composition among the groups.

8.3 Conclusions

Several conclusions may be drawn from the results of the study. These are:

1. The change in tear protein composition noted was not due to the hydrogel lens wear and/or care systems but may be due to seasonal and environmental conditions.
2. The micro-techniques of gel electrophoresis and immunoelectrophoresis adapted for small samples are a novel contribution to the study of the lacrimal and other glandular fluids where secretions are scanty.

3. Acrylamide gel micro-electrophoresis can be used to separate tear proteins into 13 observable fractions.

4. There is some evidence for *in vitro* interaction between papain and hydrogel lenses, and papain and Thimerosal. However, there is no statistically significant evidence to show that Chlorhexidine and/or Thimerosal is responsible for adverse eye reactions observed. These preservatives do not alter the concentrations of lysozyme and IgA as was previously suggested by Johnsson *et al* (1978).

8.4 Suggestions and Recommendations for Further Investigations

From this study, the following suggestions and recommendations are made for further investigations on tear proteins.

1. A long term study on the effects of Chlorhexidine and Thimerosal on the antibacterial proteins should be undertaken. In addition to lysozyme and IgA, specific tear pre-albumin (STP) and lactoferrin should be studied. Preliminary studies on lactoferrin show that it is approximately half the concentration of lysozyme and that is is not affected by Chlorhexidine and Thimerosal preserved hydrogel lens solutions.

2. Isoelectric focusing should be utilized to provide a more definitive separation of the intermediate protein fractions (PER 2 and PER 3). This, in addition to crossed immunoelectrophoresis, would enable the identification of those fractions which showed change during this study.

3. The antiproteinases should be investigated as to their role when residual papain is present in the eye. Radioimmunodiffusion may be the technique necessary to quantify low concentrations of these proteins.

4. IgE plays an important role in the eye's response to allergens. Therefore, it may be of clinical significance to study the effects of Thimerosal, a known sensitizer, on IgE. Because of the low concentration of this protein in tears, a radioimmuno diffusion method should be employed.

Appendix A

EXPERIMENTAL SECTION

A.1 Introduction

Enzymatic cleaning has proven to be an effective method of removing denatured tear proteins from hydrogel contact lenses. Since all enzymes are proteins then it is possible that papain replace the digested tear proteins coating on the lens. This residual papain may be bound to the lens surfaces following heat or chemical disinfection. In the latter case, Thimerosal may interact with the sulphydryl group of papain and the elevated concentration of the mercuri-papain complex could exacerbate the red eye syndrome. This chain of events may reflect changes in the tear protein composition.

To test this hypothesis an *in vitro* experiment was designed to clarify:-

1. whether papain binds to the hydrogel lens with either heat or chemical disinfection and
2. whether papain forms a complex with Thimerosal.

The results of these preliminary experiments formed the basis for the design of the human study.

A.2 Determination of Papain Adsorption on Hydrogel Lenses

A.2.1 Materials and Methods

Twenty-four (24) new Bausch and Lomb Soflens^{R} contact lenses which had never been worn were divided into 4 groups each containing 6 lenses. All lenses were of identical parameters (-3.00 B3) and were made from a 38.5% water content HEMA hydrogel (polymacon).

All lenses were handled with surgical gloves and tweezers during each treatment procedure. The procedures were as follows:

1. *GROUP A*

Each lens was friction cleaned with freshly prepared saline and rinsed several times with saline. Saline was prepared by dissolving the Bausch and Lomb salt tablet in the appropriate volume of distilled water to make a 0.9% saline solution.

The lenses were then soaked (3 per vial) in 10 ml of freshly made Bausch and Lomb Soflens^{R} Enzymatic (papain) cleaner and mixed constantly for 2 hours.

After removal from the papain solution each lens was again friction cleaned with saline, rinsed several times with saline and heat disinfected in the Bausch and Lomb Professional Aseptron^{R} unit for one hour.

The entire procedure was repeated 24 times to simulate 6 months or 24 weekly papain cleaning cycles which a patient would do with his/her lenses.

The lenses were then purged by storing in freshly prepared saline using the Bausch and Lomb salt tablets. Purging was repeated 3 times over a period of 48 hours.

2. *GROUP B*

Each lens was friction cleaned with Preflex^{R}, a surfactant cleaner which contains 0.002% Thimerosal, 0.1% edetate disodium and surface active agents (Burton Parson-Alcon Pharm.) The lenses were rinsed several times with Boil-N-Soak^{R}., a rinsing solution which contains 0.001% Thimerosal, 0.1% edetate in a buffered normal saline solution.

The lenses were then soaked (3 per vial) for one hour in 10 ml. Flexcare^{R}, a disinfecting solution which contains 0.005% Chlorhexidine digluconate, 0.001% Thimerosal, 0.1% Edetate disodium in a buffered saline solution.

The entire procedure was repeated 24 times. The lenses were then purged as described in Group A. Purging was necessary to remove the absorbed Flexcare^{R} which might interfere with the protein assay procedure.

3. *GROUP C*

Each lens was treated in the same manner as those in Group B but two additional procedures were introduced. After each lens was cleaned with Preflex^{R} and rinsed with the Thimerosal preserved saline, they were soaked (3 per vial) in 10 ml of freshly prepared Soflens^{R} Enzymatic (papain) cleaner and mixed constantly for 2 hours.

After removal from the papain solution, the lenses were rinsed and friction cleaned with the Thimerosal preserved saline before soaking in Flexcare^{R} for one hour.

The whole procedure was repeated 24 times before the lenses were purged as described in Group B.

4. *GROUP D*

Each lens was treated in the same manner as those in Group A but the papain cleaning step was omitted. Instead, the lenses were soaked in freshly prepared saline for the two hour period before heat disinfection. Group D lenses acted as the controls.

5. *TOTAL PROTEIN ASSAY*

All lenses in Groups A, B, C and D were purged and stored in non-preserved saline for at least 48 hours before they were assayed for protein adsorption. The protein was solubilized by heating each lens in 0.6N NaOH (0.5ml/lens) in a 70^{0}C waterbath for one hour before the Lowry method for total protein determination was performed (See Appendix B).

A.2.2 Results

Table 11 shows the mean protein concentration measured for each of these groups of lenses. The mean difference between Group A and Group D (control lenses) is 1.094 ug per lens.

The mean difference between Group B and Group D lenses is 1.700 ug per lens. Since there was no enzyme used with Group B lenses, one must assume that there is a reaction between a non-protein source of nitrogen groups in Preflex^{R} and/or Flexcare^{R} bound to the lens and the reagents used for protein determination.

The mean difference between Group B and Group C lenses is 0.263 ug per lens. The difference in this case appears to be not significant.

Table 11: Residual Papain Assay of Total Protein on Hydrogel Lenses

	Total Protein (µg/lens) Conc.			
Lens #	A	B	C	D
1	1.875	1.875	3.162	0.625
2	2.500	2.500	1.875	*****
3	1.500	1.875	2.500	0.625
4	1.500	3.187	2.500	0.625
5	1.937	*****	2.000	0.625
6	0.625	1.875	3.125	0.312
Mean	1.656	2.264	2.527	0.562
S.D.	0.625	0.583	0.479	0.140

***** Lens torn during cleaning procedure.

Analysis of variance (ANOVA) was used to compare this statistical sample of unequal size. The SAS 2x2 factorial model was employed. The results indicate a significant difference between heat and chemical ($p=0.0001$) and between enzyme and no enzyme with either mode of treatment ($p=0.007$).

A.2.3 Conclusion

This study shows that the treatment of Bausch and Lomb Soflens contact lens with the enzyme (papain) cleaner and heat results in the absorption of papain on the lens. However, there is no evidence of papain absorption with chemical disinfection.

A.3 Determination of Mercuri-Papain Complex

A.3.1 Materials and Methods

1. Soflens^{R} enzymatic cleaning tablets

These are commercially available papain tablets made by Allergan Pharmaceuticals, California, for the cleaning of hydrogel contact lenses.

2. Boil-N-Soak^{R}

This is a buffered saline solution which contains 0.001% Thimerosal and 0.1% Edetate disodium. This preserved saline is made by Burton Parson-Alcon Pharmaceuticals.

3. Mercury working standards

Mercury standards were made from a stock mercury reference (BDH) containing 1000 ppm (1ml = 1 mg Hg = 4.98 mmol/L) by diluting the stock solution with deionized distilled water to make 1, 5 and 10 ppm respectively. Edetate disodium was added to each working standard to make a 0.1% w/v solution corresponding to its concentration in the Boil-N-Soak^{R} solution.

4. Preparation of papain solutions

All samples were made up in duplicate unless otherwise stated.

a. Papain in saline

One Soflens^{R} enzymatic cleaning tablet (papain) was dissolved in 10 ml freshly made saline. Saline was prepared by dissolving the Bausch and Lomb salt tablet in the appropriate volume of deionized distilled water.

b. Papain in Boil-N-Soak^{R}

One Soflens^{R} enzymatic cleaning tablet (papain) was dissolved in 10 ml of Boil-N-Soak^{R}.

The papain solutions were kept in screw-capped vials at room temperature for 24 hours before they were analysed.

5. Determination of Mercury (Hg)

The mercury standards, samples of Boil-N-Soak^R and papain solutions were analysed by aspirating 20 ul of each into the graphite furnace of the flameless atomic absorption spectrophotometer (Pye Unicam, Model Sp 9-01). All samples were mixed before reading in the spectrophotometer. The mercury absorbance levels were recorded on a chart recorder. Three readings of each were taken and averaged.

After samples of the Papain-Boil-N-Soak^R solution were read, the mixture was centrifuged at 15000 rpm to precipitate the papain and the supernatant was analysed for mercury. Then the supernatant and precipitate were reconstituted in a vortex mixer and reanalysed as previously described.

The concentration of mercury in the samples could be determined from a calibration curve by plotting the peak heights (absorbance) of the standards against their respective concentrations (ppm). However, in this experiment a programmable calculator (Texas Instrument, Model 59) was used to compute the mercury concentration of the unknown solutions from the correlation coefficient for the standard calibration curve.

A.3.2 Results

Table 12 shows the levels of mercury present in the samples. The mercury level in the Papain-Boil-N-Soak^R mixture ranges from 4.10 to 4.23 ppm. This level is quite similar to that obtained for Boil-N-Soak^R (4.3 ppm).

The mercury level in the supernatant after centrifuging shows only trace amounts present (0 to 0.3). This result is similar to that obtained from the Papain-Saline solution.

Analysis of the reconstituted supernatant and precipitate mixture shows that the mercury level returns to an average value of 3.94 ppm. This suggests that the mercury removed from the supernatant was absorbed by the papain rather than by the glass vial and/or stopper.

Table 12: Mercury Content of a Papain-Boil-N-Soak Solution.

Sample	Absorbance (mean peak height cm.)	Conc. (ppm)
Standard #1 (1 ppm)	2.8	1.00
Standard #2 (5 ppm)	12.5	5.00
Standard #3 (10 ppm)	17.5	10.00
Papain & Saline (No Thimerosal)	0.0	0.00
Boil-N-Soak	10.8	4.30
Papain & Boil-N-Soak Solution #1	10.6	4.23
Papain & Boil-N-Soak Supernatant #1	0.0	0.00
Papain & Boil-N-Soak Reconstituted #1	9.7	3.90
Papain & Boil-N-Soak Solution #2	10.3	4.11
Papain & Boil-N-Soak Supernatant #2	0.8	0.30
Papain & Boil-N-Soak Reconstituted #2	10.0	4.00

A.3.3 Conclusions

This study shows that when papain is mixed with a solution containing Thimerosal, an organic mercurial antimicrobial agent, a complex is formed.

The results from these *in Vitro* experiments indicate that the enzymatic papain cleaner is absorbed by the hydrogel lens and that it forms a mercuri-papain complex in a solution containing Thimerosal.

Bosmann *et al.* (1980) reported the presence of residual papain activity on chemically disinfected lenses while heat disinfected lenses were devoid of this activity. This suggests that the combination of papain with the chemical disinfectant may be the cause of the adverse eye reactions reported. However, they showed that the amount of inhibitory activity in the tears was more than enough to inhibit this small but measurable residual papain activity which is bound to the lens after chemical disinfection.

Whether residual papain or its mercury complex causes the inflammatory response warrants an investigation. This may be possible with a controlled clinical study in which the tear proteins could be monitored during the use of various hydrogel lens care systems.

Appendix B

DETERMINATION OF TOTAL TEAR PROTEIN CONCENTRATION

B.1 Introduction

The Lowry *et al.* (1951) method for total protein concentration was modified to accommodate a 2 ul tear sample. The colour reaction which develops between the copper-carbonate-protein complex and the Folin Phenol reagent was read at 750 nm in a spectrophotometer against known standards.

B.2 Materials

Stock Reagents

Unless stated otherwise all reagents were analytical grade, obtained from British Drug House Ltd.,

1. Cupric Sulphate
2. 2N Folin-Ciocalteou Phenol Reagent
3. Sodium Tartrate
4. Protein Standard Solution 8% (w/v) (Sigma)

   Certified to contain:

Albumin	5.0 g/dL
Globulin	3.0 g/dL

5. Human Serum Albumin IV (Sigma)

B.3 Working Reagents

1. Cupric sulphate 1% (w/v)

$CuSO_4.5H_2O$	1.0 g	
Distilled water to		100. ml

2. Sodium tartrate 2% (w/v)

Sodium tartrate	2.0 g
Distilled water to	100.0 ml

3. Sodium carbonate in 0.1N NaOH 2% (w/v)

Sodum carbonate	20.0 g
0.1N sodium hydroxide to	1.0 L

4. Human serum albumin iv (Sigma) 1% (w/v)

Human serum albumin	0.1 g
Distilled water	10.0 ml

This reagent should be kept frozen in 1.0 ml aliquots

5. Protein calibration standard 1% (v/v)

Certified protein standard (Sigma) 8%	1.0 ml
Distilled water	7.0 ml

This standard should be kept frozen in 0.5 ml aliquots.

6. Alkaline copper solution (Reagent A)

1% Cupric sulphate	1.0 ml
2% Sodum tartrate	1.0 ml
2% Sodium carbonate in 0.1N NaOH to	100.0 ml

7. 1N Folin-Ciocalteau phenol reagent (Reagent B)

2N Folin - Ciocalteau phenol reagent	1.0 ml
Distilled water	1.0 ml

B.4 Method

A 2 ul tear sample was added to 3 ml of reagent A. The test tubes were mixed rapidly with a vortex mixer, then allowed to stand for 10 minutes at room temperature. Next, 0.3 ml of reagent B was added to each tube and mixed rapidly. This rapid mixing was essential because reagent B is unstable in an alkaline solution. The tubes were incubated at room temperature for 30 minutes, then read against a reagent blank at 750 nm. in a D.B. spectrophotometer (CE 373, Cecil Instruments).

The total protein concentration was determined from a calibration curve of the 1% certified protein solution. The volumes used were 0.5, 1.0, 2.0, 4.0 and 5.0 ul. These volumes correspond to 5, 10, 20, 40 and 50 ug/tube respectively. The standards were done in duplicate and were treated by the same procedure as the tear samples.

The protein concentration of the tear sample was read directly from the calibration curve in Figure 13 or calculated with the aid of a programmable calculator.

Figure 13: Comparison of Calibration Curve of Certified Protein Control vs Serum Albumin

Appendix C

ELECTROPHORESIS: THEORY AND METHODS

C.1 Theory

Electrophoresis is a sensitive analytical method based on the principle that changed particles, usually ions, in solution will migrate toward one electorde in an electrical field (Smith 1976). If these particles are differently charged, they will migrate in opposite directions. The positively charged particles mirgrate to the cathode (-ive pole) the negatively charged ones to the anode (+ive pole). The movement of these charged particles through the conducting medium is termed "Electrophoretic mobility".

The rate of migration of mixtures of charged particles by this technique will depend upon the strength of the electrical field, the molecular size, shape net charge and the degree of ionization. It also depends on the conducting medium which could be paper, starch, agarose, acrylamide gel or cellulose acetate. Thus a mixture of tear proteins will migrate at different rates through the conducting medium. This results in the separation of similar proteins into moving bands or fractions. The sharpness of the separation will depend upon the concentration of these fractions and the supporting medium.

C.2 Methods

There are two principal types of electrophoresis (1) moving boundary electrophoresis and (2) zone electrophoresis (Smith, 1976).

C.2.1 Moving Boundary Electrophoresis

The method of moving boundard electrophoresis was introduced by Piction and Linder in 1892 and refined by Tiselius in 1937 (Block *et al.*, 1955). This technique employs a quartz U-tube filled with a buffer solution of known ionic strength and density. The position and size of the different fractions are determined by observing the change in refractive index which occurs at the boundaries between similarly charged particles. This technique has the following disadvantages: (1) the boundaries are unstable (2) separation is incomplete (3) a large sample volume is required and (4) fixing of the substances at the positions to which they have migrated is not possible because diffusion occurs when the current is switched off. This problem does not occur when the solution is held in a stabilizing medium such as in zone electrophoresis.

C.2.2 Zone Electrophoresis

The principle of separating charge particles in an electrical field is similar to that of boundary electrophoresis however the supporting medium, which may be paper or gel, enhances the separation of these particles into discrete zones. This permits detection and quantification by chemical and physical methods.

Filter paper electrophoresis was widely used for several years until other supporting media were developed. These supporting media are starch, agar, agarose, polyacrylamide gel and cellulose acetate (Smith, 1976). Of these, agarose and polyacrylamide gel have been found to give best separation. It was for this reason they were selected for this study.

The development of immunological techniques combined with histochemical stains, enzyme reaction and radioiostope labelling have made it possible to detect and quantify proteins present in micro quantities (Laurell *et al*, 1966; Mancini *et al* 1965). Since the tear volume is quite small any analysis of the protein components would require the most sensitive technique to detect changes in the protein composition. Therefore it was for this reason polyacrylamide gel electrophoresis and agarose gel electrophoresis were selected as the techniques of choice.

Appendix D

ACRYLAMIDE GEL ELECTROPHORESIS

D.1 Introduction

The technique of disc gel electrophoresis by Ornstein and Davis (1962) was modified to accommodate the low concentration of tear proteins.

The principle of electrophoretic analysis is based on the fact that proteins, being amphoteric, can behave either as acids or bases in an electrical field. Thus when a 2 ul of tears is placed on a vertical gel column medium of a constant pore size, and a constant current is applied between the cathode and anode of the column, the proteins are separated according to their charge density and molecular size. After separation of the migrated protein fractions, the gel is fixed, stained with a protein stain and destained. The characteristic protein bands are quantified by a scanning densitometer.

D.2 Materials

Stock Reagents

Unless stated otherwise all reagents were analytical grade, obtained from British Drug House (BDH) Ltd., U.K.

1. Ammonium Persulphate
2. Boric Acid
3. Cyanogum 41
4. Citric Acid (anhydrous)
5. 2-dimethylaminoethyl cyanide.
6. di-sodium tetraborate.

7. Sucrose

8. Tris hydroxyethyl methylomine

D.3 Working Reagents

All working reagents were freshly prepared for daily use on lens stated otherwise. Working reagents were kept at 4°C when not in use.

1. Ammonium persulphate 10% (w/v)

Ammonium persulphate	1.0 g
Distilled water	10.0 ml

2. 2-Dimethylaminoethyl cyanide 10% (w/v)

2-dimethylaminoethyl cyanide 87% (w/v)	1.0 ml
Distilled water	7.7 ml

3. Borate buffer pH 8.5 (Block et al, 1958)

Sodium tetra borate	17.5 g
Boric acid	9.3 g
Distilled water to	2.0 L
The pH was adjusted to	8.5

4. Sucrose 30% (w/v)

Sucrose	3.0 g
Distilled water	10.0 ml

This was kept frozen in 1 ml aliquots.
Each day one aliquot was used then discarded.

5. Tris-citric acid buffer pH8.6 (Schultze and Hermans, 1966).

0.0591 M tris hydroxymethyl methylamine	0.717 g
0.00705 M citric acid	0.135 g
Distilled water	100.000 ml
pH adjusted to	8.6

6. Cyanogum 41 (acrylamide and N,N-1methylene-bisacrylamide) 5% (w/v)

Cyanogum 41	5.0 g
Tris-citric acid buffer	100.0 ml

This pH adjusted to 8.6 if necessary, before the solution was filtered and stored at 4°C.

D.4 Method

1. Preparation of Acrylamide Gels

The 5% cyanogum 41 in tris-citric acid buffer was brought two room temperature. Twenty (20)ml of this solution was degassed by bubbling nitrogen gas for 15 minutes and 0.2 ml of 2-dimethylaminoethyl cyanide was added and mixing. Then 0.2 ml of 10% ammonium persulphate was added and mixed gently.

Approximately 15 ml of the mixture was poured into a shell type vial. The height of the fluid column within the electrophoresis tube was adjusted to 65 mm by pouring the 5% cyanogum 41 mixture into the shell vial before polymerization occurred.

Next, 20 ul of distilled water was carefully layered on the surface of the polymerizing gel to ensure formation of a smooth flat gel surface. This was best done with a 1.0 ml disposable syringe with a 23 gauge needle attached. The top of the needle was placed against the inner wall of the tube, close to the top of the gel solution, and the water was then slowly and and evenly layered on top of the gel solution.

Polmerization was complete in 15 to 20 minutes and the gels were stored at 4^0C.

2. Preparation of the Electrophoresis Chamber

A Buchler Polyanalyst (Buchler instruments) vertical gel column electrophoresis apparatus was employed (Fig. 14). The upper chamber was modified by changing the grommets to a smaller size in order to hold the much smaller electrophoresis gel tubes. The lower reservoir was filled with 600 ml of borate buffer pH 8.5. Eight (8) of the prepared gels tubes were removed from the shell vial and the outer surface of each tube was rinsed with buffer solution to remove adhering pieces of gel. The water overlay on the gel column surface was removed with a 1.0 ml syringe with a 23 gauge needle attached and replaced with 20 ul of 30% sucrose.

The gel tubes were fitted into the grommets in the upper reservoir and adjusted so that they all protruded the same distance into the lower reservoir buffer when the upper chamber was placed on top of the lower chamber.

3. Application of Sample

The tear sample was slowly expressed under the surface of the sucrose layer from a disposable microcapillary tube (Drummond Scientific). Next, 0.5 ul of 1.0% bromaphenol blue solution was added. Finally, the remaining space in the tube was filled with the upper reservoir buffer. Care was taken to prevent mixing of the buffer with the sucrose (Fig. 15A).

4. Power Supply Adjustment

LKB constant power supply, Model 2103, was used with the current set at 1.25 mA per gel tube. A maximum of eight tube could be run at a time. Migration of the bromophenol blue indicator took 10 to 12 minutes to reach the anode (+). The current was switched off when the indicator was approximately 5 mm from the anode edge of the gel.

5. Fixing and Staining Proteins

After electrophoretic separation of the proteins, the gels were removed from the tubes with a teflon plunger (Fig. 15B), fixed and stained with 0.05%, Coomassie brilliant blue R250 in 10% acetic acid and destained overnight in 7% acetic acid (Chrambach *et al*, 1967). The gels were stored in a dark cupboard in screw-cap vials containing 10 ml, 7% acetic acid until they were analyzed by densitometry. The protein fractions are seen as dense blue bands in the destained gels.

Figure 14: Vertical Gel Column Electrophoresis Apparatus. This apparatus consists of:

a. LKB constant power supply, Model 2103

b. Buchler Polyanalyst electrophoresis chamber modified to hold micro gels

CODE:

i. EL Electrical Leads

ii. LE Lid Electrode Assemlby

iii. PCE Platinum Cathode Electrode

iv. UBR Upper Buffer Reservoir

v. GT Gel Tube

vi. PAC Platinum Anode Electrode

vii. LBR Lower Buffer Reservoir

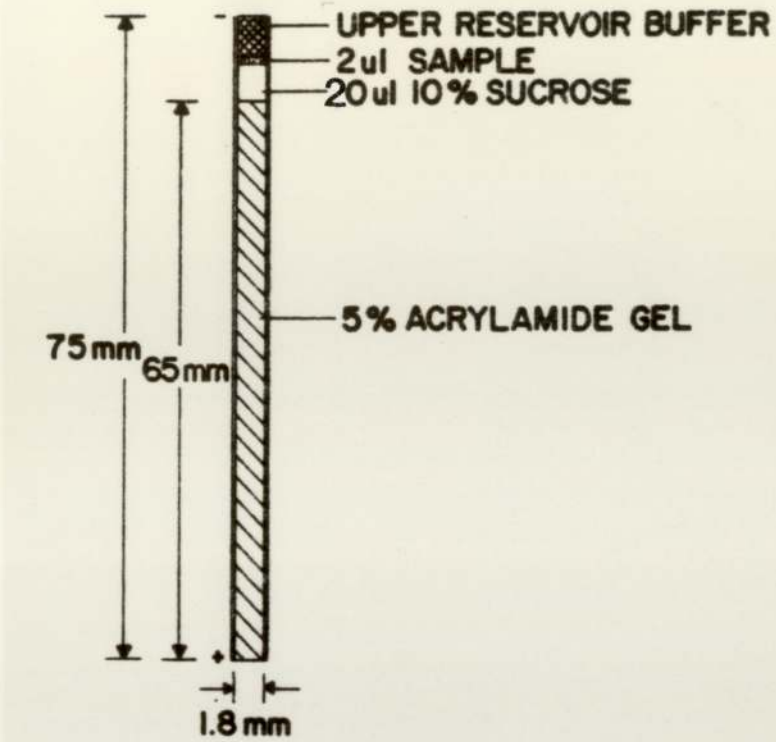
viii. LAS Level Adjustment Screw

ix. WAJ Water Cooling Jacket


EL
LE
UBR
PCE
WCJ
GT
LBR
LAS
PAE
MAINS
ON
OFF
kV
mA
VOLTAGE
V
POWER
W
CURRENT
mA
AMMETER
GND
LEAKAGE
HIGH
VOLTAGE
x200
LKB
2103 POWER SUPPLY

Fig. 14

Figure 15: Preparation and Staining of the Acrylamide Gels.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2ul SAMPLE
20ul 10% SUCROSE
5% ACRYLAMIDE GEL
75mm
65mm
1.8mm

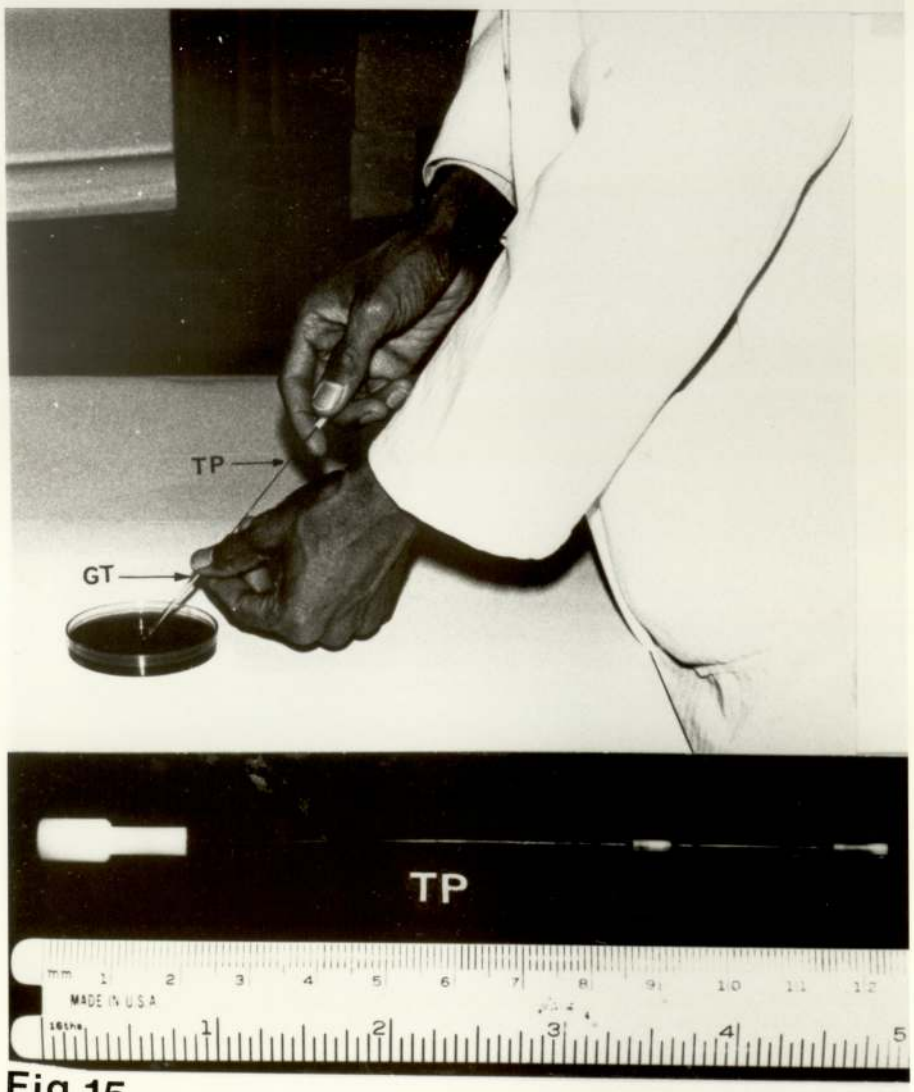
TP
GT
TP
MADE IN U.S.A.

Fig.15

D.5 Quantification of Protein Fractions

D.5.1 Scanning Densitometry

The gels were scanned at 580 nm using a Photo-Volt densitometer with a sample stage modified to accommodate these microgels. The density of the various protein fractions were graphically traced on a chart recorder (Fig. 16).

The protein fractions were differentiated into Groups I,II,III and IV in the other of the relative mobilities in the gel from the anode (+) to the cathode (-) end of the gel respectively. The area of group demarcation was taken as the deepest troughs between the protein fractions of of each group (Fig. 17B).

D.5.2 Analysis of Densitometric Tracing

The area under the peaks in each group was calculated with the aid of a microcomputer (Commodore PET) which was connected to a digitizing pad (Summagraphics BIT. PAD ONE) as shown in Figure 17A, 17B and described by William (1983). The graph of the protein fractions was taped to the surface of the digitizing pad and the cursor was used to trace the preprogrammed points for each group of fractions. The computer then calculated the X and Y coordinates and displayed the area and percentage of each group of protein fractions. This information was transfered to the University of Waterloo Mainframe Computer for storage until needed for statistical analysis.

Figure 16: Photo-Volt Densitometer for Scanning Acrylamide Gels.

Fig.16

Figure 17: Digitizing Pad(DP) and Commodore PET Microcomputer.

Figure A:
Digitizing Pad (DP) (Summagraphic BIT PAD ONE) and Commodore Pet Microcomputer. This microcomputer unit was used to calculate the percentage protein distributution for each set of proteins (PER 1, PER 2, PER 3, PER 4).

Figure B:
Cusor (C) Tracing the Preprogrammed Points on the Densitometric Tracing (DT) on Digitizing Table.

A

B

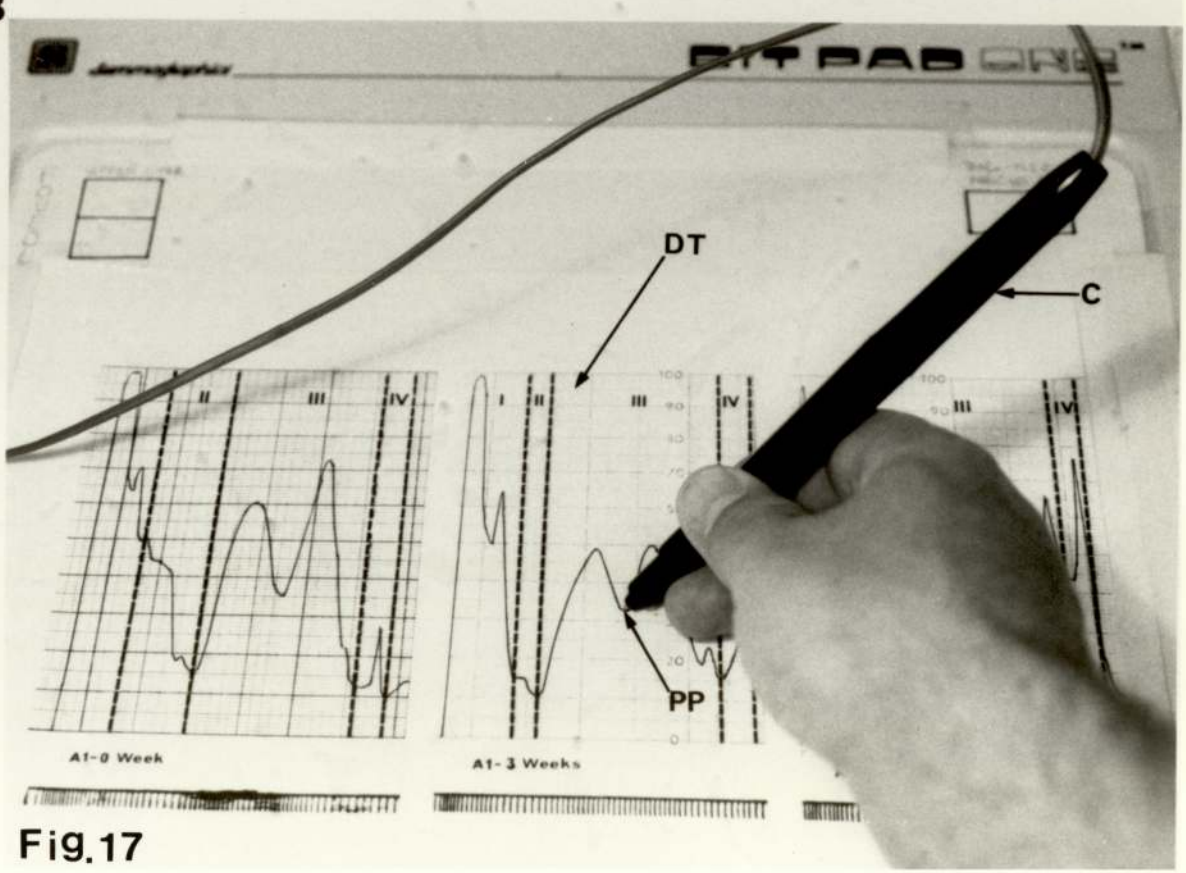

Fig.17

Appendix E

AGAROSE GEL IMMUNOELECTROPHORESIS (ROCKET IMMUNOELECTROPHORESIS)

E.1 Introduction

This technique developed by Laurell (1966) involves the electrophoresis of a specific tear antigen eg. Lysozyme of IgA, in an agarose gel medium containing the corresponding antibody. Migration of the antigen in the electrical field results in a long rocket-like immunoprecipitate.

The gel is washed, stained and destained. The length of the rocket measured is linearly correlated to the amount of antigen.

E.2 Materials

Stock Reagents

Unless stated otherwise all reagents were analytical grade, obtained from British Drug House Ltd.

1. Barbitone
2. Calcium Lactate
3. Coomassie Brilliant Blue R (Sigma)
4. Sodium Barbitone

Working Reagents

The following reagents were stored at $4^{o}C$

1. Monospecific immunoglobulins (DAKO) produced in the rabbit

a. Antihuman Colostrum IgA (alpha-chains and secretory piece). Lot 019E

b. Antihuman Lysozyme (Muramidase) Lot 0288

c. Antihuman Lactoferrin Lot 0788

2. Worthington Lysozyme Reagent Set (Worthington diagnostics) Lot 70E937

3. Immunostics^{TM} Reference Normal Serum (Seward) (See page 144)

4. Pooled Normal Serum

5. 0.12M pH8.6 Barbitone Buffer

Sodium barbitone	20.60 g
Barbitone	3.68 g
Calcium lactate	1.23 g
Distilled water	1.00 L

1ml of 0.5% thymol in isopropanol was added to prevent the growth of micro-organisms.

This barbitone buffer was diluted 1:1 to make the 0.06M solution required for the 1% agarose gel and reservoir buffer.

6. Coomassie Blue Stain 0.5% (W/V)

Coomassie brilliant blue R (Sigma)	5g
Methanol	450ml
Acetic acid	100ml
Distilled water	450ml

Coomassie blue powder was added to the methanol-acetic acid mixture and mixed with a magnetic stirrer. The solution was filtered into an amber bottle the next day.

7. Destaining Solution

Methanol	250 ml
Acetic acid	100 ml
Distilled water	450 ml

E.3 Methods

1. Preparation of 1% Agarose Gel

To 100 ml of 0.06 M barbitone buffer of pH8.6 was added 1gm of agarose. This suspension was gently boiled while being stirred until all the agarose was dissolved. 20 ml aliquots of the hot agarose solution was pipetted into screw-capped vials, cooled and stored at 4°C.

2. Preparation of Agarose-antiserum mixture

A vial containing 20 ml of the molten 1% agarose was placed in a 55°C waterbath and allowed to liquify. 15 ml of the liquified agarose was pipetted into a clean vial mounted in the waterbath and a specific volume of the antiserum was added and gently mixed. Care was taken to avoid bubbles.

In this study the volume of the following antisera required to give optimum results as shown below:

Antiserum	Volume (ul/15ml agarose)
Colostrum IgA	25
Lysozyme	300

3. Preparation of Antiserum-agarose Gel Plates

In order to obtain an antiserum-agarose gel of uniform thickness, a mould was made (Fig. 18). This consisted of two cleaned glass plates measuring 20 x 10 x 0.3 cm between which was placed a U-shaped frame made from a 1 mm thick teflon sheet. The three pieces of the mould were tightly held together by means of bulldog clips.

The mould was heated to approximately 45°C with an incandescent lamp before injecting the antiserum-agarose mixture.

A 10 ml disposable syringe was used to withdraw the antiserum-agarose mixture from the vial in the 55°C waterbath. The mould was held in a slanting position and the contents of the syringe were injected at the lower corner of the slit between the two glass plates. This method of injecting the mixture prevented the inclusion of air bubbles.

IMMUNOSTICS REFERENCE PREPARATIONS

The specific protein content in International Units (IU) has been obtained by comparison with international and national standards using IMMUNOSTICS antisera. The specific protein concentration in mg/litre is based on a comparison with a variety of standards, the accuracy of which cannot be guaranteed in the absence of international agreement.

STORAGE

IMMUNOSTICS reference preparations should be stored at +4°C.

PRODUCT DETERIORATION

Use before expiry date printed on the label. Do not use if the preparation shows any sign of contamination or is strongly turbid. Some deterioration in the more labile proteins (β lipoprotein, C3, C4, C5) can be expected with time.

1) **Normal serum pool.** Obtained from healthy donors who had no history of recent illness or hepatitis. It has been calibrated for the following proteins.

Protein	% WHO Standard 67/86	Conc. in mg/l
IgG	126	9 520
IgA	111	1 570
IgM	149	1 170
Protein	**% British Standard 74/520**	**Conc. in mg/l**
Complement C3	112	900
Complement C4	103	213
Complement C5	91	*
Albumin	134	44 400
α 2 Macroglobulin	178	2 460
Transferrin	107	2 735
α 1 Antitrypsin	120	2 040
β Lipoprotein	129	*
Orosomucoid	104	550
TBG	92	10 3
Prealbumin	122	295
Haptoglobin	53·5	614
Ceruloplasmin	120	325
Properdin Factor B	-	169

*Not established

2) **Kappa preparation** contains approximately 500 mg/l of Kappa light chains.

3) **Lambda preparation** contains approximately 500 mg/l of Lambda chains. The Kappa and Lambda preparations have been obtained from pools of Bence Jones proteins and partially purified on Sephadex G 100 to remove cross-reacting

4) **IgD preparation** contains 82 mg/l of polyclonal IgD.

5) **IgE preparation** contains 5mg/l of polyclonal IgE.

6) **IgA secretory component preparation** contains secretory component equivalent to 5,200 mg/l of secretory IgA.

7) α **Fetoprotein preparation** contains 50 mg/l of α FP.

8) **C-Reactive Protein preparation** contains 47 mg/l of C-RP.

9) **Pregnancy Serum Pool** contains 780 mg/l of Pregnancy associated α 2 glycoprotein (PAG) and 74 mg/l of Pregnancy specific β 1 glycoprotein (SP1).

PRECAUTIONS

IMMUNOSTICS reference preparations have been tested and found negative for Hepatitis B surface antigen (HBs Ag) at both donor and final pool levels, by passive haemagglutination (PHA) and radioimmunoassay (RIA). However, these tests cannot guarantee the absence of the causative agent of viral hepatitis. These products should therefore be handled in the same manner as any potentially infective biological material.

REFERENCES

1) Alexsen, N.H., J. Kroll and B. Weeke, 1973. A manual of quantitative immunoelectrophoresis. Methods and Application. Universitetsforlaget, Oslo. (Also published in Scand. J. Immunol. 2: Suppl. 1)

2) Laurell, C.B., 1972. Electrophoretic and electroimmunochemical analysis of proteins. Universitetsforlaget, Oslo. (Also published in Scand. J. Clin. Lab. Invest. 29: Suppl. 124 21 37)

3) Thompson, R.A., 1977. Techniques in clinical immunology. Blackwell Scientific Publications.

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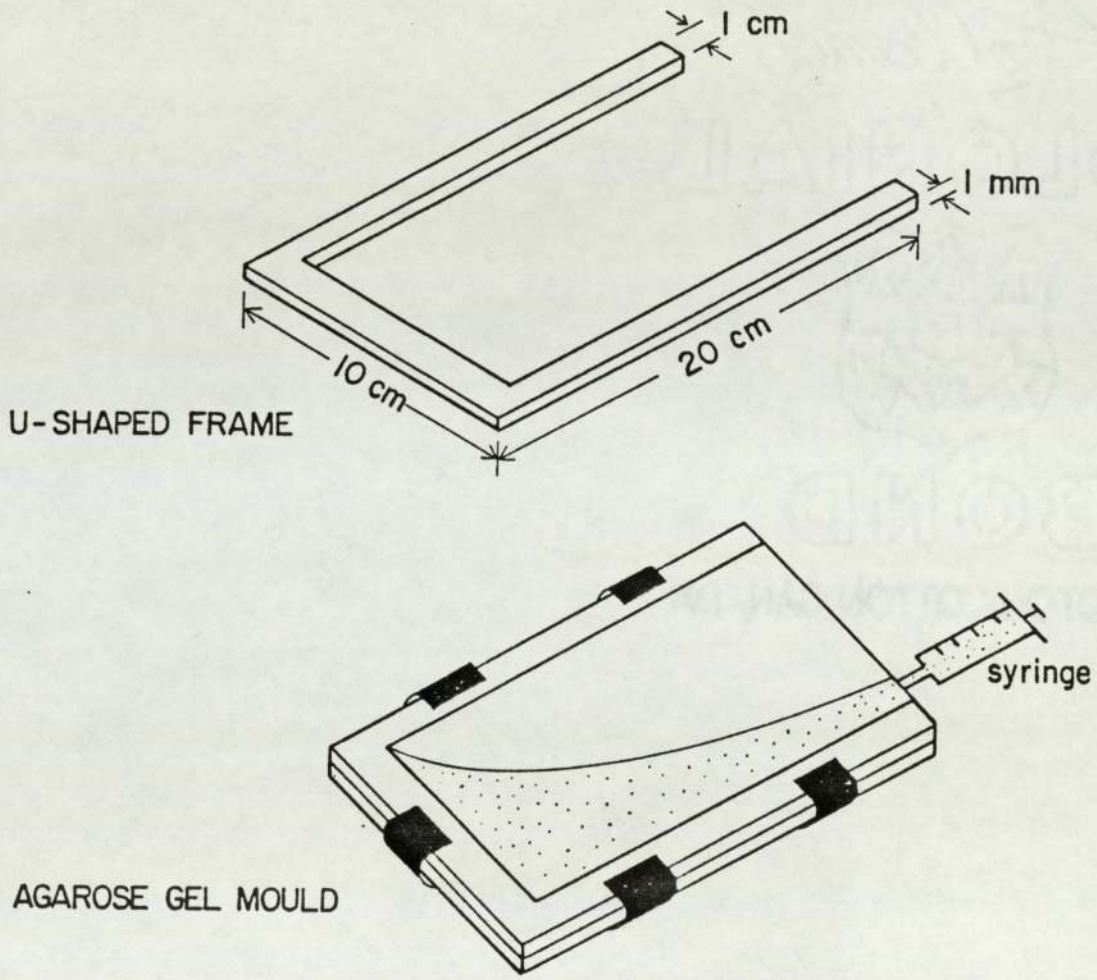


Figure 18: Preparation of Antiserum Agarose Gel in Glass Mould

Once the mould was filled, it was held in the upright position and allowed to solidify at room temperature and then stored at 4°C until the tear samples were applied.

The clamps were removed from the mould and the upper glass plate was carefully slid off. Next, the U-shaped frame was removed. The gel plate was placed on a sheet of graph paper which was used as a template for punching the sample wells 1 cm apart along a line 1 cm from the edge of the gel. The circular wells were punched out with a 3 mm gel puncher and the small cylinders of gel cut out were removed with a No. 23 gauge syringe needle.

4. Preparation of Electrophoresis Chamber

A Shandon electrophoresis unit which consisted of two electrophoresis chambers and Vokam stabilized DC power supply was employed (Fig. 19). Each buffer reservoir was filled with 500 ml of 0.06 M barbitone buffer of pH 8.6. The level of the electrode buffer in each compartment was checked to ensure that they were of equal height.

The connecting bridges were made from Whatman chromatography filter paper 3 mm measuring 20 x 10 cm. On the edge of one of the bridges was labelled 1 to 18 in pencil to correspond to the 18 sample wells.

The gel plate was placed centrally between the two reservoirs. The connecting bridges were moistened with the electrode buffer and positioned along the long edges of the gel, allowing an overlap of 5 mm onto the gel. The rest of the filter paper made contact with the reservoir buffer.

5. Application of Samples

The polarity of the two chambers were adjusted so that the sample wells for the IgA assay was at the cathode (-ive) in chamber C1 and those for the lysozyme were at the anode (+ive) in chamber C2 (Fig.19).

The power supply was switched on and the voltage adjusted to approximately 1 volt per cm before applying samples to the respective wells. This precedure was necessary to avoid diffusion of the sample from the well.

Figure 19: Shandon Apparatus for Rocket Immunoelectrophoresis. The apparatus consists of:

a. Power supply SAE 2761 (Shandon Southern).This accommodates two electrophoretic chambers.

b. Electrophoretic Chambers (Shandon Southern).

CODE:

i. AP Agarose Plate Containing Antiserum.

ii. CC Chamber Cover.

iii. FPB Filter Paper Bridges.

iv. BR Buffer Reservoir.

v. PS Polarity Switch.

vi. EL Electrical Leads.

vii. PW Paper Work.

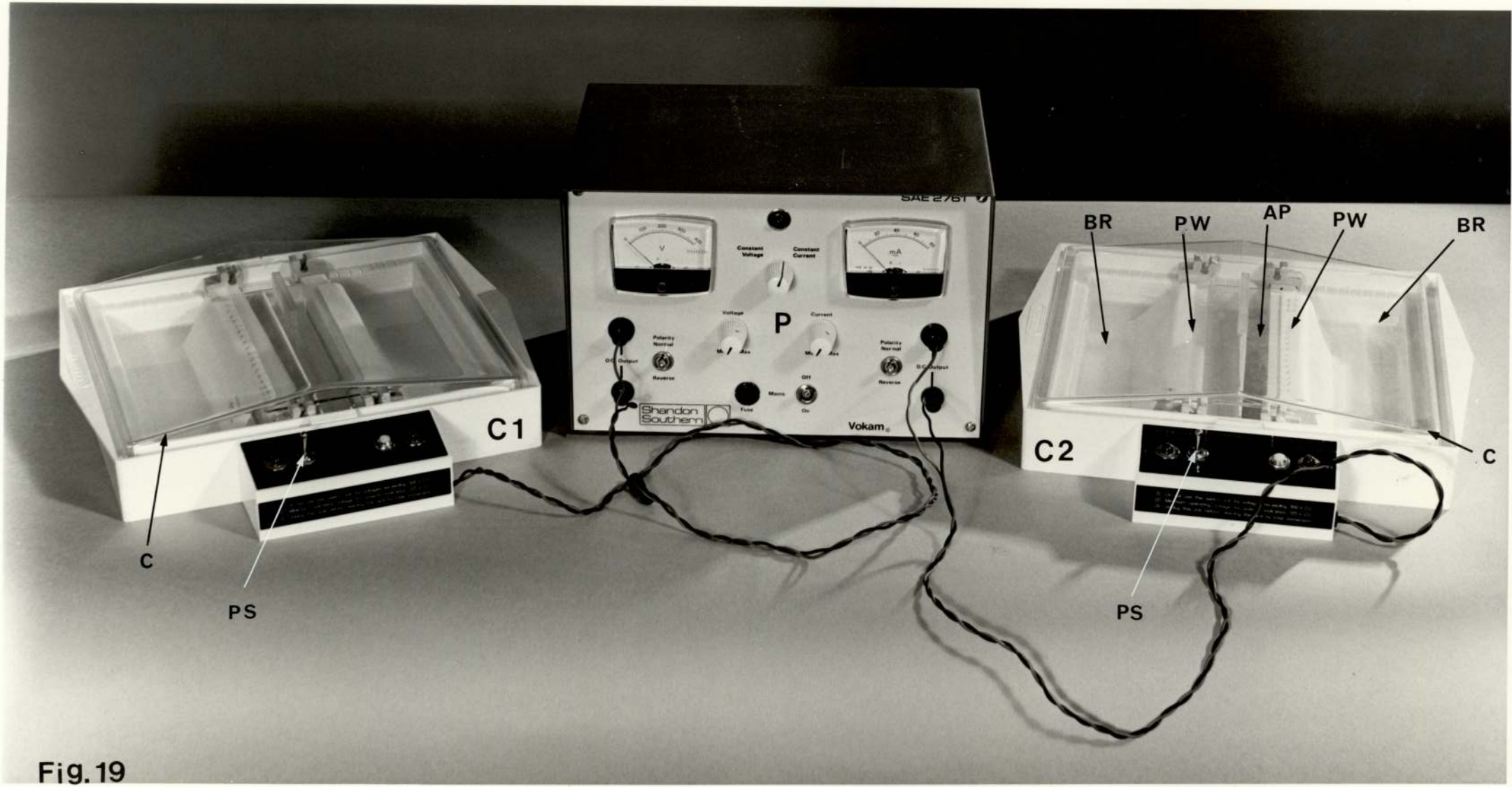


Fig. 19

The volume of the tear sample and serial dilution standards varied with each type of tear protein to be assayed. These were as follows:

1. Immunoglobulin A (IgA)

A 1 ul tear sample for each of the subjects and serial dilutions of a standard serum were applied to the appropriately labelled sample wells. The dilutions for the calibration curve were: 1:2, 1:3, 1:4, 1:5, 1:6 and 1:10. At least three of these were used for each run.

2. Lysozyme

A 1/2 ul tear sample for each subject was applied to the appropriate wells.

A commercial calibration standard was not available at the time of this study. However, known volumes of pooled tears were assayed by the spectrophometric procedure marketed by Worthington Diagnostics and corresponding volumes of the same pooled tears were used in immunoelectrophoresis method.

The concentration of the lysozyme determined by the spectrophotometric procedure multiplied by a factor of 3.5 (since human lysozyme is 3.5 times more active than egg lysozyme) was equated with the peak height of the corresponding volumes used in the immunoelectrophoresis and the calibration curve was established.

3. Power Supply Adjustment

Following the application of the tear samples and dilution standards, the power supply was switched to constant current. The current was adjusted to 8 mA.

The cover for the electrophoretic chamber was replaced and the electrophoresis ran overnight for approximately 16 hours.

4. Washing the Agarose Gel

After the electrophoresis was finished, the gel was placed in a dish containing a 0.9% saline solution. The gel was washed for 2 days with several

changes of the saline solution each day and then soaked in distilled water for one day in order to remove the salt. The distilled water wash was changed 3 times.

5. Staining and Destaining the Agarose Gel

The gel was stained with 0.5% Coomassie brilliant blue in a methanola-cetic acid mixture for 5 minutes and then washed with a methanol-acetic acid destaining solution until the background was slightly bluish. Three or four changes over a period of 15 to 30 minutes were required to complete this procedure.

6. Drying the Agarose gel

The gel was placed on an acetate sheet, blotted with soft cellulose tissue to remove the liquid phase and allowed to dry at room temperature overnight.

7. Quantification of Peak Heights

The height of the various peaks (antigen-antibody precipitate zone) can be measured directly by overlaying the acetate sheet on a sheet of metric graph paper. The peak height was measured from the tip of the peak to the top of the sample well with an accuracy of 0.5 mm.

A calibration curve was obtained by plotting the peak heights of the standard dilutions against their concentrations as shown in Figure 20A, 20B, 20C for Lysozyme and Figures 21A, 21B and 21C for IgA. By interpolation on the curve, unknown samples can be quantified. In this study a programmable calculator (Texas Instrument Model 59) was used to compute the correlation coefficient of the standard calibration curve from which the peak height of the unknown sample was input and the protein concentration calculated. This is the preferred procedure because the calibration curve data are seldom perfectly linear thus computation of the line fitted to the data approximates the data.

Figure 20: Rocket Immunoelectrophoresis Plates for Lysozyme Assay

A 0.5 ul tear sample was taken for the lysozyme assay from each subject at each visit for a period of 24 weeks. Tear lysozyme standards are R1=2ug/ul, R2=1ug/ul, R4=0.5 ug/ul.

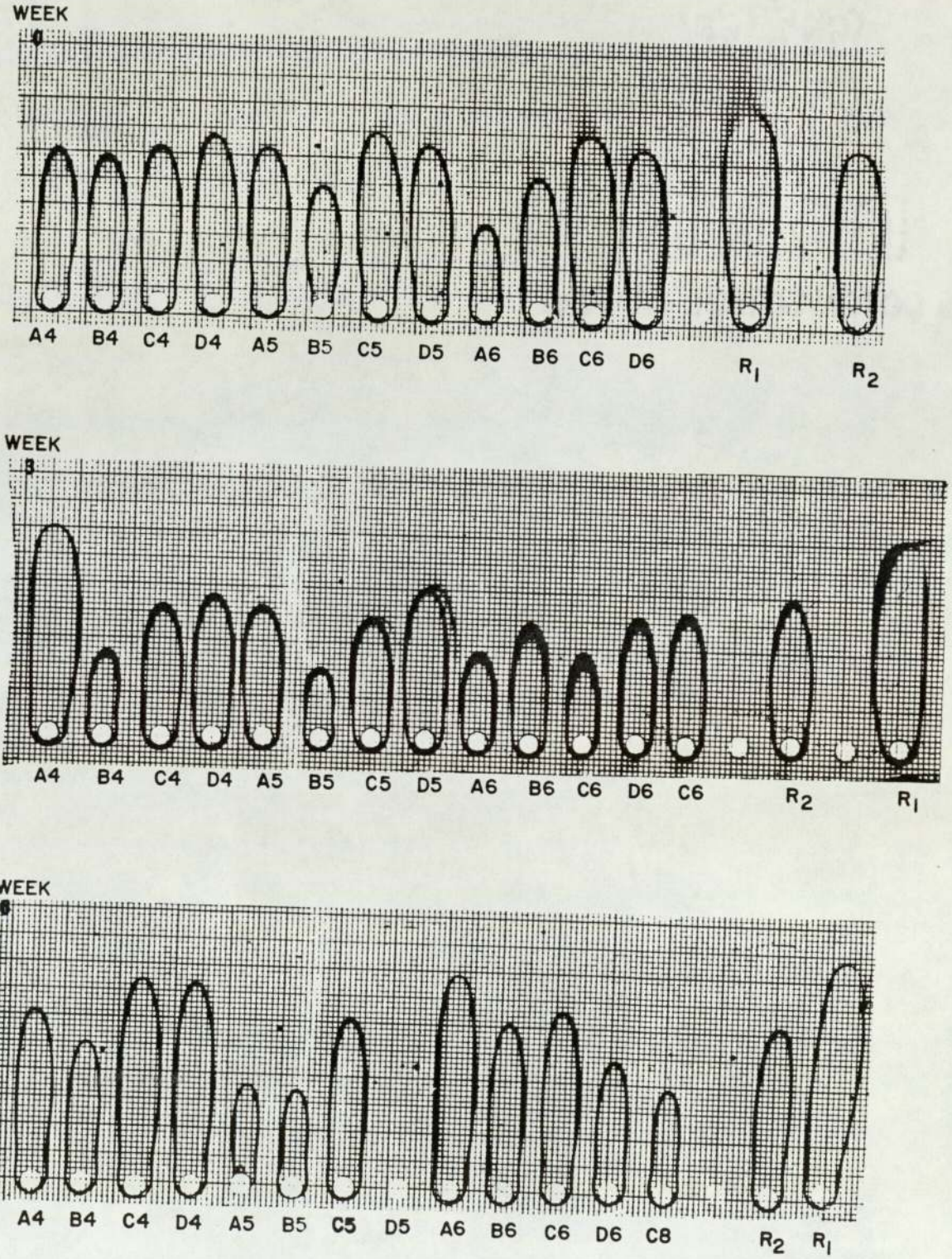


Figure 20a: Rocket Immunoelectrophoresis Plates for Lysozyme Assay

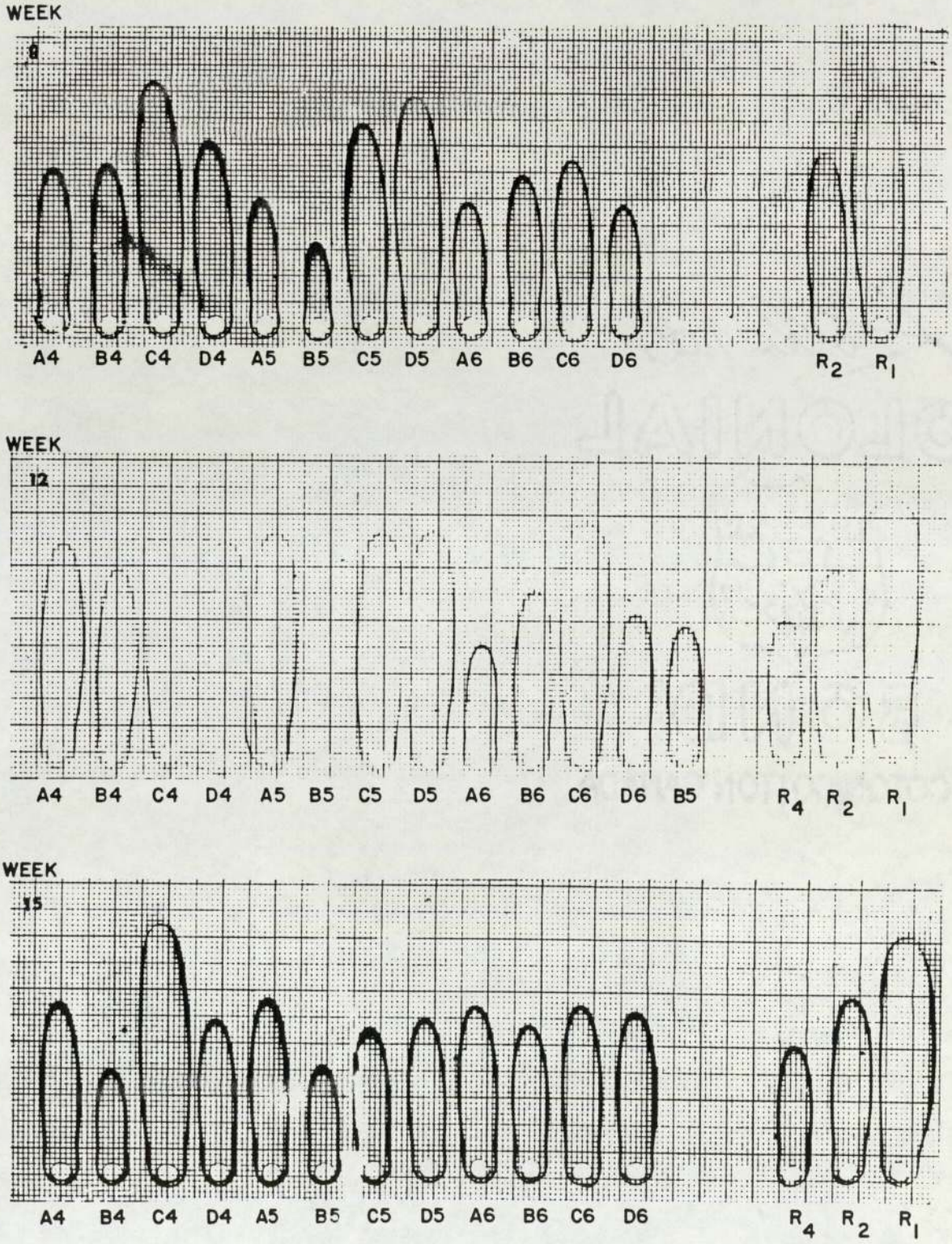


Figure 20b. Rocket Immunoelectrophoresis Plates for Lysozyme Assay

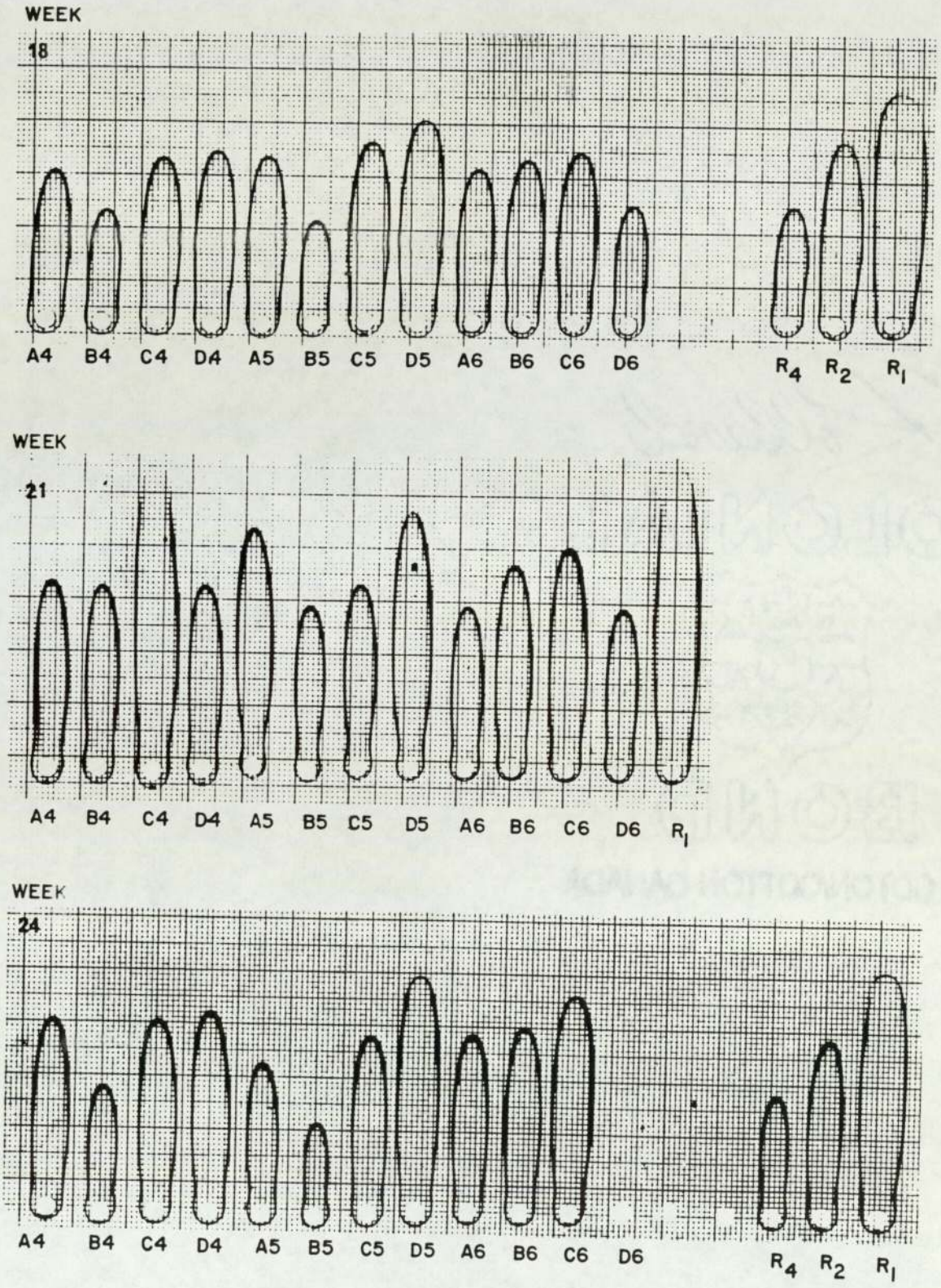


Figure 20c. Rocket Immunoelectrophoresis Plates for Lysozyme Assay

Figure 21: Rocket Immunoelectrophoresis Plates for IgA Assay

A 1 ul tear sample was taken for IgA (secretory) assay from each subject at each visit for a period of 24 weeks. A reference serum standard containing 1570 mg/L IgA was used to calibrate the pooled serum standard of 1220 mg/L. Pooled serum dilutions were:

So=neat, S_2=1:2, S_3=1:3, S_6=1:6, S_{10}=1:10.
Reference serum R_1=1:1, R_2=1:2.

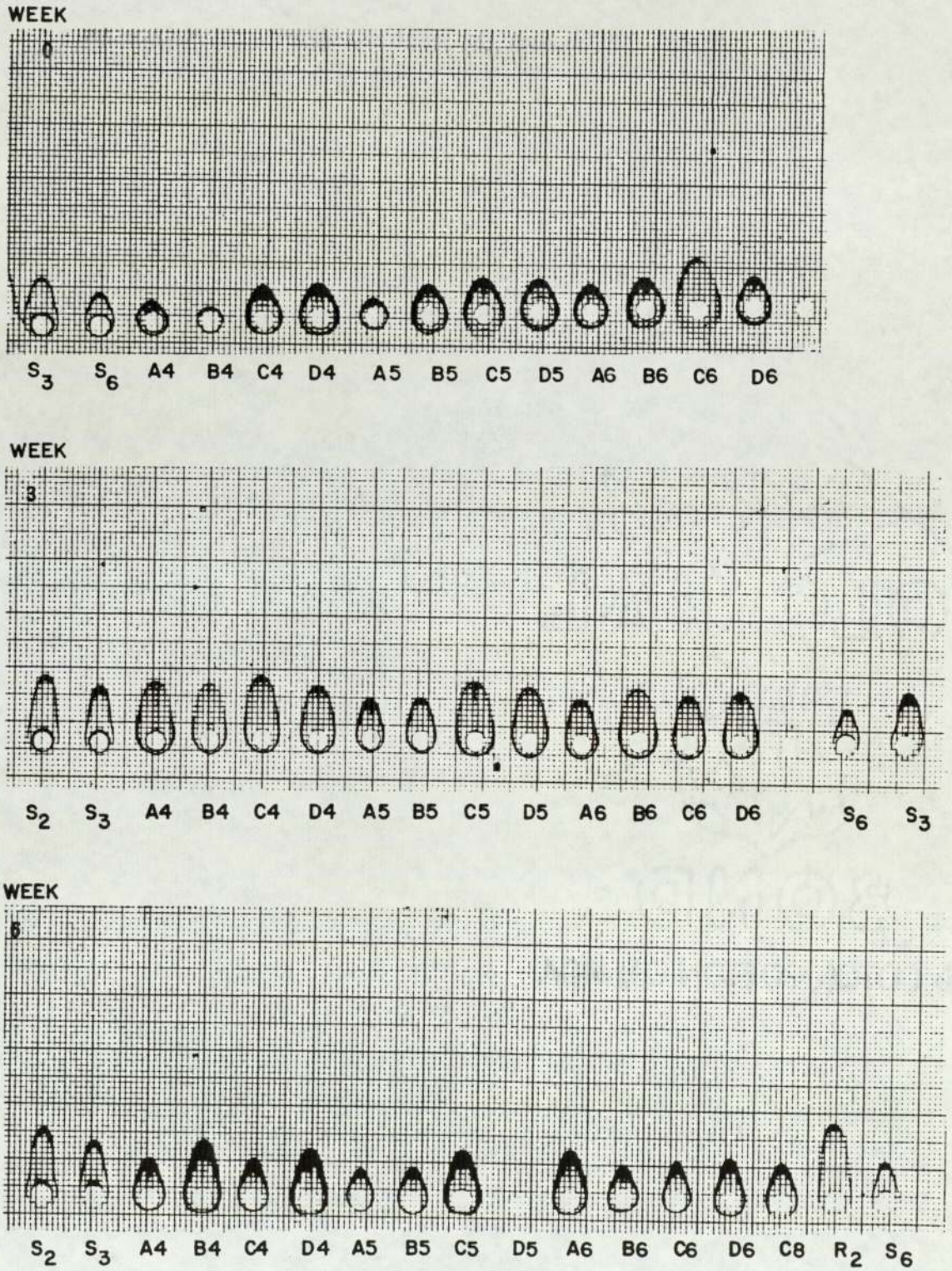


Figure 21a: Rocket Immunoelectrophoresis Plates for IgA Assay

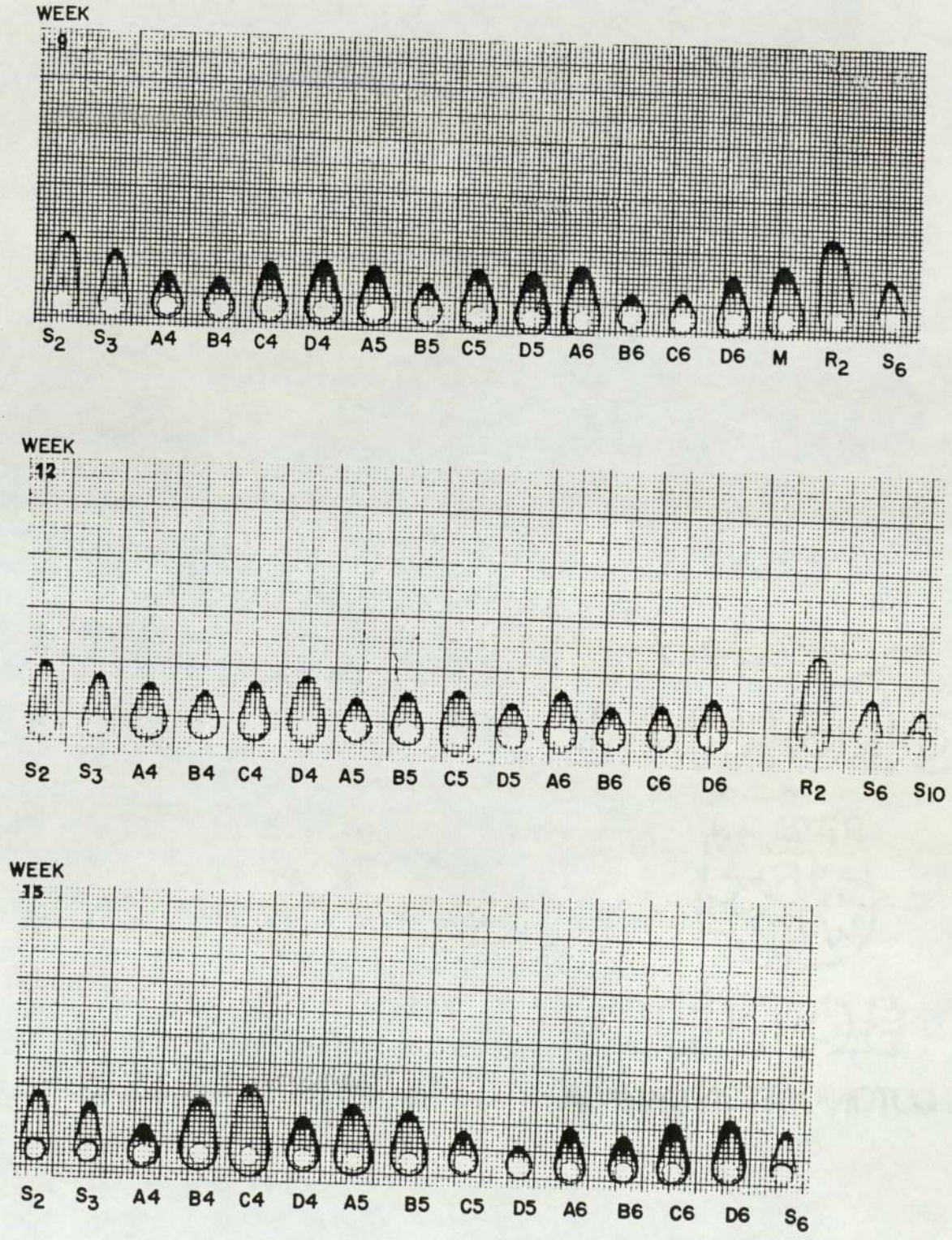


Figure 21b: Rocket Immunoelectrophoresis Plates for IgA Assay.

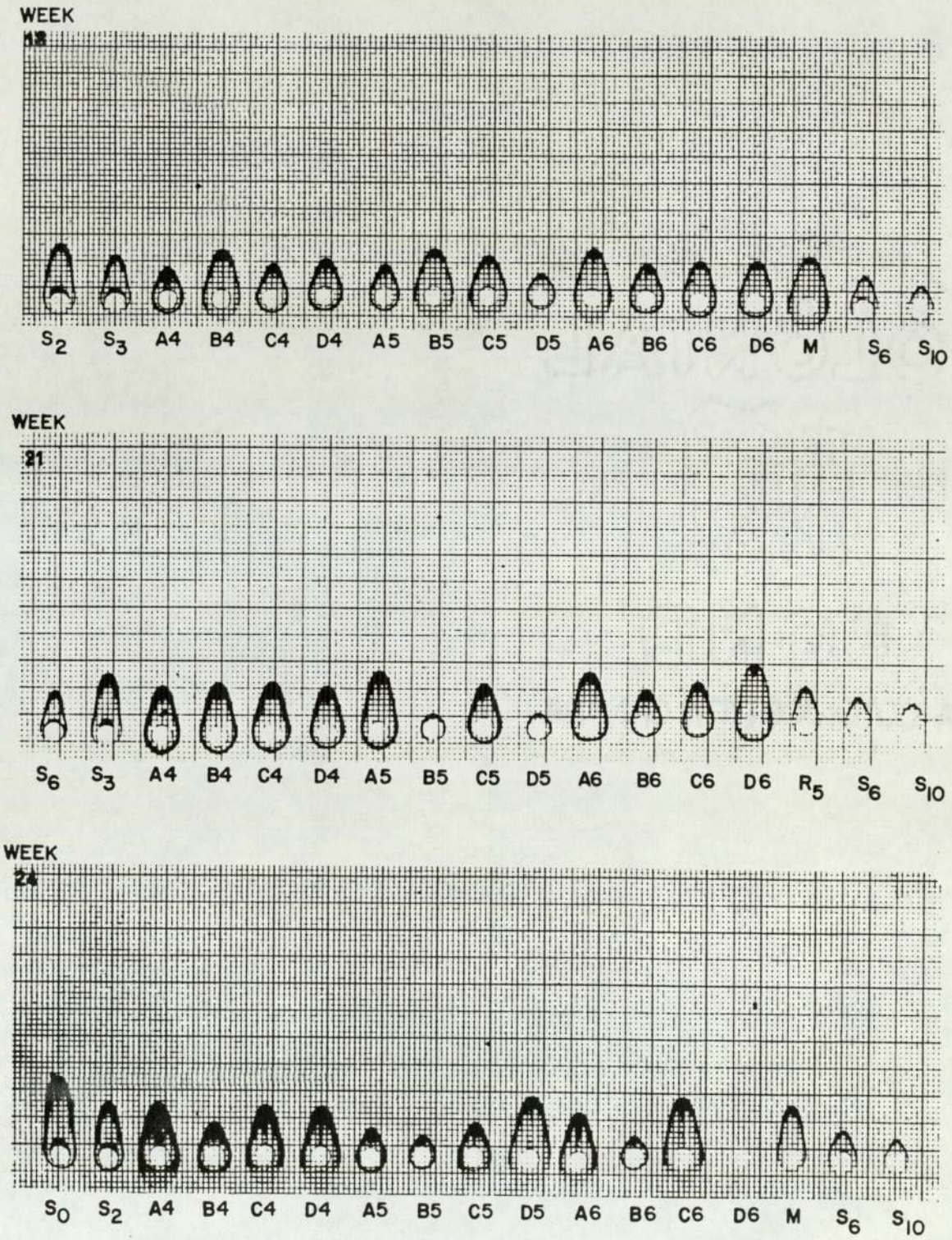


Figure 21c: Rocket Immunoelectrophoresis Plates for IgA Assay

Appendix F

STATISTICAL AND RAW DATA

Table 13: ANOVA for (Total) Tear Protein as the Dependent Variable

SAS

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: TOTAL

SOURCE	DF	PR > F	R-SQUARE	C.V.
MODEL	85	0.0001	0.522499	26.2189
ERROR	384	ROOT MSE		TOTAL MEAN
CORRECTED TOTAL	469	268.62135225		1024.53191489

SOURCE	DF	TYPE III SS	F VALUE	PR > F
GROUP	3	2894649.06449427	13.37	0.0001
ID(GROUP)	50	16221380.60632404	4.50	0.0001
VISIT	8	9310194.43009099	16.13	0.0001
GROUP*VISIT	24	1754402.09895662	1.01	0.4478

TESTS OF HYPOTHESES USING THE TYPE III MS FOR ID(GROUP) AS AN ERROR TERM

SOURCE	DF	TYPE III SS	F VALUE	PR > F
GROUP	3	2894649.06449427	2.97	0.0404

Table 14: Linear Regression Analysis for (Total) Tear Protein with Respect to Visit

SAS

DEP VARIABLE: TOTAL

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PROB>F
MODEL	1	6532045	6532045	62.791	0.0001
ERROR	432	44940274	104028		
C TOTAL	433	51472319			

ROOT MSE	322.534	R-SQUARE	0.1269
DEP MEAN	1013.894	ADJ R-SQ	0.1249
C.V.	31.81145		

VARIABLE	DF	PARAMETER ESTIMATE	STANDARD ERROR	T FOR H0: PARAMETER=0	PROB > |T|
INTERCEP	1	829.815	27.916725	29.725	0.0001
VISIT	1	15.974830	2.015987	7.924	0.0001

Table 15: ANOVA for Percentage Distribution of Protein Fractions in Set 1 (PER 1) as the Dependent Variable

SAS
PROT=1

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: PER

SOURCE	DF	PR > F	R-SQUARE	C.V.
MODEL	82	0.0001	0.454008	18.2092
ERROR	311	ROOT MSE		PER MEAN
CORRECTED TOTAL	393	4.64136736		25.48910455

SOURCE	DF	TYPE III SS	F VALUE	PR > F
GROUP	3	231.59143012	3.58	0.0142
SUBJ(GROUP)	47	3851.65205581	3.80	0.0001
VISIT	8	750.53182613	4.35	0.0001
GROUP*VISIT	24	722.21238943	1.40	0.1052

TESTS OF HYPOTHESES USING THE TYPE III MS FOR SUBJ(GROUP) AS AN ERROR TERM

SOURCE	DF	TYPE III SS	F VALUE	PR > F
GROUP	3	231.59143012	0.94	0.4279

Table 16: Linear Regression Analysis for Percentage Distribution of Protein Fractions in Set 1 (PER 1) with Respect to Visit

SAS
PROT=1

DEP VARIABLE: PER

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PROB>F
MODEL	1	85.945544	85.945544	2.765	0.0971
ERROR	392	12184.660	31.083317		
C TOTAL	393	12270.606			

ROOT MSE	5.575241	R-SQUARE	0.0070
DEP MEAN	25.489105	ADJ R-SQ	0.0045
C.V.	21.87304		

VARIABLE	DF	PARAMETER ESTIMATE	STANDARD ERROR	T FOR H0: PARAMETER=0	PROB > |T|
INTERCEP	1	26.195496	0.509271	51.437	0.0001
VISIT	1	-0.059776	0.035948	-1.663	0.0971

Table 17: ANOVA for Percentage Distribution of Protein Fractions in Set 2 (PER 2) as the Dependent Variable

SAS
PROT=2

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: PER

SOURCE	DF	PR > F	R-SQUARE	C.V.
MODEL	82	0.0001	0.500895	34.5521
ERROR	311	ROOT MSE		PER MEAN
CORRECTED TOTAL	393	4.41968984		12.79138669

SOURCE	DF	TYPE III SS	F VALUE	PR > F
GROUP	3	160.08470334	2.73	0.0432
SUBJ(GROUP)	47	2644.45353109	2.88	0.0001
VISIT	8	2989.03875620	19.13	0.0001
GROUP*VISIT	24	173.93038090	0.37	0.9974

TESTS OF HYPOTHESES USING THE TYPE III MS FOR SUBJ(GROUP) AS AN ERROR TERM

SOURCE	DF	TYPE III SS	F VALUE	PR > F
GROUP	3	160.08470334	0.95	0.4249

Table 18: Linear Regression Analysis for Percentage Distribution of Protein Fractions in Set 2 (PER 2) with Respect to Visit

SAS
PROT=2

DEP VARIABLE: PER

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PROB>F
MODEL	1	1387.549	1387.549	50.437	0.0001
ERROR	392	10784.185	27.510676		
C TOTAL	393	12171.734			

ROOT MSE	5.245062	R-SQUARE	0.1140
DEP MEAN	12.791387	ADJ R-SQ	0.1117
C.V.	41.00464		

VARIABLE	DF	PARAMETER ESTIMATE	STANDARD ERROR	T FOR H0: PARAMETER=0	PROB > |T|
INTERCEP	1	9.953091	0.479111	20.774	0.0001
VISIT	1	0.240132	0.033820	7.102	0.0001

Table 19: ANOVA for Percentage Distribution of Protein Fractions in Set 3 (PER 3) as the Dependent Variable

SAS
PROT=3

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: PER

SOURCE	DF	PR > F	R-SQUARE	C.V.
MODEL	82	0.0001	0.460542	14.4510
ERROR	311	ROOT MSE		PER MEAN
CORRECTED TOTAL	393	6.96900169		48.22521377

SOURCE	DF	TYPE III SS	F VALUE	PR > F
GROUP	3	827.48823200	5.68	0.0010
SUBJ(GROUP)	47	8099.33692649	3.55	0.0001
VISIT	8	2480.38874063	6.38	0.0001
GROUP*VISIT	24	1125.58091580	0.97	0.5119

TESTS OF HYPOTHESES USING THE TYPE III MS FOR SUBJ(GROUP) AS AN ERROR TERM

SOURCE	DF	TYPE III SS	F VALUE	PR > F
GROUP	3	827.48823200	1.60	0.2018

Table 20: Linear Regression Analysis for Percentage Distribution of Protein Fractions in Set 3 (PER 3) with Respect to Visit

SAS
PROT=3

DEP VARIABLE: PER

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PROB>F
MODEL	1	1295.880	1295.880	19.023	0.0001
ERROR	392	26703.199	68.120406		
C TOTAL	393	27999.080			

ROOT MSE	8.253509	R-SQUARE	0.0463
DEP MEAN	48.225214	ADJ R-SQ	0.0439
C.V.	17.11451		

VARIABLE	DF	PARAMETER ESTIMATE	STANDARD ERROR	T FOR H0: PARAMETER=0	PROB > |T|
INTERCEP	1	50.968152	0.753918	67.604	0.0001
VISIT	1	-0.232113	0.053218	-4.362	0.0001

Table 21:. ANOVA for Percentage Distribution of Protein Fractions in Set 4 (PER 4) as the Dependent Variable

SAS
PROT=4

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: PER

SOURCE	DF	PR > F	R-SQUARE	C.V.
MODEL	82	0.0034	0.292798	46.0643
ERROR	311	ROOT MSE		PER MEAN
CORRECTED TOTAL	393	6.21605786		13.49429499

SOURCE	DF	TYPE III SS	F VALUE	PR > F
GROUP	3	385.01550831	3.32	0.0200
SUBJ(GROUP)	47	2738.31804086	1.51	0.0227
VISIT	8	883.86559934	2.86	0.0044
GROUP*VISIT	24	960.86603123	1.04	0.4190

TESTS OF HYPOTHESES USING THE TYPE III MS FOR SUBJ(GROUP) AS AN ERROR TERM

SOURCE	DF	TYPE III SS	F VALUE	PR > F
GROUP	3	385.01550831	2.20	0.1002

Table 22: Linear Regression Analysis for Percentage Distribution of Protein Fractions in Set 4 (PER 4) with Respect to Visit

SAS
PROT=4

DEP VARIABLE: PER

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PROB>F
MODEL	1	64.307607	64.307607	1.489	0.2231
ERROR	392	16927.786	43.183128		
C TOTAL	393	16992.094			

ROOT MSE	6.571387	R-SQUARE	0.0038
DEP MEAN	13.494295	ADJ R-SQ	0.0012
C.V.	48.69752		

VARIABLE	DF	PARAMETER ESTIMATE	STANDARD ERROR	T FOR H0: PARAMETER=0	PROB > |T|
INTERCEP	1	12.883262	0.600264	21.463	0.0001
VISIT	1	0.051707	0.042372	1.220	0.2231

Table 23: ANOVA for Lysozyme as the Dependent Variable

SAS

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: LYSO

SOURCE	DF	PR > F	R-SQUARE	C.V.
MODEL	84	0.0001	0.507715	22.6484
ERROR	364	ROOT MSE		LYSO MEAN
CORRECTED TOTAL	448	60.91770239		268.97104677

SOURCE	DF	TYPE III SS	F VALUE	PR > F
GROUP	3	146042.89817047	13.12	0.0001
ID(GROUP)	49	1046792.68502067	5.76	0.0001
VISIT	3	121404.56628893	4.09	0.0001
GROUP*VISIT	24	5770.4.22749780	0.65	0.8992

TESTS OF HYPOTHESES USING THE TYPE III MS FOR ID(GROUP) AS AN ERROR TERM

SOURCE	DF	TYPE III SS	F VALUE	PR > F
GROUP	3	146042.89817047	2.28	0.0911

Table 24: Linear Regression Analysis for Lysozyme with Respect to Visit

SAS

DEP VARIABLE: LYSO

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PROB>F
MODEL	1	15868.842	15868.842	2.563	0.1101
ERROR	432	2674905	6191.909		
C TOTAL	433	2690774			

ROOT MSE	78.688684	R-SQUARE	0.0059
DEP MEAN	269.101	ADJ R-SQ	0.0036
C.V.	29.24128		

VARIABLE	DF	PARAMETER ESTIMATE	STANDARD ERROR	T FOR H0: PARAMETER=0	PROB > |T|
INTERCEP	1	260.028	6.810341	38.179	0.0001
VISIT	1	0.787380	0.491840	1.601	0.1101

Table 25: ANOVA for Percentage Lysozyme in Total Tear Protein as the Dependent Variable

SAS

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: LYSTOT

SOURCE	DF	PR > F	R-SQUARE	C.V.
MODEL	84	0.0001	0.445070	28.3096
ERROR	364	ROOT MSE		LYSTOT MEAN
CORRECTED TOTAL	448	0.07993284		0.28235275

SOURCE	DF	TYPE III SS	F VALUE	PR > F
GROUP	3	0.02362622	1.23	0.2972
ID(GROUP)	42	0.94037390	3.00	0.0001
VISIT	3	0.82941985	16.23	0.0001
GROUP*VISIT	24	0.08503875	0.55	0.9579

TESTS OF HYPOTHESES USING THE TYPE III MS FOR ID(GROUP) AS AN ERROR TERM

SOURCE	DF	TYPE III SS	F VALUE	PR > F
GROUP	3	0.02362622	0.41	0.7463

Table 26: Linear Regression Analysis for Lysozyme in Total Tear Protein with Respect to Visit

SAS

DEP VARIABLE: LYSTCT

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PROB>F
MODEL	1	0.561822	0.561822	68.523	0.0001
ERROR	432	3.541992	0.00819905E		
C TOTAL	433	4.103814			

ROOT MSE	0.090549	R-SQUARE	0.1369
DEP MEAN	0.284143	ADJ R-SQ	0.1349
C.V.	31.86723		

VARIABLE	DF	PARAMETER ESTIMATE	STANDARD ERROR	T FOR H0: PARAMETER=0	PROB > |T|
INTERCEP	1	0.338129	0.007837371	43.143	0.0001
VISIT	1	-0.00468501	0.0005659702	-8.278	0.0001

Table 27: ANOVA for IgA as the Dependent Variable

SAS

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: IGA

SOURCE	DF	PR > F	R-SQUARE	C.V.
MODEL	84	0.0001	0.556631	35.1044
ERROR	366	ROOT MSE		IGA MEAN
CORRECTED TOTAL	450	9.55260867		27.21201774

SOURCE	DF	TYPE III SS	F VALUE	PR > F
GROUP	3	1223.58050335	4.47	0.0044
ID(GROUP)	49	37217.16220949	8.32	0.0001
VISIT	9	1378.96558367	1.89	0.0605
GROUP*VISIT	24	2244.24016142	1.02	0.4328

TESTS OF HYPOTHESES USING THE TYPE III MS FOR ID(GROUP) AS AN ERROR TERM

SOURCE	DF	TYPE III SS	F VALUE	PR > F
GROUP	3	1223.58050335	0.54	0.6592

Table 28: Linear Regression Analysis for IgA with Respect to Visit

SAS

DEP VARIABLE: IGA

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PROB>F
MODEL	1	135.808	135.808	0.802	0.3709
ERROR	432	73135.373	169.295		
C TOTAL	433	73271.182			

ROOT MSE	13.011335	R-SQUARE	0.0019
DEP MEAN	27.277304	ADJ R-SQ	-0.0005
C.V.	47.70022		

VARIABLE	DF	PARAMETER ESTIMATE	STANDARD ERROR	T FOR H0: PARAMETER=0	PROB > |T|
INTERCEP	1	26.437957	1.126187	23.476	0.0001
VISIT	1	0.072841	0.081327	0.896	0.3709

Table 29: ANOVA for Percentage IgA in Total Protein as the Dependent Variable

SAS

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: IGATOT

SOURCE	DF	PR > F	R-SQUARE	C.V.
MODEL	84	0.0001	0.588526	31.9206
ERROR	366	ROOT MSE		IGATOT MEAN
CORRECTED TOTAL	450	0.00873302		0.02735856

SOURCE	DF	TYPE III SS	F VALUE	PR > F
GROUP	3	0.00037887	1.66	0.1744
ID(GROUP)	49	0.02945565	7.61	0.0001
VISIT	8	0.00779552	12.78	0.0001
GROUP*VISIT	24	0.00319191	1.74	0.0175

TESTS OF HYPOTHESES USING THE TYPE III MS FOR ID(GROUP) AS AN ERROR TERM

SOURCE	DF	TYPE III SS	F VALUE	PR > F
GROUP	3	0.00037887	0.22	0.8838

Table 30: Linear Regression Analysis for Percentage IgA in Total Protein with Respect to Visit

SAS

DEP VARIABLE: IGATOT

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PROB>F
MODEL	1	0.005054881	0.005054881	35.799	0.0001
ERROR	432	0.061000	0.0001412036		
C TOTAL	433	0.066055			

ROOT MSE	0.011883	R-SQUARE	0.0765
DEP MEAN	0.027588	ADJ R-SQ	0.0744
C.V.	43.07245		

VARIABLE	DF	PARAMETER ESTIMATE	STANDARD ERROR	T FOR H0: PARAMETER=0	PROB > |T|
INTERCEP	1	0.032709	0.001028517	31.802	0.0001
VISIT	1	-0.000444393	.0000742736?	-5.983	0.0001

Table 31: Statistical Analysis: I. The mean, SD and SEM derived from raw data on corneal thickness (Cornea), total protein (Total), lysozyme (Lyso), IgA, percentage lysozyme in total protein (PERlyso) and percentage IgA in total protein (PERIgA).

SAS

VARIABLE	LABEL	N	MEAN	STANDARD DEVIATION	STD ERROR OF MEAN
GROUP=A	VISIT (weeks)=0				
CORNEA	.	13	0.56900000	0.03251410	0.00901779
TOTAL	.	13	583.84615385	183.82684160	50.98439255
LYSO	.	13	214.00000000	70.31358332	19.50147923
IGA	.	13	23.54923077	10.43498719	2.89414472
PERLYSO		13	38.96611804	15.47084429	4.29084018
PERIGA		13	4.00259166	1.23322385	0.34203475
GROUP=A	VISIT (weeks)=3				
CORNEA	.	13	0.56269231	0.03392242	0.00940839
TOTAL	.	13	827.69230769	224.13365976	62.16349252
LYSO	.	13	244.30769231	77.22627426	21.41871474
IGA	.	13	24.82461538	12.85333460	3.56487361
PERLYSO		13	30.53145412	9.54627862	2.64766131
PERIGA		13	2.85271214	1.15919998	0.32150423
GROUP=A	VISIT (weeks)=6				
CORNEA	.	13	0.56346154	0.03176375	0.00880968
TOTAL	.	13	785.38461538	155.59645372	43.15469171
LYSO	.	13	258.76923077	59.88621690	16.60944813
IGA	.	13	27.23000000	8.80483579	2.44202207
PERLYSO		13	33.16344593	6.11033782	1.69470279
PERIGA		13	3.46386640	0.85704166	0.23770059
GROUP=A	VISIT (weeks)=9				
CORNEA	.	13	0.56007692	0.03377983	0.00936884
TOTAL	.	13	960.76923077	345.62652142	95.85954963
LYSO	.	13	230.61538462	75.55741576	20.95585668
IGA	.	11	24.68545455	12.33458014	3.71901584
PERLYSO		13	24.78422058	5.35869036	1.48623330
PERIGA		11	2.73430677	1.41402874	0.42634571
GROUP=A	VISIT (weeks)=12				
CORNEA	.	12	0.55675000	0.03831953	0.01106190
TOTAL	.	12	1025.00000000	293.92330478	84.84834957
LYSO	.	12	283.16666667	94.96969214	27.41538866
IGA	.	12	25.47083333	10.30925750	2.97602630
PERLYSO		12	27.95434190	6.70765407	1.93633294
PERIGA		12	2.57725534	1.22372670	0.35325947
GROUP=A	VISIT (weeks)=15				
CORNEA	.	13	0.56792308	0.03063076	0.00849544
TOTAL	.	12	1160.83333333	244.25985914	70.51174771
LYSO	.	12	269.00000000	62.13475093	17.93675759
IGA	.	12	29.71166667	16.00212931	4.61941683
PERLYSO		12	23.16530027	2.18858354	0.63178965
PERIGA		12	2.44405317	1.15173642	0.33247767

Table 31: Continued

SAS

VARIABLE	LABEL	N	MEAN	STANDARD DEVIATION	STD ERROR OF MEAN
GROUP=A	VISIT (weeks)=18				
CORNEA	.	13	0.57030769	0.03202963	0.00888342
TOTAL	.	13	1176.92307692	309.87590568	85.94411284
LYSO	.	13	274.46153846	108.74175477	30.15953635
IGA	.	13	30.85153846	14.34868568	3.97960938
PERLYSO		13	23.66304326	6.98785323	1.93808178
PERIGA		13	2.67340772	1.00974137	0.28005187
GROUP=A	VISIT (weeks)=21				
CORNEA	.	13	0.55976923	0.02730126	0.00757201
TOTAL	.	13	1217.69230769	321.76994489	89.24292579
LYSO	.	13	258.15384615	73.51853072	20.39037171
IGA	.	12	32.98000000	15.21235503	4.39142863
PERLYSO		13	21.72385404	5.18020481	1.43673031
PERIGA		12	2.70823872	1.40151085	0.40458133
GROUP=A	VISIT (weeks)=24				
CORNEA	.	13	0.55384615	0.02568348	0.00712332
TOTAL	.	13	1064.61538462	290.13259568	80.46830388
LYSO	.	11	274.36363636	45.15589159	13.61501359
IGA	.	13	25.46307692	10.50125341	2.91252366
PERLYSO		11	25.65770041	5.09475957	1.53612781
PERIGA		13	2.35018824	0.60502092	0.16780261
GROUP=B	VISIT (weeks)=0				
CORNEA	.	13	0.54961538	0.04339650	0.01203602
TOTAL	.	13	688.46153846	302.26495424	83.83321472
LYSO	.	12	229.66666667	55.19442220	15.93325726
IGA	.	12	24.87916667	11.04422635	3.18819353
PERLYSO		12	38.03863528	13.12122237	3.78777063
PERIGA		12	3.82571476	1.59175749	0.45950081
GROUP=B	VISIT (weeks)=3				
CORNEA	.	12	0.54933333	0.04155464	0.01199579
TOTAL	.	12	853.33333333	409.48600450	118.20842746
LYSO	.	12	233.50000000	93.04202374	26.85891873
IGA	.	12	27.96750000	11.75067745	3.39212839
PERLYSO		12	32.30712010	18.39544361	5.31030716
PERIGA		12	3.40084781	1.41418596	0.40824032
GROUP=B	VISIT (weeks)=6				
CORNEA	.	13	0.55315385	0.03905732	0.01083255
TOTAL	.	13	876.15384615	283.59414843	78.65486489
LYSO	.	12	266.66666667	82.22511597	23.73634642
IGA	.	12	25.74750000	12.91850127	3.72925009
PERLYSO		12	32.23051656	9.06385194	2.61650868
PERIGA		12	2.85303521	1.17146325	0.33817231

Table 31: Continued

SAS

VARIABLE	LABEL	N	MEAN	STANDARD DEVIATION	STD ERROR OF MEAN
GROUP=B	VISIT (weeks)=9				
CORNEA	.	13	0.54761538	0.05296310	0.01468932
TOTAL	.	13	785.38461538	285.26640720	79.11866603
LYSO	.	12	227.66666667	64.19194701	18.53061894
IGA	.	10	18.40800000	10.97541880	3.47073217
PERLYSO		12	32.48152695	10.52224459	3.03751037
PERIGA		10	2.58765773	1.31456093	0.41570067
GROUP=B	VISIT (weeks)=12				
CORNEA	.	13	0.54584615	0.04594534	0.01274294
TOTAL	.	13	1014.61538462	147.79577038	40.99117142
LYSO	.	12	259.00000000	67.09829967	19.36961069
IGA	.	12	21.53166667	9.00808408	2.60040988
PERLYSO		12	25.73062094	5.59212451	1.61430730
PERIGA		12	2.13675887	0.87825733	0.25353105
GROUP=B	VISIT (weeks)=15				
CORNEA	.	13	0.56153846	0.04672903	0.01296030
TOTAL	.	13	916.15384615	269.46052468	74.73490296
LYSO	.	12	229.33333333	90.23739734	26.04929282
IGA	.	12	30.33333333	11.21768679	3.23826724
PERLYSO		12	25.91629990	11.15873554	3.22124948
PERIGA		12	3.33930544	1.26175706	0.36423789
GROUP=B	VISIT (weeks)=18				
CORNEA	.	13	0.55900000	0.04746402	0.01316415
TOTAL	.	13	949.23076923	268.90089681	74.57969011
LYSO	.	12	226.16666667	68.63054360	19.81193141
IGA	.	12	27.12416667	13.95647615	4.02888763
PERLYSO		12	24.78971720	8.11125110	2.34151650
PERIGA		12	2.76116637	1.15823407	0.33435338
GROUP=B	VISIT (weeks)=21				
CORNEA	.	13	0.54692308	0.04307834	0.01194778
TOTAL	.	13	1116.15384615	475.66687330	131.92625398
LYSO	.	10	239.40000000	75.25394047	23.79738548
IGA	.	12	24.08083333	12.92341811	3.73066946
PERLYSO		10	25.77691996	9.73605722	3.07881162
PERIGA		12	2.22123273	0.87382841	0.25225253
GROUP=B	VISIT (weeks)=24				
CORNEA	.	13	0.54169231	0.04400452	0.01220466
TOTAL	.	13	1082.30769231	273.19571270	75.77085772
LYSO	.	11	270.36363636	78.26017215	23.59632973
IGA	.	12	21.28750000	10.66427692	3.07851158
PERLYSO		11	26.20717352	7.91217600	2.38561082
PERIGA		12	1.87280973	0.64936380	0.18745518

Table 31: Continued

SAS

VARIABLE	LABEL	N	MEAN	STANDARD DEVIATION	STD ERROR OF MEAN
GROUP=C	VISIT (weeks)=0				
CORNEA	.	14	0.54750000	0.02984511	0.00797644
TOTAL	.	14	861.42857143	286.96976906	76.69589687
LYSO	.	14	283.28571429	59.82850950	15.98984175
IGA	.	14	30.33642857	11.97708107	3.20100956
PERLYSO		14	35.59960959	11.66735291	3.11823123
PERIGA		14	3.64679084	1.20076005	0.32091662
GROUP=C	VISIT (weeks)=3				
CORNEA	.	14	0.55928571	0.03040351	0.00812568
TOTAL	.	14	927.85714286	289.54018112	77.38286839
LYSO	.	14	255.28571429	85.05421671	22.73169559
IGA	.	14	31.28714286	9.47307718	2.53178637
PERLYSO		14	29.33439020	12.33676729	3.29713975
PERIGA		14	3.50818123	0.94044801	0.25134530
GROUP=C	VISIT (weeks)=6				
CORNEA	.	13	0.54700000	0.03575845	0.00991761
TOTAL	.	14	1004.28571429	274.27426716	73.30288127
LYSO	.	14	286.57142857	65.65377051	17.54670824
IGA	.	14	24.40500000	10.92699322	2.92036178
PERLYSO		14	29.27107883	6.51236649	1.74050316
PERIGA		14	2.39786559	0.81074340	0.21668029
GROUP=C	VISIT (weeks)=9				
CORNEA	.	13	0.54800000	0.03744552	0.01038552
TOTAL	.	13	1106.15384615	388.87741131	107.85518818
LYSO	.	13	294.46153846	80.86677870	22.42840901
IGA	.	10	25.08900000	10.37945026	3.28227037
PERLYSO		13	27.99167526	6.95644931	1.92937190
PERIGA		10	2.14713816	0.60066496	0.18994694
GROUP=C	VISIT (weeks)=12				
CORNEA	.	13	0.54753846	0.03273280	0.00907844
TOTAL	.	13	1238.46153846	362.07379897	100.42120367
LYSO	.	13	329.53846154	101.17692045	28.06142881
IGA	.	13	28.30769231	9.95129653	2.75999307
PERLYSO		13	28.18557076	12.56319805	3.48440421
PERIGA		13	2.41814823	0.95758030	0.26558499
GROUP=C	VISIT (weeks)=15				
CORNEA	.	13	0.55800000	0.03785279	0.01049847
TOTAL	.	13	1159.23076923	483.79509331	134.18061660
LYSO	.	12	295.83333333	85.58019454	24.70487418
IGA	.	13	27.70153846	14.09370004	3.90888909
PERLYSO		12	25.96913665	7.21036123	2.08145200
PERIGA		13	2.42494306	0.77696664	0.21549177

Table 31: Continued

SAS

VARIABLE	LABEL	N	MEAN	STANDARD DEVIATION	STD ERROR OF MEAN
GROUP=C	VISIT (weeks)=18				
CORNEA	.	12	0.55425000	0.03434352	0.00991412
TOTAL	.	12	1062.50000000	295.60800211	85.33467980
LYSO	.	12	268.00000000	55.06359959	15.89549202
IGA	.	12	27.08666667	15.25982624	4.40513239
PERLYSO		12	26.54363735	7.27053475	2.09882260
PERIGA		12	2.45889919	1.05473252	0.30447505
GROUP=C	VISIT (weeks)=21				
CORNEA	.	12	0.55050000	0.03618513	0.01044575
TOTAL	.	12	1336.66666667	359.85687391	103.88173151
LYSO	.	11	296.36363636	73.41835292	22.13646631
IGA	.	12	30.02333333	11.33183234	3.27121823
PERLYSO		11	23.74829726	5.50758037	1.66059796
PERIGA		12	2.30513638	0.84449739	0.24378540
GROUP=C	VISIT (weeks)=24				
CORNEA	.	11	0.54063636	0.03724050	0.01122843
TOTAL	.	10	1095.00000000	237.31132857	75.04443128
LYSO	.	7	323.14285714	72.36810663	27.35257328
IGA	.	10	26.09100000	8.65423268	2.73670867
PERLYSO		7	30.28412300	7.47532156	2.82540597
PERIGA		10	2.43375314	0.82926072	0.26223526
GROUP=D	VISIT (weeks)=0				
CORNEA	.	14	0.56607143	0.03608849	0.00964505
TOTAL	.	14	786.42857143	346.66781135	92.65086979
LYSO	.	14	279.71428571	62.65832693	16.74614227
IGA	.	14	23.36500000	12.28672313	3.28376488
PERLYSO		14	38.41073703	8.98080178	2.40022024
PERIGA		14	3.04827158	1.50464830	0.40213417
GROUP=D	VISIT (weeks)=3				
CORNEA	.	14	0.56071429	0.03500424	0.00935528
TOTAL	.	14	1105.00000000	318.69324532	85.17435253
LYSO	.	14	284.42857143	83.39407310	22.28800355
IGA	.	14	32.88857143	16.01583921	4.28041308
PERLYSO		14	26.97493489	8.85741608	2.36724402
PERIGA		14	2.85459316	1.23796409	0.33085982
GROUP=D	VISIT (weeks)=6				
CORNEA	.	12	0.56375000	0.03977008	0.01148063
TOTAL	.	13	1018.46153846	279.16083040	77.42528370
LYSO	.	13	303.07692308	64.51674400	17.89372528
IGA	.	13	29.91076923	15.73154181	4.36314466
PERLYSO		13	31.16285995	8.59719452	2.38443274
PERIGA		13	2.86501027	1.48363984	0.41148765

Table 31: Continued

SAS

VARIABLE	LABEL	N	MEAN	STANDARD DEVIATION	STD ERROR OF MEAN
GROUP=D	VISIT (weeks)=9				
CORNEA	.	14	0.55928571	0.03761020	0.01005175
TOTAL	.	14	1176.42857143	471.03171774	125.88852186
LYSO	.	14	275.00000000	76.38263999	20.41411923
IGA	.	11	28.32727273	15.43092226	4.65259812
PERLYSO		14	24.77878016	6.63679587	1.77375831
PERIGA		11	2.84287632	1.59860383	0.48199719
GROUP=D	VISIT (weeks)=12				
CORNEA	.	14	0.55671429	0.03951130	0.01055984
TOTAL	.	14	1250.71428571	382.18932654	102.14439406
LYSO	.	14	321.28571429	91.65019499	24.49454493
IGA	.	14	27.83785714	14.46155018	3.86501186
PERLYSO		14	26.57001207	6.70909294	1.79308051
PERIGA		14	2.26172285	1.13090119	0.30224606
GROUP=D	VISIT (weeks)=15				
CORNEA	.	14	0.56807143	0.03555595	0.00950273
TOTAL	.	14	1127.14285714	296.29581298	79.18838695
LYSO	.	14	263.00000000	42.57662775	11.37908241
IGA	.	14	29.11142857	17.26654072	4.61467712
PERLYSO		14	24.29150458	5.34187151	1.42767521
PERIGA		14	2.52194594	1.37980355	0.36876801
GROUP=D	VISIT (weeks)=18				
CORNEA	.	14	0.56492857	0.03316964	0.00886496
TOTAL	.	14	1135.00000000	263.02237401	70.29568633
LYSO	.	14	276.85714286	88.42107053	23.63152512
IGA	.	14	30.83500000	18.33886321	4.90126736
PERLYSO		14	24.43306085	6.29372568	1.68206894
PERIGA		14	2.63933756	1.42065960	0.37968725
GROUP=D	VISIT (weeks)=21				
CORNEA	.	13	0.56030769	0.03301359	0.00915632
TOTAL	.	14	1287.14285714	393.98220521	105.29617346
LYSO	.	14	274.28571429	82.38691884	22.01883025
IGA	.	14	29.78500000	16.43191348	4.39161360
PERLYSO		14	22.41135163	7.75611317	2.07290844
PERIGA		14	2.28167687	1.35051204	0.36093952
GROUP=D	VISIT (weeks)=24				
CORNEA	.	13	0.55646154	0.03948969	0.01095247
TOTAL	.	13	1129.23076923	258.95500055	71.82119481
LYSO	.	10	291.20000000	72.81147651	23.02501056
IGA	.	13	26.54538462	14.03346869	3.89218392
PERLYSO		10	26.33177105	4.54077915	1.43592045
PERIGA		13	2.31356430	1.21436579	0.33680447

Table 32: ANOVA for Central (Cornea) Thickness as the Dependent Variable

SAS

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: CORNEA

SOURCE	DF	PR > F	R-SQUARE	C.V.
MODEL	85	0.0001	0.921546	2.0481
ERROR	383	ROOT MSE		CORNEA MEAN
CORRECTED TOTAL	468	0.01139722		0.55647122

SOURCE	DF	TYPE III SS	F VALUE	PR > F
GROUP	3	0.01270292	32.60	0.0001
ID(GROUP)	50	0.55529659	85.50	0.0001
VISIT	8	0.00938551	9.03	0.0001
GROUP*VISIT	24	0.00289245	0.93	0.5638

TESTS OF HYPOTHESES USING THE TYPE III MS FOR ID(GROUP) AS AN ERROR TERM

SOURCE	DF	TYPE III SS	F VALUE	PR > F
GROUP	3	0.01270292	0.38	0.7669

Table 33: Linear Regression Analysis for Central (Cornea) Thickness with Respect to Visit

SAS

DEP VARIABLE: CORNEA

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PROB>F
MODEL	1	0.0005871832	0.0005871832	0.433	0.5108
ERROR	432	0.585531	0.001355395		
C TOTAL	433	0.586118			

ROOT MSE	0.036816	R-SQUARE	0.0010
DEP MEAN	0.556982	ADJ R-SQ	-0.0013
C.V.	6.609858		

VARIABLE	DF	PARAMETER ESTIMATE	STANDARD ERROR	T FOR H0: PARAMETER=0	PROB > |T
INTERCEP	1	0.558727	0.003186555	175.339	0.0001
VISIT	1	-0.00015146	0.000230143	-0.658	0.5108

Table 34: Statistical Analysis II: The mean, SD and SEM derived from raw data for the distribution of protein fractions in sets PER 1, PER 2, PER 3 and PER 4.

SAS

VARIABLE	N	MEAN	STANDARD DEVIATION	STD ERROR OF MEAN
GROUP=A VISIT=0				
PER1	11	27.71282286	6.39635132	1.92857249
PER2	11	11.74400422	4.46788066	1.34711671
PER3	11	50.33835557	6.16685278	1.85937607
PER4	11	10.20481735	7.58463830	2.28685449
GROUP=A VISIT=3				
PER1	11	29.92667987	7.38186398	2.22571573
PER2	11	5.64400496	4.90198858	1.47800517
PER3	11	54.28673060	10.00545032	3.01675678
PER4	11	10.14258457	5.15338930	1.55380534
GROUP=A VISIT=6				
PER1	10	26.26856361	6.86581576	2.17116158
PER2	10	10.08688914	6.69603759	2.11747301
PER3	10	51.83943177	7.24095367	2.28979060
PER4	10	11.80511548	4.21715907	1.33358279
GROUP=A VISIT=9				
PER1	13	25.64340864	7.47561371	2.07336220
PER2	13	12.45798534	4.78039033	1.32584173
PER3	13	43.92163657	8.67084748	2.40486040
PER4	13	17.97696945	8.64411216	2.39744536
GROUP=A VISIT=12				
PER1	10	27.41857355	7.47123445	2.36261178
PER2	10	10.65697867	3.32868693	1.05262323
PER3	10	45.43130977	8.88267041	2.80894702
PER4	10	16.49313800	5.66641666	1.79187828
GROUP=A VISIT=15				
PER1	11	23.88666939	3.00324093	0.90551121
PER2	11	15.22951498	3.31459486	0.99938795
PER3	11	50.91078878	5.25056621	1.58310528
PER4	11	9.97302685	2.32839534	0.70203761
GROUP=A VISIT=18				
PER1	13	25.89820737	4.96929658	1.37823489
PER2	13	14.78870667	4.39788297	1.21975327
PER3	13	45.95162001	9.25695810	2.56741824
PER4	13	13.36146596	6.19698862	1.71873540
GROUP=A VISIT=21				
PER1	12	26.07611562	6.46197699	1.86541208
PER2	12	13.05458244	5.33017791	1.53868983
PER3	12	41.75019080	9.68111047	2.79469587
PER4	12	19.11911114	17.67055417	5.10104960
GROUP=A VISIT=24				
PER1	12	26.39788862	7.51426226	2.16918067
PER2	12	15.44725738	5.41990891	1.56459293
PER3	12	44.38694578	9.30892035	2.68725384
PER4	12	13.76790823	4.21310283	1.21621803

Table 34 continued.

SAS

VARIABLE	N	MEAN	STANDARD DEVIATION	STD ERROR OF MEAN
GROUP=B VISIT=0				
PER1	11	27.69148406	5.58898578	1.68514262
PER2	11	13.91601007	3.51914117	1.06106098
PER3	11	42.16988573	9.00768396	2.71591890
PER4	11	16.22262014	8.85749774	2.67063605
GROUP=B VISIT=3				
PER1	11	26.87066192	6.68975664	2.01703752
PER2	11	8.97038209	6.88254736	2.07516611
PER3	11	49.77547014	14.27501580	4.30407921
PER4	11	14.38348585	7.37016919	2.22218962
GROUP=B VISIT=6				
PER1	8	24.51808900	4.18059695	1.47806423
PER2	8	11.47222325	6.29021206	2.22392580
PER3	8	50.49753105	6.83511552	2.41657827
PER4	8	13.51215670	4.05201084	1.43260217
GROUP=B VISIT=9				
PER1	10	23.31773963	3.65272197	1.15509211
PER2	10	14.84270849	6.14314161	1.94263195
PER3	10	49.18130826	9.23909318	2.92165780
PER4	10	12.65824361	4.94838433	1.56481652
GROUP=B VISIT=12				
PER1	8	24.44698025	4.87299162	1.72286271
PER2	8	11.22571072	2.55710408	0.90407282
PER3	8	45.91124942	9.44116525	3.33795599
PER4	8	18.41605961	8.83569456	3.12388977
GROUP=B VISIT=15				
PER1	10	22.87873752	4.72980766	1.49569651
PER2	10	16.02745881	6.95415119	2.19909569
PER3	10	46.94635398	9.61937098	3.04191220
PER4	10	14.14744969	4.06692359	1.28607416
GROUP=B VISIT=18				
PER1	11	26.66761931	3.66513065	1.10507847
PER2	11	15.31545903	5.29173057	1.59551680
PER3	11	44.09067547	6.09886793	1.83887787
PER4	11	13.92624619	6.08852816	1.83576031
GROUP=B VISIT=21				
PER1	10	23.59017403	4.82010847	1.52425213
PER2	10	15.58520245	4.09921806	1.29628657
PER3	10	42.16174261	7.75631547	2.45276231
PER4	10	18.66288091	8.00603972	2.53173206
GROUP=B VISIT=24				
PER1	8	25.30643314	2.75371655	0.97358582
PER2	8	15.48464214	5.82812228	2.06055239
PER3	8	44.55309367	5.56712224	1.96827494
PER4	8	14.65583105	5.99787413	2.12056873

Table 34 Continued.

SAS

VARIABLE	N	MEAN	STANDARD DEVIATION	STD ERROR OF MEAN
GROUP=C VISIT=0				
PER1	13	24.57396263	4.32534275	1.19963424
PER2	13	12.34842372	4.22152493	1.17084035
PER3	13	50.28367389	7.13129600	1.97786564
PER4	13	12.79393976	9.59025939	2.65985938
GROUP=C VISIT=3				
PER1	13	29.14516543	5.72131372	1.58680692
PER2	13	6.13894002	3.69224812	1.02404538
PER3	13	52.38730456	5.82743110	1.61623859
PER4	13	12.32858999	5.36892989	1.48907323
GROUP=C VISIT=6				
PER1	11	25.58291294	3.98157012	1.20048856
PER2	11	11.58234275	4.18759019	1.26260595
PER3	11	51.76483767	4.62083732	1.39323487
PER4	11	11.06990664	3.88594357	1.17165607
GROUP=C VISIT=9				
PER1	12	26.85592732	5.08238973	1.46715954
PER2	12	11.56439445	4.84997606	1.40006749
PER3	12	47.32803603	10.06485427	2.90547316
PER4	12	14.25164219	6.30474416	1.82002287
GROUP=C VISIT=12				
PER1	11	21.32454600	7.51591589	2.26613391
PER2	11	10.74904955	7.42862213	2.23981385
PER3	11	52.96218275	10.05600206	3.03199870
PER4	11	14.96422170	4.42909701	1.33542300
GROUP=C VISIT=15				
PER1	12	25.73144180	5.81632823	1.67902933
PER2	12	15.30823343	5.07357306	1.46461439
PER3	12	48.22474440	8.40580935	2.42654814
PER4	12	10.73558037	2.64103094	0.76239996
GROUP=C VISIT=18				
PER1	9	23.87857844	4.31082175	1.43694058
PER2	9	16.70847987	6.39097070	2.13032357
PER3	9	48.47674185	5.42440685	1.80813562
PER4	9	10.93619985	5.68335303	1.89445101
GROUP=C VISIT=21				
PER1	11	28.07744944	3.34236035	1.00775956
PER2	11	13.89269457	2.94138741	0.88686167
PER3	11	46.54835294	6.81659749	2.05528148
PER4	11	11.48150305	3.84331461	1.15880296
GROUP=C VISIT=24				
PER1	10	24.22910488	7.23119540	2.28670477
PER2	10	16.66647983	4.67694377	1.47897948
PER3	10	46.69008697	7.12932434	2.25449031
PER4	10	12.41432832	2.64384005	0.83605563

Table 34 Continued.

SAS

VARIABLE	N	MEAN	STANDARD DEVIATION	STD ERROR OF MEAN
GROUP=D VISIT=0				
PER1	13	26.68125966	4.57591861	1.26913148
PER2	13	13.81944015	2.24893677	0.62374283
PER3	13	48.75299134	5.06717476	1.40538142
PER4	13	10.74630884	5.40908465	1.50021016
GROUP=D VISIT=3				
PER1	11	26.72223484	5.27610431	1.59080530
PER2	11	5.43026750	5.50800677	1.66072653
PER3	11	53.78198154	6.03001353	1.81811749
PER4	11	14.06551612	5.90094455	1.77920173
GROUP=D VISIT=6				
PER1	10	22.96352840	3.82049387	1.20814624
PER2	10	13.53330970	4.90225083	1.55022783
PER3	10	49.84244461	6.82098812	2.15698583
PER4	10	13.66071730	4.55163749	1.43935415
GROUP=D VISIT=9				
PER1	12	21.78383095	4.42507277	1.27740848
PER2	12	13.26495265	6.23629453	1.80026316
PER3	12	51.37719598	7.34985237	2.12171962
PER4	12	13.57402042	4.75260328	1.37195839
GROUP=D VISIT=12				
PER1	12	22.24006295	5.29424752	1.52831762
PER2	12	10.12329696	4.73141526	1.36584194
PER3	12	53.26626155	8.26419441	2.38566743
PER4	12	14.37037854	5.55907794	1.60476757
GROUP=D VISIT=15				
PER1	11	23.12301805	2.73243147	0.82385909
PER2	11	14.17517253	5.48608972	1.65411829
PER3	11	50.59007084	6.78735653	2.04646499
PER4	11	12.11173858	2.44512879	0.73723407
GROUP=D VISIT=18				
PER1	9	25.80352197	3.66873407	1.22291136
PER2	9	16.20171834	4.06886115	1.35628705
PER3	9	45.02498523	10.89348753	3.63116251
PER4	9	12.96977445	6.50643692	2.16881231
GROUP=D VISIT=21				
PER1	12	28.44011234	5.96139057	1.72090522
PER2	12	13.26003679	2.89874816	0.83679652
PER3	12	44.33591030	6.77211132	1.95494015
PER4	12	13.96394056	3.63056039	1.04805251
GROUP=D VISIT=24				
PER1	12	23.81119704	5.05153846	1.45825354
PER2	12	16.04643930	3.34771228	0.96640129
PER3	12	48.85740803	5.01247703	1.44697748
PER4	12	11.28495563	2.49452129	0.72010627

Table 35: Raw Data Collected From Each Subject: I. The data on lysozyme (Lyso) and IgA are expressed in mg%. Lysozyme in total protein (PERLYSO) and IgA in total protein (PERIGA) are expressed as a percentage Corneal thickness (Cornea) is expressed in mm.

SAS

OBS	GROUP	ID	VISIT	LYSO	IGA	TOTAL	CORNEA	PERLYSO	PERIGA
1	A	1	0	246	20.66	750	0.540	32.8000	2.75467
2	A	2	0	192	21.43	510	0.534	37.6471	4.20196
3	A	3	0	56	15.30	560	0.606	10.0000	2.73214
4	A	4	0	244	13.17	490	0.600	49.7959	2.68776
5	A	5	0	250	9.68	360	0.525	69.4444	2.68889
6	A	6	0	114	20.14	440	0.555	25.9091	4.57727
7	A	7	0	240	25.03	620	0.558	38.7097	4.03710
8	A	8	0	216	18.53	380	0.590	56.8421	4.87632
9	A	9	0	180	32.08	770	0.600	23.3766	4.16623
10	A	10	0	214	13.56	420	0.602	50.9524	3.22857
11	A	11	0	284	41.44	920	0.610	30.8696	4.50435
12	A	12	0	.	.	.	.	.	.
13	A	13	0	332	38.41	850	0.540	39.0588	4.51882
14	A	14	0	214	36.71	520	0.537	41.1538	7.05962
15	A	1	3	334	16.84	920	0.530	36.3043	1.83043
16	A	2	3	216	4.37	490	0.578	44.0816	0.89184
17	A	3	3	298	9.62	640	0.603	46.5625	1.50312
18	A	4	3	370	42.72	1340	0.577	27.6119	3.18806
19	A	5	3	222	27.59	740	0.516	30.0000	3.72838
20	A	6	3	136	30.90	940	0.536	14.4681	3.28723
21	A	7	3	174	9.24	620	0.534	28.0645	1.49032
22	A	8	3	168	27.73	720	0.600	23.3333	3.85139
23	A	9	3	140	34.66	810	0.600	17.2840	4.27901
24	A	10	3	246	13.40	620	0.572	39.6774	2.16129
25	A	11	3	246	41.42	970	0.602	25.3608	4.27010
26	A	12	3	.	.	.	.	.	.
27	A	13	3	294	36.81	960	0.513	30.6250	3.83437
28	A	14	3	332	27.42	990	0.554	33.5354	2.76970
29	A	1	6	220	25.92	950	0.526	23.1579	2.72842
30	A	2	6	246	17.79	730	0.525	33.6986	2.43699
31	A	3	6	270	20.33	750	0.614	36.0000	2.71067
32	A	4	6	262	24.96	880	0.600	29.7727	2.83636
33	A	5	6	134	17.52	490	0.538	27.3469	3.57551
34	A	6	6	342	34.88	1010	0.548	33.8614	3.45347
35	A	7	6	256	27.11	750	0.540	34.1333	3.61467
36	A	8	6	314	39.04	950	0.595	33.0526	4.10947
37	A	9	6	314	45.01	940	0.586	33.4043	4.78830
38	A	10	6	210	31.18	620	0.562	33.8710	5.02903
39	A	11	6	188	31.18	740	0.603	25.4054	4.21351
40	A	12	6	.	.	.	.	.	.
41	A	13	6	328	23.32	790	0.538	41.5190	2.95190
42	A	14	6	280	15.75	610	0.550	45.9016	2.58197
43	A	1	9	292	33.34	1340	0.530	21.7910	2.48806
44	A	2	9	144	10.98	820	0.536	17.5610	1.33902
45	A	3	9	84	4.88	310	0.614	27.0968	1.57419
46	A	4	9	232	20.33	950	0.558	24.4211	2.14000
47	A	5	9	182	30.28	620	0.534	29.3548	4.88387
48	A	6	9	176	37.05	690	0.542	25.5072	5.36957
49	A	7	9	256	8.77	820	0.540	31.2195	1.06951
50	A	8	9	322	29.37	960	0.600	33.5417	3.05937
51	A	9	9	348	42.93	1130	0.603	30.7965	3.79912
52	A	10	9	182	.	930	0.564	19.5699	.
53	A	11	9	278	.	1390	0.596	20.0000	.
54	A	12	9	.	.	.	.	.	.
55	A	13	9	218	22.00	920	0.504	23.6957	2.39130
56	A	14	9	284	31.61	1610	0.560	17.6398	1.96335

Table 35: Continued.

SAS

OBS	GROUP	ID	VISIT	LYSO	IGA	TOTAL	CORNEA	PERLYSO	PERIGA
57	A	1	12	302	27.21	1340	0.503	22.5373	2.03060
58	A	2	12	158	13.24	870	0.532	18.1609	1.52184
59	A	3	12	154	4.46	530	0.603	29.0566	0.84151
60	A	4	12	296	23.62	1010	0.586	29.3069	2.33861
61	A	5	12	336	15.88	1290	0.533	26.0465	1.23101
62	A	6	12	144	24.78	510	0.522	28.2353	4.85882
63	A	7	12	302	39.89	1080	0.506	27.9630	3.69352
64	A	8	12	294	28.27	1480	0.590	19.8649	1.91014
65	A	9	12	224	36.99	960	0.588	23.3333	3.85312
66	A	10	12	372	26.10	1140	0.576	32.6316	2.28947
67	A	11	12	384	36.38	950	0.606	40.4211	3.82947
68	A	12	12	.	.	.	.	.	.
69	A	13	12	432	28.83	1140	0.536	37.8947	2.52895
70	A	14	12	.	.	.	.	.	.
71	A	1	15	244	14.98	1080	0.544	22.5926	1.38704
72	A	2	15	158	3.57	750	0.535	21.0667	0.47600
73	A	3	15	194	9.63	920	0.601	21.0870	1.04674
74	A	4	15	256	17.21	1030	0.582	24.8544	1.67087
75	A	5	15	260	43.28	1370	0.540	18.9781	3.15912
76	A	6	15	242	30.24	950	0.550	25.4737	3.18316
77	A	7	15	.	.	.	0.550	.	.
78	A	8	15	394	44.79	1600	0.604	24.6250	2.79937
79	A	9	15	294	44.79	1360	0.600	21.6176	3.29338
80	A	10	15	260	35.82	1010	0.588	25.7426	3.54653
81	A	11	15	282	55.71	1270	0.612	22.2047	4.38661
82	A	12	15	.	.	.	.	.	.
83	A	13	15	302	27.91	1210	0.523	24.9587	2.30661
84	A	14	15	342	28.61	1380	0.554	24.7826	2.07319
85	A	1	18	248	34.83	1160	0.542	21.3793	3.00259
86	A	2	18	156	22.25	780	0.538	20.0000	2.85256
87	A	3	18	222	12.19	560	0.612	39.6429	2.17679
88	A	4	18	216	23.27	850	0.582	25.4118	2.73765
89	A	5	18	250	24.43	1080	0.548	23.1481	2.26204
90	A	6	18	222	41.96	1140	0.552	19.4737	3.68070
91	A	7	18	188	29.43	1140	0.522	16.4912	2.58158
92	A	8	18	272	40.34	1490	0.606	18.2550	2.70738
93	A	9	18	236	46.79	1270	0.612	18.5827	3.68425
94	A	10	18	300	39.36	1340	0.578	22.3881	2.93731
95	A	11	18	246	59.04	1340	0.608	18.3582	4.40597
96	A	12	18	.	.	.	.	.	.
97	A	13	18	506	12.74	1480	0.540	34.1892	0.86081
98	A	14	18	506	14.44	1670	0.574	30.2994	0.86467
99	A	1	21	216	26.86	1190	0.522	18.1513	2.25714
100	A	2	21	196	23.52	950	0.534	20.6316	2.47579
101	A	3	21	212	8.86	900	0.596	23.5556	0.98444
102	A	4	21	238	47.32	1100	0.573	21.6364	4.30182
103	A	5	21	308	57.24	1540	0.536	20.0000	3.71688
104	A	6	21	206	53.52	870	0.542	23.6782	6.15172
105	A	7	21	298	.	960	0.530	31.0417	.
106	A	8	21	264	34.63	1490	0.594	17.7181	2.32416
107	A	9	21	278	43.07	1560	0.580	17.8205	2.76090
108	A	10	21	178	20.66	1140	0.580	15.6140	1.81228
109	A	11	21	224	28.78	1400	0.592	16.0000	2.05571
110	A	12	21	.	.	.	.	.	.
111	A	13	21	462	36.59	1860	0.536	24.8387	1.96720
112	A	14	21	276	14.71	870	0.562	31.7241	1.69080
113	A	1	24	354	27.43	1210	0.530	29.2562	2.26694
114	A	2	24	264	13.28	880	0.526	30.0000	1.50909
115	A	3	24	234	11.45	720	0.600	32.5000	1.59028
116	A	4	24	276	38.99	1420	0.556	19.4366	2.74577
117	A	5	24	212	18.42	750	0.534	28.2667	2.45600
118	A	6	24	260	34.21	1030	0.540	25.2427	3.32136
119	A	7	24	270	18.07	890	0.536	30.3371	2.03034
120	A	8	24	354	31.11	1280	0.580	27.6562	2.43047
121	A	9	24	242	37.56	1160	0.580	20.8621	3.23793
122	A	10	24	296	35.52	1400	0.554	21.1429	2.53714
123	A	11	24	256	35.52	1460	0.592	17.5342	2.43288
124	A	12	24	.	.	.	.	.	.
125	A	13	24	.	14.73	1080	0.530	.	1.36389
126	A	14	24	.	14.73	560	0.542	.	2.63036

Table 35: Continued.

SAS

OBS	GROUP	ID	VISIT	LYSO	IGA	TOTAL	CORNEA	PERLYSO	PERIGA
127	B	1	0	246	25.64	690	0.557	35.6522	3.71594
128	B	2	0	168	22.96	480	0.522	35.0000	4.78333
129	B	3	0	216	22.19	610	0.498	35.4098	3.63770
130	B	4	0	230	5.03	550	0.627	41.8182	0.91455
131	B	5	0	184	22.46	340	0.536	54.1176	6.60588
132	B	6	0	204	22.46	360	0.608	56.6667	6.23889
133	B	7	0	260	34.79	820	0.573	31.7073	4.24268
134	B	8	0	286	33.17	940	0.600	30.4255	3.52872
135	B	9	0	126	45.94	1400	0.517	9.0000	3.28143
136	B	10	0	242	10.65	570	0.492	42.4561	1.86842
137	B	11	0	.	.	.	.	.	.
138	B	12	0	262	19 37	490	0.532	53.4694	3.95306
139	B	13	0	.	.	620	0.570	.	.
140	B	14	0	332	33.89	1080	0.513	30.7407	3.13796
141	B	1	3	344	19.46	810	0.542	42.4691	2.40247
142	B	2	3	322	22.74	780	0.536	41.2821	2.91538
143	B	3	3	304	26.02	630	0.498	48.2540	4.13016
144	B	4	3	126	40.35	620	0.620	20.3226	6.50806
145	B	5	3	100	27.59	640	0.528	15.6250	4.31094
146	B	6	3	200	37.04	1030	0.602	19.4175	3.59612
147	B	7	3	240	43.44	1660	0.578	14.4578	2.61687
148	B	8	3	134	29.12	570	0.604	23.5088	5.10877
149	B	9	3	240	41.59	1480	0.512	16.2162	2.81014
150	B	10	3	178	3.01	230	0.502	77.3913	1.30870
151	B	11	3	.	.	.	.	.	.
152	B	12	3	218	16.57	630	0.530	34.6032	2.63016
153	B	13	3	.	.	.	.	.	.
154	B	14	3	396	28.68	1160	0.540	34.1379	2.47241
155	B	1	6	146	1.27	330	0.550	44.2424	0.38485
156	B	2	6	290	24.14	1190	0.536	24.3697	2.02857
157	B	3	6	314	15.25	720	0.510	43.6111	2.11806
158	B	4	6	210	40.83	1010	0.620	20.7921	4.04257
159	B	5	6	128	20.00	590	0.525	21.6949	3.38983
160	B	6	6	256	20.00	610	0.610	41.9672	3.27869
161	B	7	6	372	48.80	1340	0.565	27.7612	3.64179
162	B	8	6	264	35.25	1210	0.600	21.8182	2.91322
163	B	9	6	318	27.11	820	0.530	38.7805	3.30610
164	B	10	6	274	20.33	820	0.486	33.4146	2.47927
165	B	11	6	.	.	.	.	.	.
166	B	12	6	224	37.14	770	0.552	29.0909	4.82338
167	B	13	6	.	.	950	0.565	.	.
168	B	14	6	404	18.85	1030	0.542	39.2233	1.83010
169	B	1	9	216	2.85	580	0.598	37.2414	0.49138
170	B	2	9	272	15.45	830	0.521	32.7711	1.86145
171	B	3	9	232	12.20	880	0.500	26.3636	1.38636
172	B	4	9	246	17.62	880	0.636	27.9545	2.00227
173	B	5	9	106	17.17	610	0.504	17.3770	2.81475
174	B	6	9	228	11.30	380	0.620	60.0000	2.97368
175	B	7	9	356	37.51	1180	0.560	30.1695	3.17881
176	B	8	9	242	37.51	820	0.607	29.5122	4.57439
177	B	9	9	154	17.44	380	0.510	40.5263	4.58947
178	B	10	9	164	.	550	0.480	29.8182	.
179	B	11	9	.	.	.	.	.	.
180	B	12	9	264	.	1080	0.520	24.4444	.
181	B	13	9	.	.	1290	0.565	.	.
182	B	14	9	252	15.03	750	0.498	33.6000	2.00400

Table 35: Continued.

SAS

OBS	GROUP	ID	VISIT	LYSO	IGA	TOTAL	CORNEA	PERLYSO	PERIGA
183	B	1	12	196	19.23	950	0.555	20.6316	2.02421
184	B	2	12	288	27.21	1080	0.520	26.6667	2.51944
185	B	3	12	292	23.22	1140	0.496	25.6140	2.03684
186	B	4	12	266	17.81	980	0.638	27.1429	1.81735
187	B	5	12	170	20.91	920	0.533	18.4783	2.27283
188	B	6	12	232	13.94	690	0.593	33.6232	2.02029
189	B	7	12	180	36.99	1210	0.562	14.8760	3.05702
190	B	8	12	224	8.52	890	0.600	25.1685	0.95730
191	B	9	12	236	36.99	880	0.514	26.8182	4.20341
192	B	10	12	278	17.68	1080	0.480	25.7407	1.63704
193	B	11	12	.	.	.	.	.	.
194	B	12	12	390	11.14	1180	0.528	33.0508	0.94407
195	B	13	12	.	.	1040	0.572	.	.
196	B	14	12	356	24.74	1150	0.505	30.9565	2.15130
197	B	1	15	140	15.34	550	0.590	25.4545	2.78909
198	B	2	15	98	20.33	570	0.546	17.1930	3.56667
199	B	3	15	248	26.75	950	0.510	26.1053	2.81579
200	B	4	15	134	43.28	1400	0.654	9.5714	3.09143
201	B	5	15	142	36.50	950	0.540	14.9474	3.84211
202	B	6	15	206	22.42	950	0.615	21.6842	2.36000
203	B	7	15	304	33.74	1010	0.566	30.0990	3.34059
204	B	8	15	204	34.63	1000	0.600	20.4000	3.46300
205	B	9	15	314	47.00	1340	0.573	23.4328	3.50746
206	B	10	15	380	25.02	1100	0.486	34.5455	2.27455
207	B	11	15	.	.	.	.	.	.
208	B	12	15	330	44.34	640	0.548	51.5625	6.92812
209	B	13	15	.	.	750	0.566	.	.
210	B	14	15	252	14.65	700	0.506	36.0000	2.09286
211	B	1	18	262	4.64	690	0.572	37.9710	0.67246
212	B	2	18	192	30.81	770	0.557	24.9351	4.00130
213	B	3	18	248	29.80	1080	0.508	22.9630	2.75926
214	B	4	18	148	43.13	1090	0.655	13.5780	3.95688
215	B	5	18	134	40.21	880	0.548	15.2273	4.56932
216	B	6	18	242	27.35	830	0.604	29.1566	3.29518
217	B	7	18	150	17.03	680	0.548	22.0588	2.50441
218	B	8	18	244	31.91	1350	0.608	18.0741	2.36370
219	B	9	18	230	29.43	1180	0.540	19.4915	2.49407
220	B	10	18	184	11.15	570	0.486	32.2807	1.95614
221	B	11	18	.	.	.	.	.	.
222	B	12	18	350	49.85	1470	0.525	23.8095	3.39116
223	B	13	18	.	.	880	0.600	.	.
224	B	14	18	330	10.18	870	0.516	37.9310	1.17011
225	B	1	21	164	8.86	780	0.572	21.0256	1.13590
226	B	2	21	220	12.21	790	0.512	27.8481	1.54557
227	B	3	21	212	20.17	1080	0.490	19.6296	1.86759
228	B	4	21	232	52.28	1430	0.628	16.2238	3.65594
229	B	5	21	210	8.23	430	0.500	48.8372	1.91395
230	B	6	21	260	30.56	750	0.596	34.6667	4.07467
231	B	7	21	298	35.66	1500	0.560	19.8667	2.37733
232	B	8	21	.	20.56	880	0.600	.	2.33636
233	B	9	21	160	19.77	820	0.532	19.5122	2.41098
234	B	10	21	218	21.72	960	0.508	22.7083	2.26250
235	B	11	21	.	.	.	.	.	.
236	B	12	21	.	37.60	2240	0.546	.	1.67857
237	B	13	21	.	.	1320	0.554	.	.
238	B	14	21	420	21.35	1530	0.512	27.4510	1.39542
239	B	1	24	224	5.51	690	0.574	32.4638	0.79855
240	B	2	24	384	39.31	1410	0.510	27.2340	2.78794
241	B	3	24	334	32.00	1230	0.500	27.1545	2.60163
242	B	4	24	172	22.25	1010	0.624	17.0297	2.20297
243	B	5	24	112	11.24	810	0.512	13.8272	1.38765
244	B	6	24	272	10.29	640	0.598	42.5000	1.60781
245	B	7	24	328	18.07	1110	0.565	29.5495	1.62793
246	B	8	24	332	29.02	1190	0.584	27.8992	2.43866
247	B	9	24	262	17.66	900	0.510	29.1111	1.96222
248	B	10	24	270	13.07	1290	0.488	20.9302	1.01318
249	B	11	24	.	.	.	.	.	.
250	B	12	24	284	35.01	1380	0.528	20.5797	2.53696
251	B	13	24	.	.	950	0.553	.	.
252	B	14	24	.	22.02	1460	0.496	.	1.50822

Table 35: Continued.

SAS

OBS	GROUP	ID	VISIT	LYSO	IGA	TOTAL	CORNEA	PERLYSO	PERIGA
253	C	1	0	246	24.49	690	0.570	35.6522	3.54928
254	C	2	0	246	16.83	1140	0.566	21.5789	1.47632
255	C	3	0	206	19.51	490	0.558	42.0408	3.98163
256	C	4	0	254	27.11	460	0.570	55.2174	5.89348
257	C	5	0	290	28.27	750	0.580	38.6667	3.76933
258	C	6	0	290	44.54	940	0.537	30.8511	4.73830
259	C	7	0	340	39.67	1080	0.555	31.4815	3.67315
260	C	8	0	200	32.08	1030	0.580	19.4175	3.11456
261	C	9	0	240	37.51	810	0.556	29.6296	4.63086
262	C	10	0	384	57.13	1480	0.540	25.9459	3.86014
263	C	11	0	380	9.49	620	0.475	61.2903	1.53065
264	C	12	0	312	32.73	830	0.504	37.5904	3.94337
265	C	13	0	336	28.24	1120	0.550	30.0000	2.52143
266	C	14	0	242	27.11	620	0.524	39.0323	4.37258
267	C	1	3	334	16.84	550	0.574	60.7273	3.06182
268	C	2	3	440	22.74	1040	0.558	42.3077	2.18654
269	C	3	3	292	26.02	1140	0.571	25.6140	2.28246
270	C	4	3	222	46.50	1270	0.604	17.4803	3.66142
271	C	5	3	200	41.77	1270	0.580	15.7480	3.28898
272	C	6	3	222	33.26	1020	0.518	21.7647	3.26078
273	C	7	3	234	35.59	1180	0.570	19.8305	3.01610
274	C	8	3	74	16.57	460	0.590	16.0870	3.60217
275	C	9	3	234	30.97	850	0.560	27.5294	3.64353
276	C	10	3	322	38.26	1210	0.590	26.6116	3.16198
277	C	11	3	176	25.61	440	0.495	40.0000	5.82045
278	C	12	3	246	26.51	730	0.527	33.6986	3.63151
279	C	13	3	300	44.32	890	0.550	33.7079	4.97978
280	C	14	3	278	33.06	940	0.543	29.5745	3.51702
281	C	1	6	358	19.06	1080	0.580	33.1481	1.76481
282	C	2	6	340	17.79	1080	0.585	31.4815	1.64722
283	C	3	6	202	17.79	810	0.552	24.9383	2.19630
284	C	4	6	324	25.46	1010	0.570	32.0792	2.52079
285	C	5	6	256	33.39	960	0.590	26.6667	3.47812
286	C	6	6	276	24.96	820	0.530	33.6585	3.04390
287	C	7	6	400	48.80	1790	0.564	22.3464	2.72626
288	C	8	6	264	37.96	1210	.	21.8182	3.13719
289	C	9	6	304	9.22	750	0.560	40.5333	1.22933
290	C	10	6	188	25.76	810	0.556	23.2099	3.18025
291	C	11	6	322	6.24	790	0.466	40.7595	0.78987
292	C	12	6	192	24.13	750	0.500	25.6000	3.21733
293	C	13	6	244	27.79	1130	0.538	21.5929	2.45929
294	C	14	6	342	23.32	1070	0.520	31.9626	2.17944
295	C	1	9	416	38.63	1820	0.624	22.8571	2.12253
296	C	2	9	376	16.27	1530	0.585	24.5752	1.06340
297	C	3	9	208	10.17	750	0.558	27.7333	1.35600
298	C	4	9	390	29.37	1200	0.551	32.5000	2.44750
299	C	5	9	320	29.37	990	0.564	32.3232	2.96667
300	C	6	9	256	13.56	580	0.520	44.1379	2.33793
301	C	7	9	348	37.51	1670	0.566	20.8383	2.24611
302	C	8	9	.	.	.	.	.	.
303	C	9	9	330	29.37	1090	0.556	30.2752	2.69450
304	C	10	9	206	.	900	0.556	22.8889	.
305	C	11	9	354	.	1130	0.474	31.3274	.
306	C	12	9	192	.	590	0.510	32.5424	.
307	C	13	9	218	15.03	880	0.546	24.7727	1.70795
308	C	14	9	214	31.61	1250	0.514	17.1200	2.52880

Table 35: Continued.

SAS

OBS	GROUP	ID	VISIT	LYSO	IGA	TOTAL	CORNEA	PERLYSO	PERIGA
309	C	1	12	376	34.80	1140	0.607	32.9825	3.05263
310	C	2	12	396	20.43	1740	0.567	22.7586	1.17414
311	C	3	12	260	19.23	1050	0.552	24.7619	1.83143
312	C	4	12	476	24.78	1600	0.564	29.7500	1.54875
313	C	5	12	328	24.78	1160	0.570	28.2759	2.13621
314	C	6	12	354	16.27	1090	0.530	32.4771	1.49266
315	C	7	12	188	21.30	1140	0.568	16.4912	1.86842
316	C	8	12	.	.	.	.	.	.
317	C	9	12	180	30.02	1260	0.565	14.2857	2.38254
318	C	10	12	420	55.08	2050	0.552	20.4878	2.68683
319	C	11	12	472	31.24	720	0.479	65.5556	4.33889
320	C	12	12	196	34.05	830	0.508	23.6145	4.10241
321	C	13	12	334	31.28	1180	0.534	28.3051	2.65085
322	C	14	12	304	24.74	1140	0.522	26.6667	2.17018
323	C	1	15	348	16.05	1340	0.592	25.9701	1.19776
324	C	2	15	402	19.62	1340	0.582	30.0000	1.46418
325	C	3	15	180	17.83	770	0.588	23.3766	2.31558
326	C	4	15	396	56.31	2180	0.582	18.1651	2.58303
327	C	5	15	206	19.81	860	0.582	23.9535	2.30349
328	C	6	15	246	35.46	1190	0.530	20.6723	2.97983
329	C	7	15	434	47.00	1920	0.572	22.6042	2.44792
330	C	8	15	.	.	.	.	.	.
331	C	9	15	284	40.37	1160	0.600	24.4828	3.48017
332	C	10	15	340	36.39	1210	0.562	28.0992	3.00744
333	C	11	15	224	10.25	480	0.473	46.6667	2.13542
334	C	12	15	.	21.61	560	0.503	.	3.85893
335	C	13	15	268	25.47	1140	0.542	23.5088	2.23421
336	C	14	15	222	13.95	920	0.546	24.1304	1.51630
337	C	1	18	346	25.77	1180	0.606	29.3220	2.18390
338	C	2	18	288	19.74	1240	0.578	23.2258	1.59194
339	C	3	18	262	33.83	1270	.	20.6299	2.66378
340	C	4	18	240	27.35	770	0.576	31.1688	3.55195
341	C	5	18	272	33.20	1080	0.565	25.1852	3.07407
342	C	6	18	250	33.20	950	0.536	26.3158	3.49474
343	C	7	18	.	.	.	0.562	.	.
344	C	8	18	.	.	.	.	.	.
345	C	9	18	196	44.31	1400	0.578	14.0000	3.16500
346	C	10	18	300	59.04	1660	0.566	18.0723	3.55663
347	C	11	18	354	13.12	920	0.470	38.4783	1.42609
348	C	12	18	166	21.65	700	0.541	23.7143	3.09286
349	C	13	18	242	3.08	750	0.545	32.2667	0.41067
350	C	14	18	300	10.75	830	0.528	36.1446	1.29518
351	C	1	21	356	28.96	1610	0.590	22.1118	1.79876
352	C	2	21	392	11.37	1340	0.574	29.2537	0.84851
353	C	3	21	.	.	.	.	.	.
354	C	4	21	400	51.04	1530	0.564	26.1438	3.33595
355	C	5	21	232	41.11	1010	0.572	22.9703	4.07030
356	C	6	21	286	38.63	1400	0.526	20.4286	2.75929
357	C	7	21	.	32.29	1980	0.584	.	1.63081
358	C	8	21	.	.	.	.	.	.
359	C	9	21	228	33.89	1210	0.585	18.8430	2.80083
360	C	10	21	270	29.49	1660	0.554	16.2651	1.77651
361	C	11	21	270	16.78	770	0.467	35.0649	2.17922
362	C	12	21	178	19.95	950	0.520	18.7368	2.10000
363	C	13	21	372	36.20	1600	0.548	23.2500	2.26250
364	C	14	21	276	20.57	980	0.522	28.1633	2.09898
365	C	1	24	.	31.09	1220	0.586	.	2.54836
366	C	2	24	454	20.58	1060	0.562	42.8302	1.94151
367	C	3	24	.	.	.	.	.	.
368	C	4	24	286	36.60	850	0.570	33.6471	4.30588
369	C	5	24	260	22.73	1140	0.567	22.8070	1.99386
370	C	6	24	320	43.78	1400	0.540	22.8571	3.12714
371	C	7	24	.	.	.	0.552	.	.
372	C	8	24	.	.	.	.	.	.
373	C	9	24	354	25.90	1480	0.552	23.9189	1.75000
374	C	10	24	.	.	.	.	.	.
375	C	11	24	350	22.25	1080	0.450	32.4074	2.06019
376	C	12	24	238	21.23	710	0.516	33.5211	2.99014
377	C	13	24	.	14.73	900	0.538	.	1.63667
378	C	14	24	.	22.02	1110	0.514	.	1.98378

Table 35: Continued.

SAS

OBS	GROUP	ID	VISIT	LYSO	IGA	TOTAL	CORNEA	PERLYSO	PERIGA
379	D	1	0	272	23.73	810	0.590	33.5802	2.92963
380	D	2	0	276	22.57	790	0.591	34.9367	2.85696
381	D	3	0	232	0.97	530	0.553	43.7736	0.18302
382	D	4	0	274	27.11	620	0.570	44.1935	4.37258
383	D	5	0	270	24.79	570	0.542	47.3684	4.34912
384	D	6	0	260	22.46	560	0.595	46.4286	4.01071
385	D	7	0	180	16.90	750	0.565	24.0000	2.25333
386	D	8	0	200	23.95	420	0.575	47.6190	5.70238
387	D	9	0	226	22.32	590	0.536	38.3051	3.78305
388	D	10	0	332	21.11	710	0.543	46.7606	2.97324
389	D	11	0	412	45.51	1730	0.616	23.8150	2.63064
390	D	12	0	332	33.89	1140	0.503	29.1228	2.97281
391	D	13	0	294	1.13	620	0.628	47.4194	0.18226
392	D	14	0	356	40.67	1170	0.518	30.4274	3.47607
393	D	1	3	428	26.02	1010	0.588	42.3762	2.57624
394	D	2	3	380	44.39	1340	0.600	28.3582	3.31269
395	D	3	3	286	1.09	690	0.541	41.4493	0.15797
396	D	4	3	242	37.99	1030	0.550	23.4951	3.68835
397	D	5	3	258	39.41	1210	0.542	21.3223	3.25702
398	D	6	3	210	37.04	940	0.597	22.3404	3.94043
399	D	7	3	192	36.97	1140	0.540	16.8421	3.24298
400	D	8	3	144	25.42	880	0.560	16.3636	2.88864
401	D	9	3	218	24.03	610	0.522	35.7377	3.93934
402	D	10	3	364	41.42	1340	0.565	27.1642	3.09104
403	D	11	3	260	52.72	1340	0.617	19.4030	3.93433
404	D	12	3	336	49.10	1710	0.506	19.6491	2.87135
405	D	13	3	278	0.52	750	0.602	37.0667	0.06933
406	D	14	3	386	44.32	1480	0.520	26.0811	2.99459
407	D	1	6	358	24.65	950	0.595	37.6842	2.59474
408	D	2	6	408	27.19	1260	0.607	32.3810	2.15794
409	D	3	6	184	0.76	770	0.554	23.8961	0.09870
410	D	4	6	314	35.87	1070	0.562	29.3458	3.35234
411	D	5	6	.	.	.	.	.	.
412	D	6	6	186	27.44	810	0.603	22.9630	3.38765
413	D	7	6	304	24.94	950	0.540	32.0000	2.62526
414	D	8	6	314	47.72	1520	.	20.6579	3.13947
415	D	9	6	264	29.28	560	0.530	47.1429	5.22857
416	D	10	6	304	43.65	1140	0.561	26.6667	3.82895
417	D	11	6	300	49.07	1070	0.603	28.0374	4.58598
418	D	12	6	328	47.45	1310	0.497	25.0382	3.62214
419	D	13	6	298	0.97	620	0.607	48.0645	0.15645
420	D	14	6	378	29.85	1210	0.506	31.2397	2.46694
421	D	1	9	424	40.66	2120	0.623	20.0000	1.91792
422	D	2	9	168	29.78	770	0.580	21.8182	3.80260
423	D	3	9	208	0.81	830	0.552	25.0602	0.09759
424	D	4	9	288	32.08	880	0.580	32.7273	3.64545
425	D	5	9	368	29.37	950	0.538	38.7368	3.09158
426	D	6	9	172	28.47	820	0.583	20.9756	3.47195
427	D	7	9	202	41.30	960	0.524	21.0417	4.30208
428	D	8	9	278	48.35	1180	0.548	23.5593	4.09746
429	D	9	9	286	37.51	810	0.540	35.3086	4.63086
430	D	10	9	354	.	1560	0.555	22.6923	.
431	D	11	9	334	.	2050	0.605	16.2927	.
432	D	12	9	292	.	1640	0.494	17.8049	.
433	D	13	9	232	0.45	820	0.600	28.2927	0.05488
434	D	14	9	244	23.32	1080	0.508	22.5926	2.15926

Table 35: Continued.

SAS

OBS	GROUP	ID	VISIT	LYSO	IGA	TOTAL	CORNEA	PERLYSO	PERIGA
435	D	1	12	404	36.40	2150	0.608	18.7907	1.69302
436	D	2	12	274	25.62	1390	0.594	19.7122	1.84317
437	D	3	12	192	0.06	900	0.553	21.3333	0.00667
438	D	4	12	318	30.59	1140	0.550	27.8947	2.68333
439	D	5	12	336	16.27	810	0.532	41.4815	2.00864
440	D	6	12	188	19.75	570	0.580	32.9825	3.46491
441	D	7	12	234	24.21	1140	0.526	20.5263	2.12368
442	D	8	12	284	44.54	1480	0.543	19.1892	3.00946
443	D	9	12	224	37.57	1000	0.522	22.4000	3.75700
444	D	10	12	420	32.64	1340	0.560	31.3433	2.43582
445	D	11	12	338	39.66	1340	0.598	25.2239	2.95970
446	D	12	12	396	44.80	1350	0.500	29.3333	3.31852
447	D	13	12	426	0.62	1300	0.626	32.7692	0.04769
448	D	14	12	464	37.00	1600	0.502	29.0000	2.31250
449	D	1	15	294	21.40	850	0.633	34.5882	2.51765
450	D	2	15	248	25.68	1150	0.594	21.5652	2.23304
451	D	3	15	180	1.78	900	0.554	20.0000	0.19778
452	D	4	15	224	29.20	960	0.580	23.3333	3.04167
453	D	5	15	220	9.38	750	0.561	29.3333	1.25067
454	D	6	15	238	38.06	920	0.592	25.8696	4.13696
455	D	7	15	268	46.12	1360	0.555	19.7059	3.39118
456	D	8	15	248	37.28	1840	0.566	13.4783	2.02609
457	D	9	15	324	44.79	1080	0.536	30.0000	4.14722
458	D	10	15	330	34.68	1160	0.556	28.4483	2.98966
459	D	11	15	316	55.71	1600	0.606	19.7500	3.48187
460	D	12	15	268	46.05	1080	0.506	24.8148	4.26389
461	D	13	15	252	0.69	1060	0.600	23.7736	0.06509
462	D	14	15	272	16.74	1070	0.514	25.4206	1.56449
463	D	1	18	330	32.82	1530	0.622	21.5686	2.14510
464	D	2	18	244	32.32	1110	0.574	21.9820	2.91171
465	D	3	18	208	1.11	950	0.574	21.8947	0.11684
466	D	4	18	258	30.28	870	0.560	29.6552	3.48046
467	D	5	18	314	14.50	900	0.557	34.8889	1.61111
468	D	6	18	156	34.36	900	0.584	17.3333	3.81778
469	D	7	18	154	37.36	1010	0.555	15.2475	3.69901
470	D	8	18	160	17.03	880	0.564	18.1818	1.93523
471	D	9	18	222	46.79	1070	0.536	20.7477	4.37290
472	D	10	18	364	51.82	1610	0.556	22.6087	3.21863
473	D	11	18	336	52.48	1340	0.601	25.0746	3.91642
474	D	12	18	382	59.04	1450	0.510	26.3448	4.07172
475	D	13	18	330	0.52	940	0.608	35.1064	0.05532
476	D	14	18	418	21.26	1330	0.508	31.4286	1.59850
477	D	1	21	372	38.59	1340	0.604	27.7612	2.87985
478	D	2	21	304	32.31	1650	0.560	18.4242	1.95818
479	D	3	21	160	1.74	820	0.550	19.5122	0.21220
480	D	4	21	234	47.32	1140	0.566	20.5263	4.15088
481	D	5	21	330	8.85	820	0.544	40.2439	1.07927
482	D	6	21	204	60.34	1180	0.588	17.2881	5.11356
483	D	7	21	220	30.19	1540	.	14.2857	1.96039
484	D	8	21	184	35.66	1160	0.550	15.8621	3.07414
485	D	9	21	250	31.77	1180	0.534	21.1864	2.69237
486	D	10	21	350	33.01	1930	0.562	18.1347	1.71036
487	D	11	21	258	33.37	1640	0.610	15.7317	2.03476
488	D	12	21	204	23.48	820	0.496	24.8780	2.86341
489	D	13	21	322	1.82	880	0.596	36.5909	0.20682
490	D	14	21	448	38.54	1920	0.524	23.3333	2.00729
491	D	1	24	.	29.26	1270	0.615	.	2.30394
492	D	2	24	424	36.57	1270	0.580	33.3858	2.87953
493	D	3	24	204	0.95	660	0.548	30.9091	0.14394
494	D	4	24	294	36.12	1320	0.559	22.2727	2.73636
495	D	5	24	354	46.17	1310	0.572	27.0229	3.52443
496	D	6	24	.	.	.	.	.	.
497	D	7	24	336	36.32	1400	0.547	24.0000	2.59429
498	D	8	24	270	33.20	1360	0.548	19.8529	2.44118
499	D	9	24	270	30.42	1010	0.524	26.7327	3.01188
500	D	10	24	282	13.07	1340	0.540	21.0448	0.97537
501	D	11	24	168	22.76	620	0.613	27.0968	3.67097
502	D	12	24	310	36.32	1000	0.480	31.0000	3.63200
503	D	13	24	.	0.74	1010	0.598	.	0.07327
504	D	14	24	.	23.19	1110	0.510	.	2.08919

Table 36: Raw Data Collected from Each Subject: II. The data on the distribution of the sets of protein fractions was measured by the Digitizing Pad. Each set of protein fractions is expressed as a percentage of the total area of the electrophoretograph.

SAS

OBS	GROUP	SUBJ	VISIT	SUM	PER1	PER2	PER3	PER4
1	A	1	0	4000	28.1500	17.2250	51.3500	3.2750
2	A	2	0	.	.	.	.	.
3	A	3	0	2373	35.1454	9.4817	50.8639	4.5091
4	A	4	0	.	.	.	.	.
5	A	5	0	1543	37.2003	7.3234	51.1342	4.3422
6	A	6	0	2673	33.6700	12.3831	40.6659	13.2810
7	A	7	0	4413	25.9461	17.6071	45.9325	10.5144
8	A	8	0	1468	28.6104	10.9673	41.6894	18.7330
9	A	9	0	3620	25.6630	11.1326	58.0110	5.1934
10	A	10	0	3170	16.3722	16.5300	58.5489	8.5489
11	A	11	0	4476	20.4870	14.6783	53.3512	11.4835
12	A	12	0	4278	22.4638	3.6933	45.8859	27.9570
13	A	13	0	2242	31.1329	8.1624	56.2890	4.4157
14	A	1	3	3122	33.8245	2.6586	56.3741	7.1429
15	A	2	3	2167	34.6562	11.2137	46.8389	7.2912
16	A	3	3	3828	34.0125	5.6426	55.1202	5.2247
17	A	4	3	.	.	.	.	.
18	A	5	3	4579	23.9790	4.4551	58.3315	13.2343
19	A	6	3	2987	28.6910	15.6679	51.4563	4.1848
20	A	7	3	.	.	.	.	.
21	A	8	3	4127	23.0676	5.5731	60.2375	11.1219
22	A	9	3	3186	27.1500	2.3854	56.5286	13.9360
23	A	10	3	1191	47.9429	10.3275	29.4710	12.2586
24	A	11	3	4384	25.4562	0.0000	58.1661	16.3777
25	A	12	3	3894	22.9841	4.1602	54.6995	18.1561
26	A	13	3	2727	27.4294	0.0000	69.9303	2.6403
27	A	1	6	3564	30.6397	2.8900	55.6397	10.8305
28	A	2	6	2715	34.4015	5.9669	56.5009	3.1308
29	A	3	6	.	.	.	.	.
30	A	4	6	1885	31.9894	12.2546	39.0981	16.6578
31	A	5	6	3423	23.0500	14.4610	47.2393	15.2498
32	A	6	6	.	.	.	.	.
33	A	7	6	3420	34.4152	5.9064	45.7018	13.9766
34	A	8	6	4088	12.5245	26.6879	45.1566	15.6311
35	A	9	6	4874	26.1182	8.2684	51.2310	14.3824
36	A	10	6	.	.	.	.	.
37	A	11	6	1923	23.3489	6.8123	58.7624	11.0764
38	A	12	6	4411	25.5498	8.2748	58.2634	7.9120
39	A	13	6	4194	20.6485	9.3467	60.8011	9.2036
40	A	1	9	4800	28.7083	6.9375	46.1250	18.2292
41	A	2	9	1724	35.6148	8.9907	34.3387	21.0557
42	A	3	9	1911	42.5432	13.8148	31.7635	11.8786
43	A	4	9	3458	32.8514	15.9630	38.7796	12.4060
44	A	5	9	3483	20.0115	13.7238	52.1677	14.0970
45	A	6	9	4167	25.2700	14.0389	34.2453	26.4459
46	A	7	9	3375	19.7333	5.5704	31.5852	43.1111
47	A	8	9	5026	18.9614	17.9268	52.6064	10.5054
48	A	9	9	5445	26.0790	9.8623	50.7805	13.2782
49	A	10	9	3957	20.6217	20.9502	43.4167	15.0114
50	A	11	9	5556	17.2426	15.5868	51.7279	15.4428
51	A	12	9	4084	22.0372	13.0020	49.2654	15.6954
52	A	13	9	4618	23.6899	5.5868	54.1793	16.5440
53	A	1	12	4355	35.3617	9.1160	43.2377	12.2847
54	A	2	12	3412	29.4842	11.2837	45.9848	13.2474
55	A	3	12	1768	42.3643	9.0498	27.9412	20.6448
56	A	4	12	3722	28.1032	11.8485	37.7754	22.2730

Table 36: Continued.

SAS

OBS	GROUP	SUBJ	VISIT	SUM	PER1	PER2	PER3	PER4
57	A	5	12	4421	27.4825	4.8405	61.1400	6.5370
58	A	6	12	3744	18.3226	16.5598	40.4915	24.6261
59	A	7	12	4824	25.4353	7.6078	50.3109	16.6459
60	A	8	12	.	.	.	.	.
61	A	9	12	3562	19.8203	12.1842	47.6137	20.3818
62	A	10	12	3471	28.4932	9.9395	50.4754	11.0919
63	A	11	12	3727	19.3185	14.1401	49.3426	17.1988
64	A	12	12	.	.	.	.	.
65	A	13	12	.	.	.	.	.
66	A	1	15	3484	24.5121	18.6567	50.9759	5.8553
67	A	2	15	.	.	.	.	.
68	A	3	15	3312	21.4372	8.9070	59.9034	9.7524
69	A	4	15	3929	23.8483	17.3836	46.4749	12.2932
70	A	5	15	3371	26.2830	18.4218	42.7766	12.5185
71	A	6	15	4490	27.4610	11.4031	49.2650	11.8708
72	A	7	15	.	.	.	.	.
73	A	8	15	3497	18.1870	13.7261	59.5939	8.4930
74	A	9	15	2990	21.6722	12.9766	54.7826	10.5686
75	A	10	15	3475	23.4245	18.5612	49.2662	8.7482
76	A	11	15	3906	24.3472	15.0026	48.0287	12.6216
77	A	12	15	4803	22.6109	18.2594	48.8653	10.2644
78	A	13	15	2903	28.9700	14.2267	50.0861	6.7172
79	A	1	18	4569	24.9070	16.2180	46.5091	12.3659
80	A	2	18	1512	37.5000	10.3175	24.0741	28.1085
81	A	3	18	2884	30.0971	18.1692	36.9972	14.7365
82	A	4	18	4345	23.3372	19.2405	46.9735	10.4488
83	A	5	18	3932	25.7630	11.9023	49.0844	13.2503
84	A	6	18	4498	20.9649	12.7612	50.2223	16.0516
85	A	7	18	2648	29.6073	18.3157	38.2175	13.8595
86	A	8	18	4335	17.3702	21.8685	57.3702	3.3910
87	A	9	18	4645	24.8009	12.0990	44.1981	18.9020
88	A	10	18	3439	26.1122	13.8994	43.0358	16.9526
89	A	11	18	5688	21.9058	19.6906	47.0464	11.3572
90	A	12	18	4969	25.5182	9.9014	57.4562	7.1242
91	A	13	18	2643	28.7930	7.8698	56.1862	7.1510
92	A	1	21	2348	32.2828	11.1584	42.5894	13.9693
93	A	2	21	4006	30.2047	13.3300	41.3130	15.1523
94	A	3	21	3811	25.6626	13.3823	46.6544	14.3007
95	A	4	21	3934	24.6823	19.1408	40.5186	15.6584
96	A	5	21	2941	29.6158	17.6471	43.9986	8.7385
97	A	6	21	3130	30.9904	19.2652	30.4792	19.2652
98	A	7	21	.	.	.	.	.
99	A	8	21	3420	7.2222	2.5731	16.1696	74.0351
100	A	9	21	2988	28.0790	4.8862	47.0214	20.0134
101	A	10	21	1982	27.3966	10.6963	45.0050	16.9021
102	A	11	21	3257	26.9266	18.1762	45.5941	9.3030
103	A	12	21	5246	24.4949	14.5444	50.7244	10.2364
104	A	13	21	3585	25.3556	11.8550	50.9344	11.8550
105	A	1	24	1451	35.2860	22.3983	33.9076	8.4080
106	A	2	24	2030	34.5320	18.6700	30.0493	16.7488
107	A	3	24	1634	33.4761	18.4823	32.3745	15.6671
108	A	4	24	5476	17.9328	15.2666	54.9671	11.8335
109	A	5	24	2499	26.2105	15.6863	37.4550	20.6483
110	A	6	24	4390	21.4579	10.7289	53.0979	14.7153
111	A	7	24	2986	32.1835	8.1380	48.8948	10.7837
112	A	8	24	3045	18.1281	26.0755	38.6535	17.1429
113	A	9	24	.	.	.	.	.
114	A	10	24	2911	17.5885	12.5386	54.8265	15.0464
115	A	11	24	2911	17.7946	16.7296	51.3569	14.1189
116	A	12	24	3182	35.1037	8.9252	51.0371	4.9340
117	A	13	24	2703	27.0810	11.7277	46.0229	15.1683

Table 36: Continued.

SAS

OBS	GROUP	SUBJ	VISIT	SUM	PER1	PER2	PER3	PER4
118	B	1	0	1917	34.5331	15.5451	44.6531	5.2686
119	B	2	0	1873	33.1020	11.4789	48.4250	6.9941
120	B	3	0	2215	30.4289	8.8036	42.9345	17.8330
121	B	4	0	2650	34.4151	17.0189	42.4906	6.0755
122	B	5	0	1572	25.6361	14.8219	30.2163	29.3257
123	B	6	0	2149	22.3360	15.0302	34.4812	28.1526
124	B	7	0	4531	18.7155	14.4560	51.0042	15.8243
125	B	8	0	3764	26.8332	10.8927	47.3167	14.9575
126	B	9	0	884	31.7873	20.7014	26.1312	21.3801
127	B	10	0	.	.	.	.	.
128	B	11	0	2192	26.2318	9.3522	56.2044	8.2117
129	B	12	0	3439	20.5874	14.9753	40.0116	24.4257
130	B	1	3	1320	34.6970	11.2121	28.1818	25.9091
131	B	2	3	2328	32.6890	1.3746	54.8969	11.0395
132	B	3	3	.	.	.	.	.
133	B	4	3	3671	20.1035	12.8575	48.6244	18.4146
134	B	5	3	4052	26.7522	24.2103	41.2883	7.7493
135	B	6	3	3385	16.7208	12.6440	62.6588	7.9764
136	B	7	3	4341	20.0645	3.3402	68.4174	8.1778
137	B	8	3	3494	34.8025	8.2427	38.2370	18.7178
138	B	9	3	4888	22.3200	4.6645	53.6007	19.4149
139	B	10	3	1724	44.2227	12.9350	27.3202	25.5220
140	B	11	3	2547	29.7212	0.0000	62.1516	8.1272
141	B	12	3	4254	23.4838	7.1932	62.1533	7.1697
142	B	1	6	.	.	.	.	.
143	B	2	6	3873	30.2866	0.0000	53.0855	16.6279
144	B	3	6	.	.	.	.	.
145	B	4	6	5424	22.8245	15.6711	43.6578	17.8466
146	B	5	6	3208	24.4701	16.1160	40.7107	18.7032
147	B	6	6	2394	20.7185	11.1111	57.9365	10.2339
148	B	7	6	.	.	.	.	.
149	B	8	6	4013	27.0870	14.0045	44.2562	14.6524
150	B	9	6	3865	17.5679	16.7141	53.0401	12.6779
151	B	10	6	.	.	.	.	.
152	B	11	6	1932	28.6232	3.5714	59.1097	8.6957
153	B	12	6	5312	24.5670	14.5896	52.1837	8.6596
154	B	1	9	1899	24.9078	6.8984	45.5503	22.6435
155	B	2	9	3519	29.6107	6.0529	53.3959	10.9406
156	B	3	9	.	.	.	.	.
157	B	4	9	5105	24.7209	17.9824	46.4643	10.8325
158	B	5	9	3376	24.3187	23.3116	41.3211	11.0486
159	B	6	9	2993	17.3070	19.3451	52.7230	10.6248
160	B	7	9	5967	21.7027	15.0327	50.7960	12.4686
161	B	8	9	5603	22.5058	16.7946	50.3302	10.3694
162	B	9	9	2296	26.9164	21.9948	31.1411	19.9477
163	B	10	9	.	.	.	.	.
164	B	11	9	4502	22.5677	12.0613	53.3319	12.0391
165	B	12	9	5752	18.6196	8.9534	66.7594	5.6676
166	B	1	12	.	.	.	.	.
167	B	2	12	5042	17.0766	6.6839	58.4292	17.8104
168	B	3	12	5134	26.5875	10.7908	47.5653	15.0565

Table 36. Continued.

SAS

OBS	GROUP	SUBJ	VISIT	SUM	PER1	PER2	PER3	PER4
169	B	4	12	2626	31.6070	10.3960	38.8804	19.1165
170	B	5	12	1977	25.2908	12.6454	34.9519	27.1118
171	B	6	12	1836	18.8453	12.4728	34.8584	33.8235
172	B	7	12	5554	23.5506	14.1340	43.9503	18.3651
173	B	8	12	5220	29.1571	13.8123	50.6322	6.3985
174	B	9	12	.	.	.	.	.
175	B	10	12	.	.	.	.	.
176	B	11	12	.	.	.	.	.
177	B	12	12	4126	23.4610	8.8706	58.0223	9.6461
178	B	1	15	2190	26.9406	13.6073	50.5023	8.9498
179	B	2	15	2302	18.8966	3.7359	65.9427	11.4248
180	B	3	15	4486	22.7820	16.2060	46.2996	14.7124
181	B	4	15	3921	23.8970	8.6458	48.3550	19.1023
182	B	5	15	2617	31.2572	19.9083	35.7661	13.0684
183	B	6	15	.	.	.	.	.
184	B	7	15	4170	17.7458	14.7962	59.1607	8.2974
185	B	8	15	1444	23.4072	13.7119	42.1745	20.7064
186	B	9	15	4412	15.5938	28.7398	41.8631	13.8033
187	B	10	15	3745	26.8625	19.7864	35.8879	17.4633
188	B	11	15	.	.	.	.	.
189	B	12	15	2990	21.4047	21.1371	43.5117	13.9465
190	B	1	18	2481	28.1741	7.5373	54.0911	10.1975
191	B	2	18	4181	22.9371	13.7527	47.1418	16.1684
192	B	3	18	2473	29.6401	13.9507	42.9034	13.5059
193	B	4	18	4332	20.4524	14.1736	51.3389	14.0351
194	B	5	18	2371	28.1316	14.8039	33.5302	23.5344
195	B	6	18	2541	25.8560	7.0051	43.0146	24.1244
196	B	7	18	.	.	.	.	.
197	B	8	18	4131	31.7115	13.6771	39.9661	14.6454
198	B	9	18	2313	29.9611	23.8651	41.9801	4.1937
199	B	10	18	2057	29.7521	20.0292	40.2528	9.9660
200	B	11	18	4172	23.0105	21.5244	40.0767	15.3883
201	B	12	18	4132	23.7173	18.1510	50.7018	7.4298
202	B	1	21	1128	27.8369	17.7305	34.0426	20.3901
203	B	2	21	3197	28.1827	11.2293	51.2043	9.3838
204	B	3	21	5361	19.3621	12.0873	33.3706	35.1800
205	B	4	21	3238	20.8771	12.3533	49.4750	17.2946
206	B	5	21	4644	16.6236	14.5564	42.6357	26.1843
207	B	6	21	.	.	.	.	.
208	B	7	21	5123	22.2331	16.2795	42.2799	19.2075
209	B	8	21	4221	31.8408	13.6224	36.8870	17.6498
210	B	9	21	.	.	.	.	.
211	B	10	21	2663	26.2861	24.8967	39.2039	9.6132
212	B	11	21	3697	19.2318	14.2007	56.0725	10.4950
213	B	12	21	3641	23.4276	18.8959	36.4460	21.2304
214	B	1	24	1362	29.0749	4.7724	40.6021	25.5507
215	B	2	24	2585	26.5764	16.4410	39.2263	17.7563
216	B	3	24	4088	24.5841	13.6742	44.1781	17.5636
217	B	4	24	.	.	.	.	.
218	B	5	24	3459	24.7181	16.7100	50.1012	8.4707
219	B	6	24	2588	24.0726	11.8238	50.3091	13.7944
220	B	7	24	4568	25.1970	16.4405	52.3862	5.9764
221	B	8	24	2945	28.1154	19.0153	38.6757	14.1935
222	B	9	24	.	.	.	.	.
223	B	10	24	.	.	.	.	.
224	B	11	24	3192	20.1128	25.0000	40.9461	13.9411
225	B	12	24	.	.	.	.	.

Table 36. Continued.

SAS

OBS	GROUP	SUBJ	VISIT	SUM	PER1	PER2	PER3	PER4
226	C	1	0	3634	28.3159	10.4018	55.1734	6.1090
227	C	2	0	3648	24.0406	13.8432	57.1546	4.9616
228	C	3	0	3141	31.5823	5.9535	53.0723	9.3919
229	C	4	0	1564	19.0537	13.8107	41.5601	25.5754
230	C	5	0	4661	20.0386	18.4295	48.2085	13.3233
231	C	6	0	3562	20.2695	9.9663	33.9697	35.7945
232	C	7	0	5738	19.9373	13.2450	55.9254	10.8923
233	C	8	0	3188	25.6901	16.2798	49.3099	8.7202
234	C	9	0	4224	23.6979	13.3523	50.9706	11.9792
235	C	10	0	2833	21.8143	9.4246	45.9230	22.8380
236	C	11	0	2742	31.4004	4.6681	60.7586	3.1729
237	C	12	0	3236	25.4326	18.4487	47.4969	8.6218
238	C	13	0	2125	28.1882	12.7059	54.1647	4.9412
239	C	1	3	1526	29.4889	4.8493	52.8178	12.8440
240	C	2	3	3108	31.0489	4.9228	55.0837	8.9447
241	C	3	3	3161	36.8554	2.6574	45.5236	14.9636
242	C	4	3	5172	23.5692	7.9466	46.2104	22.2738
243	C	5	3	5410	29.3900	7.9298	50.5360	12.1442
244	C	6	3	3065	22.1860	5.2202	51.6150	20.9788
245	C	7	3	2838	26.4271	3.5588	56.4834	13.5307
246	C	8	3	1639	37.5229	7.6876	48.0171	6.7724
247	C	9	3	4144	22.1766	5.9846	59.7490	12.0898
248	C	10	3	3776	23.2786	4.5816	63.1621	8.9778
249	C	11	3	2516	36.8839	5.0079	53.8553	4.2528
250	C	12	3	1895	33.7731	16.8865	42.6913	6.6491
251	C	13	3	4858	26.2865	2.5731	55.2902	15.8501
252	C	1	6	2934	30.6408	5.8964	49.8637	13.5992
253	C	2	6	3531	29.5667	6.9952	51.7984	11.6398
254	C	3	6	.	.	.	.	.
255	C	4	6	3883	26.9379	9.9665	52.6655	10.4301
256	C	5	6	3727	25.1140	11.5374	48.0011	15.3475
257	C	6	6	4129	30.7338	13.2235	48.4863	7.5563
258	C	7	6	5596	16.7262	18.6383	51.7691	12.8663
259	C	8	6	2981	24.9916	13.9886	43.8108	17.2090
260	C	9	6	5449	23.4171	18.0033	50.6515	7.9281
261	C	10	6	.	.	.	.	.
262	C	11	6	2296	24.5209	9.9739	61.3240	4.1812
263	C	12	6	1077	23.6769	7.4280	55.7103	13.1848
264	C	13	6	3488	25.0860	11.7546	55.3326	7.8268
265	C	1	9	6303	22.6083	8.8529	49.9921	18.5467
266	C	2	9	4875	22.8718	11.8359	53.8256	11.4667
267	C	3	9	3860	26.9948	13.7047	49.1451	10.1554
268	C	4	9	3666	23.4861	9.9018	58.1560	8.4561
269	C	5	9	5273	27.4227	12.4597	52.5887	7.5289
270	C	6	9	1274	34.9294	17.8964	19.0738	28.1005
271	C	7	9	.	.	.	.	.
272	C	8	9	4518	25.4980	0.0000	53.3422	21.1598
273	C	9	9	3652	24.3702	10.7886	49.8083	15.0329
274	C	10	9	5347	24.7241	15.2422	45.8575	14.1762
275	C	11	9	3819	28.1749	11.5475	51.5842	8.6934
276	C	12	9	3240	22.5000	18.2407	40.7099	18.5494
277	C	13	9	1879	38.6908	8.3023	43.8531	9.1538
278	C	1	12	6319	19.8133	9.9541	56.7495	13.4831
279	C	2	12	5471	19.9598	7.1651	58.0333	14.8419
280	C	3	12	4227	12.5384	0.0000	70.8067	16.6548

Table 36. Continued.

SAS

OBS	GROUP	SUBJ	VISIT	SUM	PER1	PER2	PER3	PER4
281	C	4	12	.	.	.	.	.
282	C	5	12	4681	28.1777	6.5157	51.4206	13.8859
283	C	6	12	.	.	.	.	.
284	C	7	12	4613	14.0906	11.7060	61.6085	12.5948
285	C	8	12	4673	14.6373	24.0317	40.7661	20.5649
286	C	9	12	5409	16.9717	22.1298	38.5099	22.3886
287	C	10	12	4360	26.4908	11.1697	47.6606	14.6789
288	C	11	12	2128	38.3929	4.5113	50.9398	6.1560
289	C	12	12	2965	22.7993	15.9528	43.4064	17.8415
290	C	13	12	5271	20.6982	5.1034	62.6826	11.5158
291	C	1	15	3729	18.7182	5.7120	68.9997	6.5701
292	C	2	15	3660	20.7923	15.2732	54.3716	9.5628
293	C	3	15	2720	28.9338	15.9191	42.4632	12.6838
294	C	4	15	4519	21.9739	16.7294	47.2892	14.0075
295	C	5	15	3279	26.7155	19.7926	39.9207	13.5712
296	C	6	15	3362	23.2302	23.4384	42.4152	10.9161
297	C	7	15	5458	19.7325	20.5203	49.4687	10.2785
298	C	8	15	.	.	.	.	.
299	C	9	15	3272	22.2800	18.0012	49.2665	10.4523
300	C	10	15	2777	30.0324	13.6118	48.8297	7.5261
301	C	11	15	1361	37.6929	8.5231	45.7752	8.0088
302	C	12	15	1135	33.3040	15.2423	36.5639	14.8899
303	C	13	15	4170	25.3717	10.9353	53.3333	10.3597
304	C	1	18	5252	23.6101	16.8126	53.3130	6.2643
305	C	2	18	4708	23.4282	17.9482	56.3934	2.2302
306	C	3	18	.	.	.	.	.
307	C	4	18	3508	26.9669	16.5906	47.7765	8.6659
308	C	5	18	4435	27.8241	12.4014	44.0586	15.7159
309	C	6	18	2555	21.0176	26.5753	38.4344	13.9726
310	C	7	18	.	.	.	.	.
311	C	8	18	.	.	.	.	.
312	C	9	18	3677	19.8259	20.1795	48.4362	11.5583
313	C	10	18	5758	20.4411	23.2199	45.8666	10.4724
314	C	11	18	.	.	.	.	.
315	C	12	18	3873	19.6230	5.6545	53.1113	21.6112
316	C	13	18	2092	32.1702	10.9943	48.9006	7.9350
317	C	1	21	4772	26.4250	16.1358	47.2967	10.1425
318	C	2	21	2847	28.2051	12.9961	50.4742	8.3246
319	C	3	21	.	.	.	.	.
320	C	4	21	3593	25.9672	12.1069	54.5505	7.3755
321	C	5	21	4061	26.1758	17.6065	44.0039	12.2137
322	C	6	21	2814	32.8003	15.3518	34.2573	17.5906
323	C	7	21	4181	22.9610	11.6479	50.8252	14.5659
324	C	8	21	.	.	.	.	.
325	C	9	21	2659	33.7721	8.3114	40.6168	17.2997
326	C	10	21	3090	25.3074	12.9450	54.7896	6.9579
327	C	11	21	2693	31.4519	15.4103	41.5893	11.5485
328	C	12	21	3016	28.5477	18.2361	40.4509	12.7653
329	C	13	21	4233	27.2384	12.0718	53.1774	7.5124
330	C	1	24	1879	22.4055	18.0415	46.5141	13.0389
331	C	2	24	2889	15.9225	17.9647	57.0093	9.1035
332	C	3	24	.	.	.	.	.
333	C	4	24	2144	22.5746	10.7743	52.3787	14.2724
334	C	5	24	3892	25.6166	19.9126	44.5529	9.9178
335	C	6	24	5484	25.0365	21.0430	41.3931	12.5274
336	C	7	24	5343	18.9032	23.1144	46.5469	11.4355
337	C	8	24	.	.	.	.	.
338	C	9	24	4666	24.8607	16.2023	49.2070	9.7300
339	C	10	24	.	.	.	.	.
340	C	11	24	1722	16.3763	10.2207	55.5168	17.8862
341	C	12	24	2295	29.9346	19.0414	39.0850	11.9390
342	C	13	24	2029	40.6604	10.3499	34.6969	14.2928

Table 36: Continued.

SAS

OBS	GROUP	SUBJ	VISIT	SUM	PER1	PER2	PER3	PER4
343	D	1	0	.	.	.	.	.
344	D	2	0	4942	32.8410	8.5998	52.3068	6.2525
345	D	3	0	2780	26.6187	13.3094	52.2302	7.8417
346	D	4	0	3469	22.1101	15.2205	46.7282	15.9412
347	D	5	0	4277	22.7496	14.1454	45.2888	17.8162
348	D	6	0	2456	26.5472	13.9251	47.1091	12.4186
349	D	7	0	2578	31.8852	14.5849	40.5353	12.9946
350	D	8	0	3069	30.6289	11.2414	47.5073	10.6224
351	D	9	0	2247	25.5007	13.7517	41.8781	18.8696
352	D	10	0	2972	26.1440	14.5020	54.8452	4.5087
353	D	11	0	5005	22.3377	15.8641	51.1688	10.6294
354	D	12	0	3614	32.6785	17.9303	44.3830	5.0083
355	D	13	0	4674	18.1001	14.2276	52.6102	15.0620
356	D	14	0	2591	28.7148	12.3504	57.1980	1.7368
357	D	1	3	.	.	.	.	.
358	D	2	3	4238	31.1468	3.0911	45.5168	20.2454
359	D	3	3	2718	32.4503	6.7329	50.7726	10.0442
360	D	4	3	2130	30.3286	17.7465	42.3474	9.5775
361	D	5	3	5746	26.6968	8.5799	60.3028	4.4205
362	D	6	3	4250	25.4118	11.5529	57.0118	6.0235
363	D	7	3	4656	19.7809	5.3265	59.4502	15.4424
364	D	8	3	3629	33.1496	0.0000	53.1000	13.7503
365	D	9	3	.	.	.	.	.
366	D	10	3	4949	25.5809	3.3340	50.4344	20.6506
367	D	11	3	5669	20.4269	3.3692	57.8056	18.3983
368	D	12	3	.	.	.	.	.
369	D	13	3	5228	18.5348	0.0000	60.2525	21.2127
370	D	14	3	1899	30.4371	0.0000	54.6077	14.9552
371	D	1	6	.	.	.	.	.
372	D	2	6	4628	27.7874	10.6525	39.8012	21.7589
373	D	3	6	.	.	.	.	.
374	D	4	6	3204	22.7840	16.1673	46.2547	14.7940
375	D	5	6	4653	24.7152	9.5852	49.0866	16.6129
376	D	6	6	3886	20.1750	23.2887	46.1143	10.4220
377	D	7	6	3278	26.2965	15.8633	43.2276	14.6126
378	D	8	6	.	.	.	.	.
379	D	9	6	3386	21.5889	16.4796	47.2534	14.6781
380	D	10	6	.	.	.	.	.
381	D	11	6	4226	18.9778	8.7080	57.3592	14.9550
382	D	12	6	5284	22.1991	16.6730	50.5110	10.6170
383	D	13	6	4412	16.7498	9.7235	59.6782	13.8486
384	D	14	6	3064	28.3616	8.1919	59.1384	4.3081
385	D	1	9	.	.	.	.	.
386	D	2	9	5550	26.1081	1.7477	61.1892	10.9550
387	D	3	9	2762	23.5337	11.7306	57.7842	6.9515
388	D	4	9	4498	23.8328	13.2281	49.3108	13.6283
389	D	5	9	3620	31.7956	8.9779	49.7514	9.4751
390	D	6	9	4711	19.3802	25.5784	37.4231	17.6183
391	D	7	9	.	.	.	.	.
392	D	8	9	6035	20.7788	13.5874	48.3347	17.2991

Table 36: Continued.

SAS

OBS	GROUP	SUBJ	VISIT	SUM	PER1	PER2	PER3	PER4
393	D	9	9	3560	18.3989	19.6910	46.1236	15.7865
394	D	10	9	6238	20.8080	17.2972	47.4832	14.4117
395	D	11	9	6458	16.1660	13.3168	56.9836	13.5336
396	D	12	9	4080	21.9608	17.2059	44.9510	15.8824
397	D	13	9	6218	15.6964	7.3014	54.9694	22.0328
398	D	14	9	2070	22.9469	9.5169	62.2222	5.3140
399	D	1	12	.	.	.	.	.
400	D	2	12	3528	25.0850	8.7585	44.8696	21.2868
401	D	3	12	3260	20.4601	6.7485	62.2393	10.5521
402	D	4	12	.	.	.	.	.
403	D	5	12	3188	28.7327	4.7365	56.4931	10.0376
404	D	6	12	3585	27.4198	9.7908	49.4003	13.3891
405	D	7	12	4809	24.9532	9.9605	49.7817	15.3046
406	D	8	12	2499	29.0516	12.0848	39.0556	19.8079
407	D	9	12	3863	18.1465	15.9979	46.9324	18.9231
408	D	10	12	5183	22.1107	10.9782	56.9168	9.9942
409	D	11	12	4864	16.8791	12.9523	56.6817	13.4868
410	D	12	12	5869	11.4841	19.9864	48.5432	19.9864
411	D	13	12	5210	18.9060	3.8580	59.8464	17.3896
412	D	14	12	3412	23.6518	5.6272	68.4349	2.2860
413	D	1	15	.	.	.	.	.
414	D	2	15	3851	20.6440	20.1766	49.8831	9.2963
415	D	3	15	4541	21.1627	15.8555	54.4594	8.5224
416	D	4	15	2582	27.1882	12.0062	47.6762	13.1294
417	D	5	15	.	.	.	.	.
418	D	6	15	2808	24.2877	20.5128	39.4587	15.7407
419	D	7	15	3157	22.1096	22.6164	42.3503	12.9237
420	D	8	15	3726	25.7112	14.6001	45.0081	14.6806
421	D	9	15	.	.	.	.	.
422	D	10	15	4983	17.4192	13.3253	60.5057	8.7497
423	D	11	15	3420	24.8538	9.3275	51.3743	14.4444
424	D	12	15	3685	24.8304	8.6567	55.0882	11.4247
425	D	13	15	4019	22.5180	4.6280	60.3135	12.5404
426	D	14	15	5358	23.6282	14.2217	50.3733	11.7768
427	D	1	18	.	.	.	.	.
428	D	2	18	4945	22.1234	17.3104	45.5207	15.0455
429	D	3	18	3622	26.4771	18.8570	49.4478	5.2181
430	D	4	18	3606	24.4870	8.9018	56.6833	9.9279
431	D	5	18	.	.	.	.	.
432	D	6	18	1136	31.4261	17.2535	23.5915	27.7289
433	D	7	18	4161	30.1851	17.5679	42.1053	10.1418
434	D	8	18	.	.	.	.	.
435	D	9	18	.	.	.	.	.
436	D	10	18	4682	24.0282	17.6634	48.0350	10.2734
437	D	11	18	4603	20.0521	14.8599	55.4856	9.6024
438	D	12	18	3376	27.9917	22.3341	32.4052	17.2690
439	D	13	18	.	.	.	.	.
440	D	14	18	3307	25.4611	11.0674	51.9504	11.5210
441	D	1	21	.	.	.	.	.
442	D	2	21	5292	23.6206	18.5752	42.7249	15.0794
443	D	3	21	3243	28.2763	13.0743	48.9670	9.6824
444	D	4	21	2278	37.1378	10.5795	42.0105	10.2722
445	D	5	21	2321	28.2637	10.5989	40.6290	20.5084
446	D	6	21	.	.	.	.	.
447	D	7	21	2042	37.0225	14.4466	28.9422	19.5886
448	D	8	21	3812	32.0042	12.4607	38.3526	17.1826
449	D	9	21	2917	20.2263	17.6551	47.9945	14.1241
450	D	10	21	5835	24.9529	16.1097	47.3350	11.6024
451	D	11	21	3378	24.1859	12.4926	50.6809	12.6406
452	D	12	21	2101	34.2218	11.4707	43.3603	10.9472
453	D	13	21	2747	20.2403	9.2829	55.4423	15.0346
454	D	14	21	2586	31.1292	12.3743	45.5916	10.9049
455	D	1	24	.	.	.	.	.
456	D	2	24	3740	25.9893	13.0481	49.8930	11.0695
457	D	3	24	1638	28.1441	14.1636	49.4505	8.2418
458	D	4	24	4948	20.1698	16.8957	49.3129	13.6217
459	D	5	24	4790	20.0835	13.0480	53.7161	13.1524
460	D	6	24	.	.	.	.	.
461	D	7	24	3845	24.7594	23.7191	42.1586	9.3628
462	D	8	24	3318	31.7963	13.6528	43.1887	11.3623
463	D	9	24	3247	22.2051	20.1109	47.3052	10.3788
464	D	10	24	3115	14.0289	14.5104	58.5233	12.9374
465	D	11	24	1696	19.8703	13.9741	51.5920	14.5637
466	D	12	24	3931	27.0415	19.1554	40.6767	13.1264
467	D	13	24	5184	21.7593	16.1265	50.4437	11.6705
468	D	14	24	3540	29.8870	14.1525	50.0282	5.9322

Appendix G

POSTER AND CLINICAL EVALUATION FORMS

THE UNIVERSITY
OF ASTON
IN BIRMINGHAM

DEPARTMENT OF OPHTHALMIC OPTICS

Soft Lens Research
VOLUNTEERS REQUIRED

Volunteer subjects are required for a soft contact lens study starting October 15th 1979.

The purpose of this study is to investigate the efficacy of commercially available cleaners prescribed for the care and maintenance of these lenses.

Interested persons should be spectacle wearers. Volunteers will be fitted with lenses at a reduced fee. Half of this fee will be refunded if all visits necessary for data collection are kept.

Contact lens solutions will be provided at no cost.

Interested persons should contact:

Murchison Callender

Soft Lens Research
Room 188
Main Building
Tel: 021 359 5355/Direct
021 359 3611/Ext. 517

Appendix G.1

THE UNIVERSITY OF ASTON IN BIRMINGHAM

SOFT LENS RESEARCH

CLINICAL EVALUATION OF
SOFT CONTACT LENSES

AGE: _____

CONFIDENTIAL (To be completed by patient)

Mr.
Miss
Mrs.

Official
Patient
No: _____

Surname _____ Other names _____

Home Address _____ Term Address _____

_____ _____

_____ _____

Tel: _____ Tel: _____

Occupation: _____

Name of regular Optician (if any) Family Doctor

_____ _____

Address _____ Address _____

_____ _____

_____

Visual "correction"
Wearing at present: Spectacles ☐ Contact Lenses ☐ Both ☐ None ☐

_____

If spectacles worn:- Age when spectacles first prescribed _____

Full time ☐ Part time ☐ All distances ☐ Long distance ☐ Close work ☐

_____

If contact lenses worn:- Every day ☐ Intermittantly ☐ Infrequently ☐ Discontinued ☐

_____

When was your last eye examination?

_____

Have you any complaints on the performance of your present lenses?

_____

Family History: Are there any instances of eye diseases* in a

Grandparent ☐
Parent ☐
Brother/sister ☐
None known ☐

e.g. *Glaucoma ☐
Retinal Detachment ☐
Cataracts ☐
Blindness or very poor sight of unknown origin. ☐

Appendix G.2

Personal History

General Health:

- Good ☐
- Indifferent ☐
- Poor ☐

Any past:

- Eye disease ☐
- Eye injury ☐
- Neither ☐

Any eye treatment other than glasses

- Yes ☐
- No ☐

Do you suffer from any of the following conditions:-

- Frequent colds ☐
- Catarrh ☐
- Sinus trouble ☐
- Hay fever ☐
- Asthma ☐
- Food allergies ☐
- Drug allergies ☐
- Boils, abscesses ☐
- Pimples, Acne ☐
- Lip cold sores ☐
- Headaches/Migraine ☐
- Dandruff ☐
- Red eyes ☐
- Red eyelids ☐
- Scaly eyelashes ☐
- Styes ☐
- Sore or gritty eyes ☐
- Itching eyes ☐
- Watering eye(s) ☐
- Sticky eyes ☐
- Discharging eyes ☐
- Intolerance to light ☐
- Double vision ☐
- Intermittent "steamy" vision ☐

Are you at present taking any regular pills, tablets or medicines prescribed by your doctor?
(please state what, if known)

- Yes ☐
- No ☐

Other relevant comments:

Appendix G.2

UNIVERSITY OF ASTON IN BIRMINGHAM

DEPARTMENT OF OPHTHALMIC OPTICS

SOFT LENS RESEARCH

DECLARATION to be signed by experimental patients on initial registration.

I have read the noted for guidance and information of patients attending the Soft Lens Research Clinic, and have received and read a written description of the experiment in which I am to take part.

I hereby agree to act as a volunteer experimental subject.

Signed. . . . . .

Date. . . . . .

NAME
(Block Capitals) . . . . . .

ADDRESS . . . . . .

. . . . . .

. . . . . .

. . . . . .

Appendix G.3

DEPARTMENT OF OPHTHALMIC OPTICS

SOFT LENS RESEARCH

COLLECTION OF TEAR FLUID SAMPLES

The purpose of this experiment is to collect samples of tear fluid for subsequent analysis. The tears are collected by means of a sterile glass capillary, one end of which is placed immediately above your lower lid. It will aid the experimenter and lessen the risk of accidents if you keep your eye as still as possible during this procedure.

J R Larke

DEPARTMENT OF OPHTHALMIC OPTICS

SOFT LENS RESEARCH

PATIENT FITTING AND 'BASELINE' DATA

Prior to the fitting of soft contact lenses various measurements will be made of your eyes. Generally this will consist of the use of various optical instruments, although a sample of tear fluid will also be collected, and a nylon thread will touch upon your eye in order to measure corneal sensibility.

You will be asked to fill in a number of questionnaires and photographs will be taken of your eye.

The fitting of soft contact lenses will involve the use of a number of lenses, and you may not find this a particularly pleasant process. Please remember that if you want a lens removed at any time this will be done immediately.

J R Larke

THE UNIVERSITY OF ASTON IN BIRMINGHAM
THE DEPARTMENT OF OPHTHALMIC OPTICS

CONTACT LENS PROGRESS CHECK #___ HARD____ SOFT____ CARE SYSTEM _____

PATIENT_____ Date_____ Time worn today_____ hrs.
Max. wearing time ____ hrs.

HISTORY

SUMMARY OF VISIT

Complaints:

Relevant Findings:

Diagnosis:

Treatment:

MODIFICATIONS

	O.D.	O.S.
B.C.		
S.C.R/W.		
P.C.R/W.		
O.Z.D.		
DIA.		
POWER		
BLEND		

ADDITIONAL INFORMATION:

PLAN FOR NEXT VISIT:

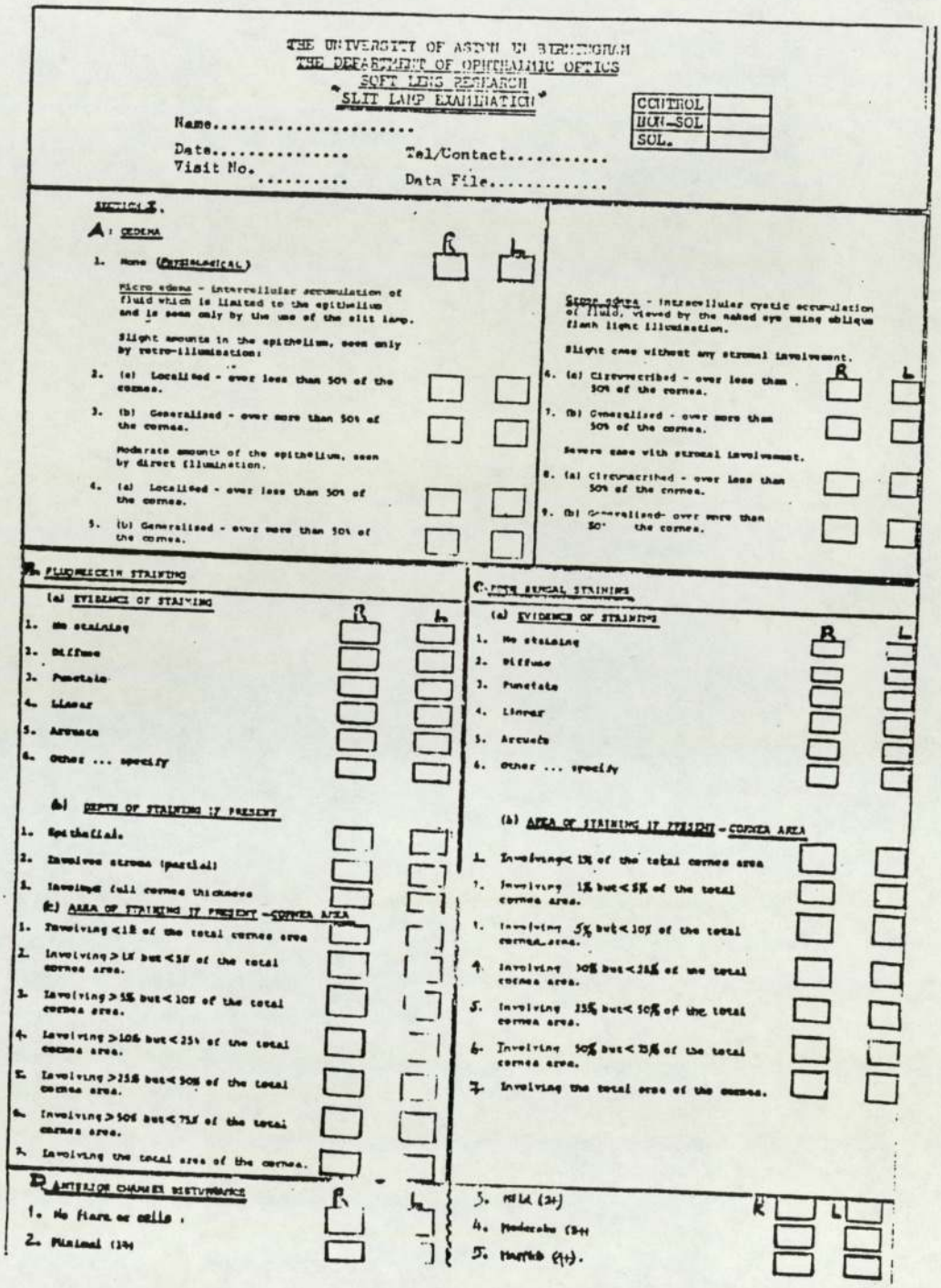
	RIGHT EYE	LEFT EYE
With Lenses On		
Visual Acuity	20/	20/
Retinoscopy	20/	20/
Subjective (best sphere)	20/	20/
Fluorescein Pattern (H.C.L.)		
Keratometry & mire appearance (S.C.L.)		
	Lag / Position	Lag / Position
Sup. gaze_____ St. ahead_____	◯	_____ _____ ◯
Biomicroscopy	◯	◯
With Lenses Off		
Biomicroscopy	◯	◯
Pachometry	mm	mm
Keratometry		
Retinoscopy	20/	20/
Subjective (sphero-cyl)	20/	20/
Verification of lenses (B.C./Dia./O.Z./Power)		
Inspection for nicks, spots, cloudy patches, mucus deposits		
Photography of unusual biomicroscope findings		

Student Clinician

Supervising Clinician

Optometry June78 1854A-1

Appendix G.5



THE UNIVERSITY OF ASTON IN BIRMINGHAM
THE DEPARTMENT OF OPHTHALMIC OPTICS
SOFT LENS RESEARCH
"SLIT LAMP EXAMINATION"

CONTROL	
NON-SOL	
SOL.	

Name.....
Date..... Tel/Contact.....
Visit No. ..... Data File.....

SECTION E.

A: OEDEMA

1. None (PHYSIOLOGICAL)

Micro edema - intercellular accumulation of fluid which is limited to the epithelium and is seen only by the use of the slit lamp.

Slight amounts in the epithelium, seen only by retro-illumination:

2. (a) Localised - over less than 50% of the cornea.
3. (b) Generalised - over more than 50% of the cornea.

Moderate amounts of the epithelium, seen by direct illumination.

4. (a) Localised - over less than 50% of the cornea.
5. (b) Generalised - over more than 50% of the cornea.

Gross edema - intracellular cystic accumulation of fluid, viewed by the naked eye using oblique flash light illumination.

Slight case without any stromal involvement.

6. (a) Circumscribed - over less than 50% of the cornea.
7. (b) Generalised - over more than 50% of the cornea.

Severe case with stromal involvement.

8. (a) Circumscribed - over less than 50% of the cornea.
9. (b) Generalised- over more than 50% of the cornea.

B. FLUORESCEIN STAINING

(a) EVIDENCE OF STAINING

1. No staining
2. Diffuse
3. Punctate
4. Linear
5. Arcuate
6. Other ... specify

(b) DEPTH OF STAINING IF PRESENT

1. Epithelial.
2. Involves stroma (partial)
3. Involves full cornea thickness

(c) AREA OF STAINING IF PRESENT - CORNEA AREA

1. Involving <1% of the total cornea area
2. Involving > 1% but < 5% of the total cornea area.
3. Involving > 5% but < 10% of the total cornea area.
4. Involving > 10% but < 25% of the total cornea area.
5. Involving > 25% but < 50% of the total cornea area.
6. Involving > 50% but < 75% of the total cornea area.
7. Involving the total area of the cornea.

C. ROSE BENGAL STAINING

(a) EVIDENCE OF STAINING

1. No staining
2. Diffuse
3. Punctate
4. Linear
5. Arcuate
6. Other ... specify

(b) AREA OF STAINING IF PRESENT - CORNEA AREA

1. Involving< 1% of the total cornea area
2. Involving 1% but< 5% of the total cornea area.
3. Involving 5% but< 10% of the total cornea area.
4. Involving 10% but< 25% of the total cornea area.
5. Involving 25% but< 50% of the total cornea area.
6. Involving 50% but< 75% of the total cornea area.
7. Involving the total area of the cornea.

D. ANTERIOR CHAMBER DISTURBANCE

1. No flare or cells.
2. Minimal (1+)
3. Mild (2+)
4. Moderate (3+)
5. Marked (4+).

Appendix G.6

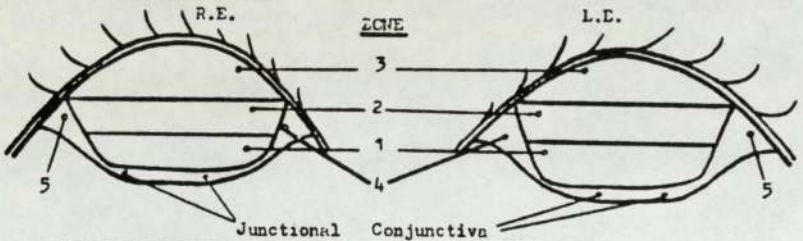
THE UNIVERSITY OF ASTON IN BIRMINGHAM
THE DEPARTMENT OF OPHTHALMIC OPTICS
Soft Lens Research

Examination of the Tarsal Conjunctiva of the upper eyelids.

CONTROL	
NON-SOL	
SOL	

Patient.....
Tel/Contact.....
Visit No..... DATE...../...../.....
Data File.....

TARSAL CONJUNCTIVA



Classification of upper tarsal conjunctival types	R	L
.A. SMOOTH appearance(where the conjunctiva is devoid of papillae and had a smooth surface).		
.B. UNIFORM PAPILLARY appearance(in which small(4 to 8 per mm.) microscopically elevated papillae were detected over zones 1,2 and 3.)		
.C. NON-UNIFORM papillary appearance(in which some of the papillae were 0.4 to 0.8mm. in diameter resulting in non-uniform appearance		
.D. GIANT PAPILLARY appearance(in which papillae 1mm. in diameter or greater were present in zones 1,2 or 3).		
.E. Severity of GIANT PAPILLAE		
.a. 0		
.b. 1		
.c. 2		
.d. 3		
.e. 4		

.F. Description of zones 4 and 5.....

.G. Other Comments

Appendix G.7

THE UNIVERSITY OF ASTON IN BIRMINGHAM
THE DEPARTMENT OF OPHTHALMIC OPTICS

SOFT LENS RESEARCH

Instructions for Group A

1. Hygiene. Before putting on or taking off your lenses, always wash your hands. Rinse them thoroughly and dry them with a lint-free towel. Before handling your lenses avoid oily substances such as hand creams, lotions, or cosmetics.

2. Putting on Lenses. Remove right lens from storage container, place it in the palm of the left hand, and rinse with freshly made saline solution before putting on the lens. Check to make sure your lens is not inside out. Repeat for left lens. Discard solution in the storage container so that fresh solution may be used in the evening.

3. Cleaning Lenses. When removing lenses in the evening, remove right lens, place in palm of left hand and place a few drops of saline on the lens. Rub lens thoroughly with index finger of right hand, being careful not to contact the lens with your fingernail. Do one side of the lens and then the other. Rinse lens with saline and place lens in case filled with fresh saline. Repeat for left lens.

   * Use the Bausch and Lomb protein remover tablets twice per week in addition to daily cleaning with saline.

4. Heat Disinfection of Lenses. Place the lens case in the recessed compartment of the Bausch and Lomb heat unit, close the cover and press the button to start the disinfecting cycle.

5. Eye Drops. Eye drops or other eye medications or solutions intended for hard contact lenses must NOT be used by wearers of soft lenses. If used, medicants or preservatives will be absorbed by your lenses and serious damage to the eyes could result.

6. Wearing Schedule. You may wear your lenses up to eight hours per day during the first week and in most cases full time wear will be reached by the end of the second week. Once full time wear is achieved lenses may be worn during all waking hours, but must NOT be worn during sleep.

7. Sports Wear. Soft lenses may be worn for sporting and athletic activities and are superior to hard contact lenses for such activities as they are not easily dislodged from the eyes.

8. Swimming. Soft lenses should not be worn for swimming or other water sports unless a mask is worn. Otherwise the lenses may become contaminated with microorganisms if worn in fresh water or in salt water, or if worn in chlorinated water, chlorine may be absorbed by the lenses and can result in severe eye irritation.

9. Water. Fresh water or tap water will lower the salt content of soft lenses and will cause them to adhere tightly to the tissues of the eyes. This can cause damage to the eye if removal of lenses is attempted before the tears have a chance to bring the salt content back to normal.

10. Eye Make-up and Hair Sprays. If eye make-up or hair sprays come into contact with your soft lenses they can cause severe eye irritation or can permanently destroy the lenses.

11. Blurring of Vision. If vision blurs while wearing your lenses this is usually due to drying of the lenses and should clear up after blinking several times while moving the eyes back and forth. Check to make sure your lenses are in the proper eyes.

12. Pain, Discomfort, Redness. If these symptoms occur, remove lenses, clean with saline, and rinse with saline, and put them back on. If symptoms persist, remove lenses and telephone the clinic for an appointment.

Appendix G.7A

THE UNIVERSITY OF ASTON IN BIRMINGHAM
THE DEPARTMENT OF OPHTHALMIC OPTICS

SOFT LENS RESEARCH

Instructions for Group B

1. Hygiene. Before putting on or taking off your lenses, always wash your hands. Rinse them thoroughly and dry them with a lint-free towel. Before handling your lenses avoid oily substances such as hand creams, lotions, or cosmetics.

2. Putting on Lenses. Remove right lens from storage container, place it in the palm of the left hand, and rinse with flexcare solution before putting on the lens. Check to make sure your lens is not inside out. Repeat for left lens. Discard solution in the storage container so that fresh solution may be used in the evening.

3. Cleaning Lenses. When removing lenses in the evening, remove right lens, place in palm of left hand and place a few drops of preflex on the lens. Rub lens thoroughly with index finger of right hand, being careful not to contact the lens with your fingernail. Do one side of the lens and then the other. Rinse lens with flexcare and place lens in storage container filled with fresh flexcare. Repeat for left lens.

4. Lens Storage. Flexcare contains antibacterial agents which will keep your lens free of contamination only if fresh flexcare is used daily, and if lenses are properly cleaned with preflex and rinsed with flexcare before overnight storage. We recommend that you periodically check your solutions expiration dates to guard against adverse effects and insure proper hygiene of your lenses.

5. Eye Drops. Eye drops or other eye medications or solutions intended for hard contact lenses must NOT be used by wearers of soft lenses. If used, medicants or preservatives will be absorbed by your lenses and serious damage to the eyes could result.

6. Wearing Schedule. You may wear your lenses up to eight hours per day during the first week and in most cases full time wear will be reached by the end of the second week. Once full time wear is achieved lenses may be worn during all waking hours, but must NOT be worn during sleep.

7. Sports Wear. Soft lenses may be worn for sporting and athletic activities and are superior to hard contact lenses for such activities as they are not easily dislodged from the eyes.

8. Swimming. Soft lenses should not be worn for swimming or other water sports unless a mask is worn. Otherwise the lenses may become contaminated with microorganisms if worn in fresh water or in salt water, or if worn in chlorinated water, chlorine may be absorbed by the lenses and can result in severe eye irritation.

9. Water. Fresh water or tap water will lower the salt content of soft lenses and will cause them to adhere tightly to the tissues of the eyes. This can cause damage to the eye if removal of lenses is attempted before the tears have a chance to bring the salt content back to normal.

10. Eye Make-up and Hair Sprays. If eye make-up or hair sprays come into contact with your soft lenses they can cause severe eye irritation or can permanently destroy the lenses.

11. Blurring of Vision. If vision blurs while wearing your lenses this is usually due to drying of the lenses and should clear up after blinking several times while moving the eyes back and forth. Check to make sure your lenses are in the proper eyes.

12. Pain, Discomfort, Redness. If these symptoms occur, remove lenses, clean with preflex, and rinse with flexcare, and put them back on. If symptoms persist, remove lenses and telephone the clinic for an appointment.

Appendix G.7B

THE UNIVERSITY OF ASTON IN BIRMINGHAM
THE DEPARTMENT OF OPHTHALMIC OPTICS

SOFT LENS RESEARCH

Instructions for Group C

1. Hygiene. Before putting on or taking off your lenses, always wash your hands. Rinse them thoroughly and dry them with a lint-free towel. Before handling your lenses avoid oily substances such as hand creams, lotions, or cosmetics.

2. Putting on Lenses. Remove right lens from storage container, place it in the palm of the left hand, and rinse with Flexcare solution before putting on the lens. Check to make sure your lens is not inside out. Repeat for left lens. Discard solution in the storage container so that fresh solution may be used in the evening.

3. Cleaning Lenses. When removing lenses in the evening, remove right lens, place in palm of left hand and place a few drops of Preflex on the lens. Rub lens thoroughly with index finger of right hand, being careful not to contact the lens with your fingernail. Do one side of the lens and then the other. Rinse lens with Flexcare and place lens in storage container filled with fresh Flexcare. Repeat for left lens.

   * Use the Bausch and Lomb protein remover tablets twice per week in addition to daily cleaning with Preflex.

4. Lens Storage. Flexcare contains antibacterial agents which will keep your lens free of contamination only if fresh Flexcare is used daily, and if lenses are properly cleaned with preflex and rinsed with Flexcare before overnight storage. We recommend that you periodically check your solutions expiration dates to guard against adverse effects and insure proper hygiene of your lenses.

5. Eye Drops. Eye drops or other eye medications or solutions intended for hard contact lenses must NOT be used by wearers of soft lenses. If used, medicants or preservatives will be absorbed by your lenses and serious damage to the eyes could result.

6. Wearing Schedule. You may wear your lenses up to eight hours per day during the first week and in most cases full time wear will be reached by the end of the second week. Once full time wear is achieved lenses may be worn during all waking hours, but must NOT be worn during sleep.

7. Sports Wear. Soft lenses may be worn for sporting and athletic activities and are superior to hard contact lenses for such activities as they are not easily dislodged from the eyes.

8. Swimming. Soft lenses should not be worn for swimming or other water sports unless a mask is worn. Otherwise the lenses may become contaminated with microorganisms if worn in fresh water or in salt water, or if worn in chlorinated water, chlorine may be absorbed by the lenses and can result in severe eye irritation.

9. Water. Fresh water or tap water will lower the salt content of soft lenses and will cause them to adhere tightly to the tissues of the eyes. This can cause damage to the eye if removal of lenses is attempted before the tears have a chance to bring the salt content back to normal.

10. Eye Make-up and Hair Sprays. If eye make-up or hair sprays come into contact with your soft lenses they can cause severe eye irritation or can permanently destroy the lenses.

11. Blurring of Vision. If vision blurs while wearing your lenses this is usually due to drying of the lenses and should clear up after blinking several times while moving the eyes back and forth. Check to make sure your lenses are in the proper eyes.

12. Pain, Discomfort, Redness. If these symptoms occur, remove lenses, clean with reflex, and rinse with lexcare, and put them back on. If symptoms persist, remove lenses and telephone the clinic for an appointment.

Appendix G.7C

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