ASPECTS OF TEAR FLUID COMPOSITION IN HYDROGEL CONTACT LENS WEAR

BY

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SUMMARY

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This study was designed to investigate the interactions of hydrogel contact lenses and the levels of proteins within the pre-ocular tear film (P.O.T.F.). Firstly it was necessary to establish a standardised technique for the qualitative and quantitative assessment of these lacrimal proteins. This was achieved using polyacrylamide gel electrophoresis, incorporating Sodium Dodecyl sulfate (S.D.S.), to raise the sensitivity level of the technique.

After the electrophoresis had been completed the gels were stained for protein fractions using Coomassie Brilliant Blue and qualitative results obtained using a densitometer.

A "waking hours" study was undertaken to assess any circadian relationship which could influence the experimental design of the hydrogel lens studies. No such time-linked variation was established with the lacrimal protein levels of non-contact lens wearers.

Thus four groups were designed, one group being a control. All groups contained ten adult male volunteer subjects. Each of the three contact lens groups were fitted using different water content hydrogels:- (a) Bausch & Lomb Soflens, (b) Sauflon 85 and (c) Sauflon 70. Both (a) and (c) are daily wear lenses and (b) is an extended wear lens. A 5µl sample of tears was taken from each subject at pre-determined data collection points throughout a twelve week period.

Using an analysis of variance technique the results showed that all three types of hydrogels were related to a significant change in the tear protein profiles. Trends in both the total, and individual amounts of protein, such as albumin, globulin and lysozyme were monitored. Over the initial stages of adaptation to contact lenses wear, a sharp decrease in the total amount of protein was observed. This was followed by the establishment of more stable values towards the final eight weeks of each study. A more complex response was obtained for the individual sub-groups of proteins.

Key Words

ELECTROPHORESIS, HYDROGEL CONTACT LENS, PROTEINS, TEARS.

To my Mother

ABBREVIATIONS

$\overset{o}{A}$	Angstrom
B.U.T.	Break up Time of Tears
^{o}C	Degrees Centigrade
CM	Centimetre
DIAM	Diameter
gm/ml	Grams per microlitre
H_1	Alternative Hypothesis
H.E.M.A.	Hydroxyethyl Methacrylate
Ho	Null Hypothesis
L	Litre
mm	Millimetre
M.M.A.	Methyl Methacrylate
N.M.	Nanometre
OH	Hydroxyl
PMMA	Poly Methyl Methacrylate

P.O.T.F.	Pre Ocular Tear Film
P.V.P.	Poly-Vinyl Pyrrolidone
S.D.S.	Sodium Dodecyl Sulphate
sec	Seconds
T.M.	Trade Mark
V.P.	Vinyl Pyrrolidone
α	Alpha
β	Beta
γ	Gamma
μ	Micro

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CHAPTER 1

Introduction - A Brief Outline of the Problems

The use of contact lenses as an alternative means to spectacles, for correcting ocular refractive errors, has been well established for many years. The reasons are many and varied for this demand. They cover a wide spectrum; from the more transient whims of fashion and vanity, to the real advantages for those participating in sport, and in extreme cases to the only available means of achieving an efficient visual correction e.g. to maintain single binocular vision for an unilateral aphakic.

The hydrogel or polyhema, soft contact lens has received a mixed reception since its introduction in the late 1960's. To some, it is the only serious competitor to emerge, so far, to the conventional, methylmethacrylate lens, whilst to others it is merely an essential clinical supplement.

Generally the initial biotolerance levels to these hydrogel lenses are considerably higher than for the conventional hard lenses. Hence the almost immediate increase in both patient and practitioner acceptability of these hydrogel contact lenses.

Unfortunately these high initial levels of biocompatability are not easily maintained and even within a few months of wear, problems such as the loss of visual performance and comfort levels begin to occur. Many sources have been cited in explanation of these deleterious effects. These embrace such apparently unrelated items as cosmetic and cellular debris, environmental pollutants, proteins, lipids, bacteria, antigen-antibody complexes and even build-up of the preservatives from the cleaning solutions themselves.

A common vehicle for most of these potentially offensive irritants is the lacrimal tear fluid. This has revived considerable interest in this formerly almost disregarded body fluid. A reasonable hypothesis is that lacrimal tear fluid changes in composition, as do other body fluids, with the introduction of a foreign body such as a hydrogel.

At the beginning of this study, a comprehensive literature survey did not reveal a great deal of recent research on the composition of the lacrimal fluid. Existing research had been conducted using fairly good qualitative techniques,

but the sensitivity had been sufficiently inadequate to require tear volumes in the order of Mls. for quantification purposes. Samples of this magnitude would take a very long time under normal production rates. (1.2µl/min according to Fatt 1978). Therefore tear production was stimulated using a variety of methods, from almost inadvertent mechanical irritation, to the use of contrived stimulii such as onions and sneezing powder.

Thus it was decided to establish whether any diurnal or "waking hours", variation existed in the protein composition of this fluid, and then determine the influence of hydrogel contact lens wear.

A sensitive and repeatable methodology for the collection, analysis and classification of non-stimulated tear fluid had to be designed.

CHAPTER 2

Anatomy, Physiology & Biochemistry of the Cornea, Lacrimal Apparatus and the Pre-Ocular Tear Fluid.

A BRIEF SYNOPSIS OF CORNEAL ANATOMY AND PHYSIOLOGY

The cornea is the main refractive element of the human visual system. It consists mainly of the fibrous protein collagen, and is distinguished from skin by its optical clarity and lack of keratinisation. This transparant, supportive, avascular membrane consists of five layers. The most anterior of these is the epithelium, which is approximately 50-70 µm thick in the fixed, adult eye, and is continuous with the conjunctiva where it becomes folded and thickens considerably. The life cycle of corneal epithelial cells in man has been estimated from the results of ISOTOPE studies of enucleated eyes, by Cogan & Kinsey (1942), to take about seven days. The inner most layer of the epithelium consists of closely packed columnar, basal cells, where new cells are continually produced and then are forced to migrate anteriorly by successive additions. They flatten considerably on their journey towards the front surface, where they finally desquamate into the lacrimal fluid. (Maurice 1962(a)).

Posterior to the basal cell layer, lies a very thin "membrane" about 10 µm thick. Using an electron-microscope it is seen to consist of fine collagen fibres, about 250 µm in diameter, (JAKUS 1962) and arranged in a randomly orientated meshwork. It would appear to have a common origin with the stroma, due to the similarity of its structure, and the fact that the two layers cannot be separated. (Maurice 1962(a)).

The Stroma (SUBSTANTIA PROPRIA) is approximately 480-500μm thick, and constitutes about 90% of the total corneal thickness. It consists of KERATOCYTES, (of which the FIBROCYTES are PHAGOCYTIC), collagen fibres and Mucoid matrix. The fibril diameters increase from about 19nm anteriorly to 34nm in the posterior layers. In each layer they lie parallel to each other, but the fibrillar orientation of adjacent layers shows a spiral arrangement (JAKUS (1964)). The Matrix occupies both inter fibrillar and inter lamellar spaces. It consists of water, protein, glycoprotein, mucopolysaccharides and inorganic salts. Maurice (1962(a)).

The fourth layer is DESCEMET'S membrane (Posterior Limiting Membrane), which is only about half the thickness of the Anterior Limiting Membrane. Electron microscopy studies reveal that the anterior part of this layer shows a very fine, granular appearance in whichever plane it is sectioned, showing no sign of a patterned organisation. It's thickness more than doubles with age from about 5nm to 12nm (JAKUS 1964).

The deepest layer of the cornea does not have the distinctive thin basement membrane, usually associated with endothelia. It consists of a single layer of hexagonal cells, initially presenting a regular, smooth surface to the anterior chamber. With advancing age,

some cells degenerate and are less frequently replaced by mitosis. (KAUFMAN et al 1977). These spaces due to cell loss are filled by the thinning and spreading of adjacent cells, thus resulting in a more irregular layer and a reduced cell population in older age groups (SHERRARD 1976).

The literature relating to the physiology of the cornea is profuse and extensive, embracing several areas of contention, which have undoubtedly been provoked, at least in part, by the increasing interest and expansion in the field of contact lens practice.

Apart from providing a regular, high power refractive medium, the cornea is most remarkable in its transparancy. It transmits more than 90% of the light incident upon it, and has had several differing theories proposed in explanation of this remarkable quality. It had been accepted for years that all parts of the cornea had the same refractive index, thus accounting for its transparancy. Subsequently, direct measurement of the refractive indices of the two major components (collagen 1.47 and Matrix 1.34) disproved this theory (NAYLOR 1953). Maurice's calculations (1957) were based on a system of collagen fibres of refractive index 1.55 and 0.10 volume fraction, distributed in a ground substance of refractive index 1.345 and 0.90 volume. Maurice (1957

and 1962) proposed from electron microscope studies that the fibrils behaved as a series of diffraction gratings, spaced regularly in three dimensions at about 300Å, to form a lattice in which the inter-fibre space is less than the wavelength of visible light, consequently most scattered light is eliminated by destructive interference. This theory is almost universally accepted as the most reasonable explanation to date, supported by the fact that adverse conditions which produce any disruption of the normal regular spacing between the fibres is accompanied by a corresponding loss of transparancy which normally tends to reverse, as the normal spacing is recovered, However, the complete mechanisms by which this corneal transparancy is maintained are not fully understood.

The cornea in vivo, maintains a constant state of deturgesence, although it is bathed anteriorly by the pre-corneal tear film, and by the aqueous fluid posteriorly. Complex chemical and pressure relationships exist between these two fluids and the layers of the cornea. Any disturbance of "normal" homeostasis results in some oedema of the corneal tissue. The contents of the eye, which are themselves about 98% water, exert a pressure of approximately one atmosphere on the cornea, which is approximately 78% water. (COGAN & KINSEY 1942).

Thus this negative water pressure inside the cornea and the positive pressure in the tears and aqueous humour would be expected to lead to continuous swelling and therefore corneal cloudiness (Maurice 1962). Some process, perhaps a pump mechanism, maintains a fairly constant corneal thickness, Mandell & Fatt (1965). The endothelium is universally considered to be the principal site of this "pump" mechanism, but both the epithelium and endothelium have been shown to act as inert, semi-permeable membranes, with differing diffusion characteristics. These characteristics have been extensively investigated, notably by Maurice (1951) and DONN et al (1963).

An exised cornea when immersed in dilute saline or water, imbibes a considerable amount of water, e.g. in water it increases by over a factor of five, in weight, after eight hours, and resembles an opaque, coagulated piece of egg white. Electron microscopy shows under these conditions, that the fibres are of normal diameter, but they are more widely and irregularly spaced due to the swollen matrix, thus adding support for Maurice's Lattice Theory of Regular, Critical interfibril spaces. Any involved discussion regarding maintenance of corneal transparancy and metabolism is beyond the scope of this thesis, since there is such a volume of current research and new suggestions in this area, which demand and deserve more involved

debate. Due to the realisiation of the importance in maintaining a deturgesed cornea, the clinical technique of monitoring the corneal thickness by Mandell & Fatt (1965) and VON BAHR (1956) of contact lens wearers, has recently received much deserved attention as a method of assessing the optimum, minimal interference with corneal processes.

The Pre-ocular Tear Film and Associated Glands

The pre-ocular tear film is a mixture of secretions from the lacrimal gland and accessory lacrimal gland tissue, together with the secretions of the meibomian glands and mucous glands of the conjunctiva (Ehlers 1965). The lacrimal gland is about one gram in weight and lies in the superior, temporal angle of the orbit. The lateral aponeurosis of the levator tendon produces a deep indentation in the gland, dividing the anterior part of the gland into two lobes. The orbital portion is situated in the lacrimal fossa of the frontal bone, and the palpebral portion lies below the aponeurosis. This lower part is closely related to the conjunctival fornix and it may be seen by everting the top lid. In the past the lacrimal gland was classified as a serous gland, but more recent evidence, put forward by Scott & Peace (1959), shows it is in fact a sero-mucous gland in rats, and in monkeys. (Ruskell (1968)). That the serous cells are most active is shown by Kuhnell in 1968. It had been proposed that the lacrimal gland did not contribute to the normal basic

tear secretions, but that it only contributed when excessive volumes of tears were produced, as in psychic weeping, or when irritation was present. This was based on the observation that when the gland is removed or its motor nerve supply is terminated then the eye usually remains sufficiently wet for the retention of subjective comfort and an optically adequate corneal surface (RUSKELL 1972). However denervation or removal of the gland does produce a significant reduction in the secretion rate of tears, as reported by Ruskell (1969) and Golding-Wood (1964).

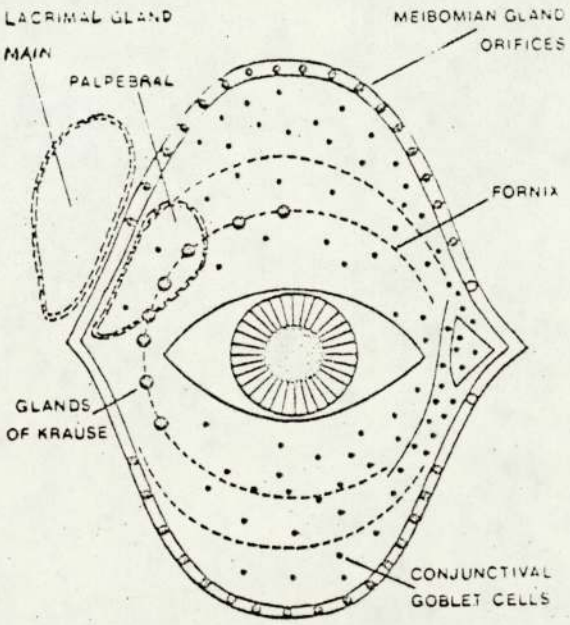


FIG 2:1 Sites of origin of tear film components. (Accessory glands only diagrammatically represented and not all types shown) (Tiffany & Bron 1978)

The openings to the meibomian glands lie immediately adjacent to the junction of the skin and conjunctiva at the lid margin. These glands run nearly the whole length of the tarsal plate, just extending beyond it at the lid margin, behind the eyelashes. These sebaceous, holocrine glands produce an oily secretion which helps prevent the overflow of tears. They number about twenty-five along the upper lid margin, and about twenty along the lower. They frequently become infected to produce internal styes, or chalazion. (LINTON et al 1961).

The conjunctival epithelium contains and supports a number of the accessory lacrimal glands. The goblet cells, which are apocrine glands are found in the tarsal and orbital conjunctiva. Maximum concentrations are found at the fornix and the plica semilumaris. There is a progressive disappearance of goblet cells in the epithelium of the bulbar conjunctiva, as the number of cell layers increase and the superficial layers of cells become flattened. Other accessory lacrimal glands, the glands of WOLFRING, lie in the upper border of the tarsal plates and their ducts open at the junction of the bulbar and tarsal conjunctiva. The glands of KRAUSE are formed by invagination of the epithelium in the fornices. There are about forty in the upper, and about eight in the lower fornix. Some contribution towards the pre ocular tear film is

made from the glands of ZEIS and MOLL. Those of ZEIS are paired, sebaceous glands which open into the eyelash follicles where they moisten the eyelashes. Sweat glands of MOLL are present in small numbers in the lid margins. They sometimes open into the lash follicles, but more commonly open directly onto the skin. The secretions from all these glands are found along with dead, corneal and conjunctival epithelial cells and debris in the tears. (MISHIMA 1965)

Conventionally the P.O.T.F. is considered to consist of a three layered structure - a superficial oily layer, a central watery layer and mucoid layer which lies immediately anterior to the corneal and conjunctival epithelium. The importance of the tear film to the underlying cornea has been investigated by several workers Smelser (1953) showed that the uptake of atmospheric oxygen through the tear film is required to maintain the normal deturgescent state of the cornea. It was also reported by Robb and Kuwabara (1962), that "Wandering" cells from the limbal vessels reach a corneal wound by way of the tear film and that these cells participate in the initial period of the wound-healing process. Obviously it also presents an optically smooth refracting surface to incident light.

The superficial oily layer covers the entire free surface of the tear fluid reservoirs, called the

marginal strips, which extend to the posterior margin of the meibomian gland openings. The closing of the orifices of these glands in rabbits, results in the absence of the superficial oily layer. This layer varies in thickness from 50 to 5,000 Å and is thickest when the palpebral aperture is narrowed. The layer has a refractive index of 1.5 (Mishma & Maurice (1961)), and shows interference colours, only when, on narrowing of the aperture, its thickness exceeds the wavelength of light. According to a classical paper by Ehlers in 1965, the Meibomian gland secretion, unlike most sebaceous glands, consists mainly of cholesterol esters with small amounts of fatty acids, triglycerides and waxes. This secretion will not form a smooth homogenous covering when added to water or saline solutions without the presence of mucins. The precise nature of this meibomian-mucin interaction has not been fully explained. BROWN and DERVICHIAN (1969), on finding that the meibomian gland secretions were insoluble in water, warmed them to approximate body temperature, at about 35°C. The mixture liquified and the surface active molecules spread on the watery surface to form a multimolecular layer of about 0.2μ, about 100 molecules thick. They also found that the meibomian gland oils, did not retard evaporation. The apparent contradiction between EHLERS (1965) in VIVO findings and those of BROWN & DERVICHIAN (1969) in vitro, could be explained by a factor present in the tear film, but absent from the model, such as albumin or a lipo-protein complex.

The aqueous or fluid middle layer of the P.O.T.F., is normally between 6 - 7 µm thick between blinks. It is a mixture mostly of the serous secretions from the lacrimal gland and the numerous glands of the conjunctiva. It is difficult to assess the relative amounts of tear fluid constituents since all methods of sample collection inevitably induce additional flow and different investigators produce differing amounts of irritation due to varied collection techniques. However it is generally accepted that the tears consist of about 98% water and 2% solids, of which proteins are the main constituents. The main cation among the inorganic ions in the tears is Sodium. The concentration of Potassium is approximately 1/6 to 1/11 of the Sodium concentration depending on age and condition of the tears. These two cations play an important role in the osmotic regulation of the extracellular and intracellular spaces, and in general, changes in the Sodium level are the reverse of changes in the Potassium levels. The chloride anion concentration is slightly higher in tears than in serum (ALTMAN AND DITTMER 1961). BOTELHO (1964) proposed that the Bicarbonate and Carbonate ions in the tears may be involved in the regulation of PH.

The deepest layer of the P.O.T.F. is called the Mucoid layer and it is assumed to be derived from the GOBLET CELLS of the conjunctiva. However EHLERS (1965) proposed

that the outermost layers of the epithelium are infiltrated by lipids which are probably produced by the tarsal glands, and that the muco-protein secretion of the conjunctival glands is not a deep film, but dispersed within the thick, central aqueous phase of the tear film. Thus there is agreement that muco-proteins and lipids are of the utmost importance in the wettability of corneal epithelium and are present near the epithelial surfaces, but disagreement as to whether they form a discreet layer or mixed indistinguishably with the adjacent watery layer. Glycoproteins are found in this posterior "mucoid" layer as well as in the tear fluid, since they are highly soluble in water. IWATA and KABASAWA (1971), fractioned three types of glycoprotein by gel filtration from tear mucoid clots, collected from the conjunctival surface of rabbit eyes. The carbohydrate-protein ratio of these three fractions is much lower than those found in other glycoproteins. However there are several inherent difficulties in glycoprotein analysis, not least of which is the problem of whether, in the isolation and purification procedures, structural changes of the glycoprotein have taken place. These and others are outlined by GOTTSCHALK (1963).

The total protein content of tears is usually given as between 0.3% - 0.7%, figures given on the following

table, are those normally quoted in textbooks, for the three main groups of proteins:-

FRACTIONS	UNSTIMULATED Tear Flow (BOTELHO (1964))	STIMULATED Tear Flow (FRANCOIS, RABAEY 1960))
ALBUMIN	58.2%	20.2%
GLOBULIN	23.9%	56.9%
LYSOZYME	17.9%	22.9%

However these values were determined using very variable electrophoretic methods for their analysis e.g., Paper and AGAR gel electrophoresis,so their accuracy cannot be considered as satisfactory. McEWEN, KIMURA & FEENEY (1958), separated six components by filter paper electrophoresis, one lysozyme, three globulins, named components I, II and III, and two albumins, one which appeared to be a specific tear albumin. They also determined the source of each protein. The lacrimal gland was the only source of LYSOZYME. SERUM ALBUMIN was produced by the GOBLET CELLS and TEAR ABLUMIN by the lacrimal gland. With the globulin group it was more difficult to localise their site of production, but it would seem from pooled samples that any of the three main sites of production i.e. lacrimal gland, accessory lacrimal gland and the goblet cells are all capable of producing any or all of the three globulin components.

PROTEIN	SOURCE (McEWEN, KUMURA & FEENEY 1958)
LYSOZYME	LACRIMAL GLAND
TEAR ALBUMIN	LACRIMAL GLAND
SERUM ALBUMIN	GOBLET CELLS
GLOBULIN GROUP	ANY ONE OR ALL SITES POSSIBLE — LACRIMAL GLAND; GOBLET CELLS; ACCESSORY LAC.GLAND

Lysozyme was first described by Fleming in 1922. It is present in many tissues in man, animals and plants including saliva, spleen, kidney, lymph glands, milk, eggs, cauliflower, and of course the tear secretion of many species. The systematic name of lysozyme is MUCOPEPTIDE N - ACETYL MURAMYLHYDROLASE: the trivial name is lysozyme of MUCOPEPTIDE GLUCOHYDROLASE or the synonym MURAMIDASE. Originally it was discovered as a substance in nasal mucous, which had the ability to LYSE or dissolve certain bacteria by hydrolysing their cell walls. The lysozyme obtained from egg-white has a molecular weight of 14,500 and consists of a single polypeptide chain of 129 amino acids. The first entirely purified human lysozyme was that from human milk by Jolle's and Jolle's (1961). Jolle's and Jolle's (1967) also compared the activity of Human tear and human milk lysozymes with that of hen egg white lysozyme and found that lysozymes from Human sources were more active by a factor of 3.5 but that their mechanisms of action were very similar. When lysozyme is examined

from a source consisting of pooled tear samples, then often two different electrophoretic mobilities are found. Apparently a "slow" and a "fast" lysozyme is possible in several animal species including man. In tears of a given individual, the mobility of lysozyme may change periodically, but both varieties are never present in one individual simultaneously. (McEWEN et al 1958).

Ericksen (1956) designated the fast moving albumin in human tears as Specific Tear Albumin. The albumin from patients with an inactive lacrimal gland is similar to serum albumin in its electrophoretic mobility. Bovine Serum albumin has a molecular weight of 66,500. Spencer and King (1971) found that albumin extracted from both Human and Bovine sources showed a hetereogeneity of isoelectric points, indicating a mixture of albumins with slight differences in formation.

The globulins have been separated into many fractions, including the very important immunoglobulins, which have been shown to be similar, but in different proportions, to blood serum (ALTMAN & DITTMER (1961)). Compared with the other tear proteins they have very large molecular weights e.g. alpha - globulin is 140,000. The immunoglobulins are thought to be important local agents of a host defence mechanism. McEwen et al (1958) found that immunoglobulin tear

fractions differed from their serum counterparts in their staining properties, suggesting that tear globulins were more likely to be found in complexes with lipids and mucopolysaccharides, than those in serum. Their range of isoelectric points is around neutrality, moving slightly towards the basic side.

Many investigators have given estimates of the amount of protein in tears. These values vary widely within the range 0.3 - 4.52 gm/100ml. Partly this wide range of values is due to the differing methods of collecting the tear samples and in their subsequent storage and analytical methods. The relationship between the protein constituents of blood serum is a very valuable tool in the diagnosis and monitoring of many systematic pathological conditions. TARNOKY and DOWDING (1967) give an interesting synopsis of distinctive patterns found in anaemia, pneumonia, glandular fever, tuberculosis, rheumatoid arthritis, ulcerative colitis and many renal and liver conditions. Disc electrophoresis shows particular promise in the differentiation and location of tumours in malignant disease.

Thus it is hardly surprising that the range of protein values for tears is found to be extensive, since careful consideration of the relevant papers will show a great mixture of sample types with indiscriminate mixing of sexes, ages etc. There seems to be a

difference in results for the comparison of "normal" tears from males and from females. TAPASZTO and VASS (1965) suggested that the tears of female subjects contain higher concentrations of proteins than do the tears of male subjects. However Norm (1965) reported that the differences between sexes were negligible, except in early adult life, when females have a higher rate of secretion. According to Henderson and Prough (1950), mechanical injuries only cause a slight increase in the protein concentration of tears, but excessive exposure to radiation, especially ultra violet, causes a large increase in the protein levels in excess of 1000mg/100ml, as was also the case for eyes which had been subjected to chemical (mostly alkaline) injuries. Tapaszto and Vass (1965) proposed two main reasons for these changes (a) over active functioning of the lacrimal gland, and (b) increased permeability of the conjunctival vessels. They also found that the tear albumin levels reduced and the serum albumin fraction increased in the tears of subjects who had bacterial or viral conjunctivitis and keratitis. The alpha globulins show very little difference between "normal and diseased" samples. Beta 2 globulin, the largest fraction in normal tears often shows a small (about 10%) reduction in pathological states. The lysozyme level would appear to be most sensitive since its concentration reduces to around 50% of its original level. When considering the values given here, it should be remembered that ocular pathologies often cause irritation

and therefore an increase in the volume of lacrimal fluids. Thus although the lysozyme concentration drops to half its normal levels the amount of lysozyme present on the eye is about the same as under normal conditions, hence the increase in volume relatively lowers the concentration. PIETSCH and PEARLMAN (1973) studied the tears of 460 healthy human volunteers and found a tendancy for the lysozyme level to decrease after ten years of age, suggesting a linear relationship between production rate and age increase. Since tear production is shown to decrease with age, Pietsch and Pearlman (1973) suggested an accompanying decreased activity of the lacrimal gland. They did emphasise that a low tear value does not indicate a low lysozyme level, since the concentration for these relatively dry-eyed subjects was usually within normal levels. They stated that very low tear volumes with accompanying very low, or negligible lysozyme levels indicated the presence of kerato-conjunctivitis sicca. Sen et al (1978) investigated the normal biological variation in tear immunoglobulin concentration related to age and sex. They found a significantly higher mean value for Iga levels in females. The concentrations of Iga for both males and females increased slightly with age. They felt the values and the relationships that they had established were too weak to use the Iga levels as a screening device, to separate the normals and the probable abnormals. They found in agreement with Bluestone et al (1975) that

IgG levels were just detectable but not quantifiable and thus no correlation with age and sex was possible. IgM was only present in about 30% of the two hundred samples and was also in very small quanitities. IgD was not detected in any samples.

The Dynamics of BLINKING and Physical Properties of the TEAR Film

As previously discussed the P.O.T.F. is produced by various glands distributed throughout and along the upper and lower eyelids. With respect to volume, most tears are produced from the lacrimal gland and eventually spread evenly over the anterior surface of the eye. Wolff (1948) suggests that lacrimal fluid passes mostly to the temporal upper fornix whence it descends to the strip of fluid at the superior lid margin. They reach the superior punctum along this strip or directly under the upper lid. He felt that the tears reached the lower lid mainly via the outer canthus and through the outer conjunctival cul de sac, with which it is continuous. Medially these strips terminate in the lacrimal lake which is often described as a reservoir of tears bathing the caruncle and bounded by the inner canthus and the free edge of the plica semilunaris. Normally the inner canthus is moist, but does not contain a pool of fluid. The puncta of the upper and lower lids are arranged in apposition to the globe thus facilitating drainage of the tears by interupting the marginal tear strips. It should be noted that the P.O.T.F. would seem to be unaffected by gravity and that its movement is solely due to movement of the eyelids during the blink mechanism. Francois and Neetens (1973) suggested two mechanisms for the passage of lacrimal fluid into the cannaliculi. One is

that pressure exerted during blinking, forces tears into the canaliculi which is unlikely to have a major effect, since both the puncta and the lumen of the canaliculi are extremely small. The other, more feasible, mechanism was the principle of capillary attraction. The blink cycle moves tears nasally, since lid closure begins at the outer canthus and then progresses to the inner canthus. Eye lid closure and muscle contraction tends to close the puncta and lumen and therefore shorten them, thus forcing the tears into the lacrimal sac. Basic principles show that the smaller the diameter of the tube (in this case the diameter of the canaliculi) the easier would be the entrance of fluid by capillarity. Perhaps widening of this canaliculus, due to loss of puysiological muscle tonus could be a factor in the explanation of epiphera in older subjects. Jones (1961) demonstrated the absence of backflow from the compressed tear sac toward the conjunctival fornix during concentration since regurgitation is prevented by the simultaneous closure of the ampulla of each canaliculus. The existence of a lacrimal pump mechanism which would necessitate closure of the "valve" of HASNER has been questioned by BRIENEN and SNELL (1967), since measurements within the lacrimal sac do not reveal any change in pressure during eyelid movement. It has been suggested that gravitation alone may account for the transfer of tears into and out of the lacrimal sac. The stability of the normal tear

film over the corneal surface has received a lot of interest lately, due mainly to an increase of awareness caused by the rise in popularity of contact lens usage.

The corneal epithelial surface is composed largely of lipophilic material which by nature is relatively hydrophobic. Very good wetting properties are only achieved due to the amphipathic properties of the absorbed mucins, mostly glycoproteins. Mishima (1965) has shown that wiping the corneal surface removes something essential to the maintenance of a stable tear film. The importance of these Mucins is further emphasised by some contact angle measurements of tear film giving a value of 47°, by LEMP et al (1970). This value would indicate that the tears would not spread spontaneously over the corneal surface. The addition of a mixture of sample proteins did not alter these measurements significantly. But the addition of Mucus to the tear sample, resulted in approximately a 50% reduction in the contact angle value indicating a greatly enhanced wettability of the surface. BROWN and Dervichian (1969) measured the normal tear film to be about 6 μm thick. If this thickness reduces then it becomes too thin to maintain the necessary curvature and contact angle, thus the liquid will retract to attain a greater thickness, compatible with equilibrium. Thus the sudden appearance of "holes" results, known as the tear break up time (B.U.T.). This could happen

for various reasons (a) Reduced tear secretion, (b) Reduced quality of wetting agents in the tears (c) Reduced blink rate or "half" blinks.

Obviously the normal volume of tear flow is an elusive quantity since any irritation to the eye can cause rapid fluctuations in the tear flow. Mishima (1966) investigated tear volume using the principle of the determination of dilution of flourescein by the tears and the rate of its concentration decay using a fuorophotometer to study the dynamic changes in tear flow and turnover rate. The average normal secretion was calculated to be 1.2µl/min, with a very wide individual variation in turnover rate, so that "normal range" would be about 0.5 - 2.2µl/min. The tear volumes present were calculated separately for (a) normal peapebral fissure (b) marginal tear strips (c) under the peapebral conjunctiva and the fornices to give a total averaging 7.0 ± 2.0µl. FATT (1978) gives a figure of 1.2µl/min for the volume of normal tear production, by examining the chemical composition of freshly produced tear samples. Adler (1965) states that the normal average period between blinks is about 2.8 secs in MALES and just under 4 secs in FEMALES. FATT (1978) gives the normal blink rate in human as 12 per minute and compared this with rabbits which only blink once every 10 or 15 minutes. Thus the difference in rates between the species is difficult to explain, in view of the similarities in tear composition (with

the exception of lysozyme) and underlying corneal physiology. It has been proposed that the blink rate is related to information processing, but no real evidence has yet been produced (ADLER 1965). If the lids are held apart to prevent blinking, then the appearance of "dry spots" in the cornea will be observed after a period of 12 - 40 seconds, in man. It is unlikely that this is caused by evaporation since at least ten minutes would be required for evaporation of the aqeuous phase of the tear film. Holly (1973) suggests that as the tear film thins, so the lipid molecules from the top surface migrate posteriorly to the mucoid layer immediately adjacent to the corneal epithelium, thus contaminating the surface and producing patches of hydrophobicity. The relationship between B.U.T. and successful contact lens wearers was investigated by FORST (1976), and a critical range of 5 - 7 seconds was found. Values below this range indicated that comfort could not be achieved and thus B.U.T. has been suggested as a diagnostic predictive test of possible success for contact lens patients. Another interesting investigation relating to B.U.T. was initiated by a survey in Canada which stated that irritation of the eyes was the most annoying aspect for non-smokers entering a smoke-filled room (SHEPHARD and LABARRE 1978). Thus BASU et al (1978) decided to investigate any possible relationship between

cigarette smoke and B.U.T. of non-smokers. The B.U.T.'s of fourteen subjects dropped from around 21 seconds to about 12 seconds after 10 minutes exposure to a smoke-filled room.

Thus it would appear that the P.O.T.F. is an interesting and complex body fluid with several unexplained qualities which have only fairly recently been viewed with any degree of scientific methodology.

CHAPTER 3

The Chemical and Physical Properties of Hydrogels

CHEMISTRY OF HYDROGELS

In 1960, Wichterle and Lim published a short paper proposing and outlining the use of synthetic hydrogels for contact lenses. Most of the original research referred to in this paper was carried out using 2-Hydroxyethyl methacrylate (H.E.M.A.) in a variety of cross-linked co-polymers which yielded transparent hydrogels of greater hydration than can be obtained with H.E.M.A. alone. These synthetic hydrogels consist of many sub-units called monomers which join together to form a polymer. If the monomers are identical then it is called a HOMOPOLYMER and if there are more than one type of monomer then it is called a COPOLYMER (REFOJO 1978). A polymer chain, consisting of hundreds or thousands of linked monomers can be joined to another such chain by the addition of a cross-linking agent. These cross-linked polymers or networks will swell but not dissolve in solvents and cannot be permanently deformed by heat or pressure similar in structure to the so called THERMOSETTING PLASTICS. Non-cross linked polymers are soluble in some solvents, and their form often can be changed with heat or pressure in which case they are called THERMOPLASTICS. Thus these hydrogels which are used for the manufacturing of contact lenses can be described as cross-linked, hydrophilic POLYMERS

which swell in water to form soft, elastic gel-like materials. These properties were responsible for much improved comfort levels in contact lens wear. This improved patient acceptance of these lenses meant that they were the first serious competitor to the conventional hard methylmethacrylate contact lenses (Tighe 1979).

The material, H.E.M.A. was not originally designed as a contact lens material but it does satisfy most of the desireable qualities for such a function. These would be:-

(a) Minimal interference with the underlying corneal metabolism.

(b) Maintenance of an acceptably stable visual environment during the blink cycle.

(c) High subjective comfort levels.

Because the cornea is avascular, its oxygen requirements for cellular respiration are taken probably from a combination of sources in the pre-ocular tear film and directly from the air (FATT and BEIBER 1968). Obviously a contact lens must produce at least a potential barrier to these sources. There is some controversy about the movement of tears under a relatively tight fitting soft lens. However Carter (1972) reported that red blood cells can be seen moving under a soft contact lens, when a drop of blood is added to the

tear pool outwith the lens, obviously suggesting a certain amount of tear pumping and exchange under the lens from the tear pool. The measurement of oxygen permeability of hydrogel lenses has been studied by Holly and Refojo (1972) and further by Refojo (1973). There is general agreement that the oxygen permeability of hydrogels increases exponentially with increasing specific water content irrespective of the type of polymer involved in the formation of the hydrogel. (TIGHE 1976) Ng and Tighe (1976) also observed when investigating the effect of temperature that the O_2 permeabilities at 34°C were found to be almost exactly double the values at 25°C and at the same H_2O content.

Ng, et al (1976) concluded that the O_2 permeabilities of hydrogels having H_2O contents in the region of 30% or below show appreciable dependence on the chemical nature of the polymer backbone together with the properties of "bound" and "free" water.

However the type of polymer does influence the amount of free and bound water within the lens materials. (FRANKLAND 1973) "Hydron" and "Soflens" materials have relatively low levels of "free" water compared with SAUFLON. The water molecule

has a partial positive charge along its dipole axis at one end, and a partial negative charge at the other. Oxygen and Hydrogen covalent bonds are polar, due to different electronegativities of the two different atoms (a slight imbalance in the distribution of their outer electrons). Thus FRANKLAND (1973) claims that since the electronegativity of oxygen is greater than that of nitrogen then bonding of oxygen with the oppositely charged part of the water molecule will be stronger, and thus more difficult to break than water which is bonded to a nitrogen site in a polymer. Since the PYRROLIDONE RING in sauflon uses a Nitrogen bonding site for water, and materials such as "SOFLENS" and "HYDRON" use oxygen bonding sites then the bound water for SAUFLON will be less tightly bound than that for the HYDROXY containing polymers. Thus, as FRANKLAND (1973) emphasised, the larger the "free" water content of the material and the weaker the nature of the water/polymer bond then the easier the movement of water within the system.

FATT (1978) measured the water flow conductivity of three high water content gels, proposed as materials for extended wear contact lenses. All measurements were made at 22°C, but according to the author, little change is made by correcting

the data to 37°C (or eye surface temperature) since although with increasing temperatures, the pore diameters would be expected to decrease, the oppositely acting effect of decreasing viscosity of saline with increasing temperature almost negates this effect. The author also stated that the flow conductivities had little practical use in the study of contact lenses, because of the extremely small pressure gradient across a soft lens in situ. Of much more clinical significance is the comparison of the estimated gel pore diameters with the diameter of selected biological materials, The pore diameters of the three investigated materials (FATT 1978), plus that determined for H.E.M.A. by Refojo (1965) are given below:-

MATERIAL	WATER CONTENT (%)	ESTIMATED (MEAN) PORE DIAM (Å)
PERMALENS	72	30
SAUFLON 85	85	24
SMITH & NEPHEW 407	79	21
HEMA	38	8

These figures show that a HEMA lens would be expected to be impermeable to even the smallest biological molecules. However the higher water content gels,

having substantially greater pore sizes could be expected to allow several bio-molecules to pass into their matrices. Of particular interest is the estimated diameter given by the author for LYSOZYME to be 30Å, indicating that it may just be possible for this important anti-bacterial agent in the tear fluid to penetrate the Permalens, at least. It should also be noted that these figures were obtained from new, fresh material and it is often suggested that during the wearing and cleaning procedures of gel lenses, there may be a certain amount of polymer matrix degradation which would irregularly increase some of these pore diameters.

The values are given as estimated Pore diameters, and although the method of estimation is not immediately clear, it must be assumed that these are "mean" values. A certain number of pores, in even the most carefully controlled manufactured polymer, must contain a certain number of pores both above and below this mean value.

An interesting comparison of Fatt's data with that of ONCLEY et al (1947) can be made:-

PROTEIN	DIAMETER Å	PROJECTED AREA END-ON (Å)^2	LENGTH Å	PROJECTED AREA SIDE-ON (Å)^2
ALBUMIN	40	1,700	115	4,600
GAMMA-GLOBULIN	44	2,000	235	10,300
FIBRINOGEN	65	4,200	475	13,000 to 30,000

The only protein which both authors give is Albumin. Fatt gives a figure of 65Å and ONCLEY et al give 40Å for the diameter of albumin. This is obviously a significant difference as the lower value would mean that even albumin may just fit into a very high water content lens which had begun to show enlargement of "pore" size due to in-use degredation. The higher figure would make this penetration much more unlikely. (Refojo 1978)

Physical and Surface Properties of Hydrogels

Another fairly obvious variable in the strive towards greater oxygen permeabilities of hydrogels is to decrease the thickness of the manufactured lenses. Most hydrogel lenses cannot be made very thin, because of increased frailty, leading to great reductions in their service life and visual stability resulting from warping of the lens during the blink cycle, RUBEN (1974) proposed a series of measurements to classify the stability of

Hydrophilic lenses by relating lens form to material. Three factors were measured:-

(1) HARDNESS - as measured by indentation or scratching of surfaces.

(2) RIGIDITY OF FORM - related to the elasticity of the material, measured by the change of form with pressure and secondary temperature produced variations.

(3) VOLUME ELASTICITY and relationship to transient or permanent loss of water from the material.

The most important parameter seemed to be LENS FORM RIGIDITY, influenced by thickness and size of lens. An index system was proposed whereby the higher the number, the more moldable or plastic will be the lens form. It was shown that elasticity and hardness are only complementary to the lens form in importance.

Tighe (1976) determined certain relationships between eyelid load and indentation of several polymers. His results correlated well with clinical studies and he stated that materials whose deformation behaviour is time-dependent and where the graph of deformation against time showed appreciably "damped" curves, could still produce acceptable

clinical behaviour. The acceptability limits which he suggested were that the overall deformation should not be larger than that of poly HEMA. Hydrogels with values larger than those considered as acceptable were typically associated with hydrogels of fairly high (greater than 50%) water content and low "effective" crosslink density. He emphasises that although water content plays a major part in controlling the mechanical properties, there are several others which also contribute to this fundamentally important aspect of hydrogels for clinical application.

Since hydrogels are materials which are sufficiently hydrophilic to absorb water into the polymer structure, it was initially assumed that wettable surfaces would not be a problem when using hydrogel contact lenses. If a contact lens material is not easily wettable, dry areas occur and beads of water form on the surface of the lens. This would cause blurred, distorted vision and comfort levels would decrease as these dry patches cause irritation to the eye. Therefore it is desireable to have a wettable surface over which tear fluid can spread evenly (ROCHER 1975). Wettability can be discussed in terms of surface energies. The nature and packing of the exposed groups of atoms at any surface determine its wettability, a characteristic feature generally independent of the nature and

arrangements of the underlying atoms and molecules. (HOLLY and REFOJO 1975). Thus the surface molecules distribute some of their electronic charges to the underlying groups of atoms to maintain normal cohesion. The remainder of these electronic charges of the outermost layer of atoms then constitute the surface energy of that object (HOFFMAN 1974). These surface free energies have a tendency to "spend themselves" e.g. when a liquid of low surface energy is placed on a solid having a high surface energy, the liquid will spread over the solid, the system tending to work towards equilibrium by using the solids surface energy to attract and fixate the liquid. Conversely if the solid surface has a lower surface energy than that of the applied liquid, then the liquid will not spread evenly and will form discreet droplets on the surface. This is the case with polymethyl-methacrylate (conventional hard lens material) having a surface energy of approximately 39 dynes/cm., and that of tear fluid having a surface energy of approximately 46 dynes/cm. These figures were produced by RANKIN and TRAGER (1970).

The measure of wettability of a surface is often given by its contact or wetting angle. This is the angle formed by the tangent of the liquid surface and the surface of the solid. PEDLEY et al (1973). See figure overleaf:-

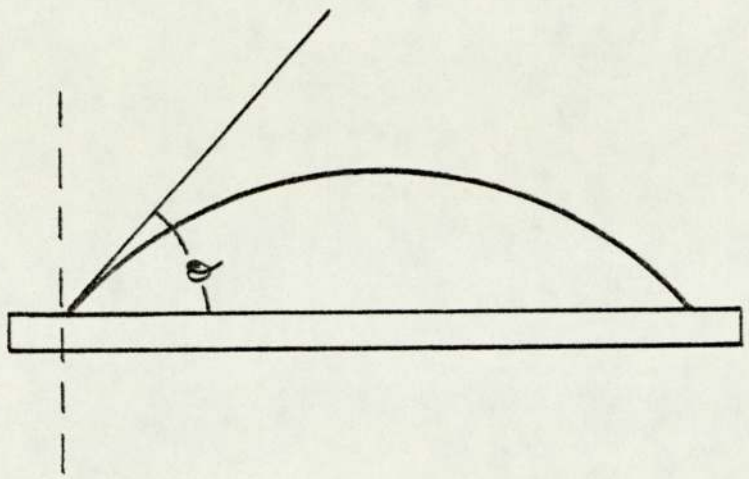


FIG 3:1

The wetting angle of a liquid on a flat, horizontal solid.

A material with a wetting angle of more than 90° is said to be hydrophobic while a material of less than 90° is partially wettable. A completely hydrophilic surface would have a wetting angle of 0° i.e. the liquid would spontaneously spread to form a regular thickness of film over the surface.

In reassessing the general idea that poly HEMA, and other hydrogels have hydrophilic surfaces it should be noted that clinical experience in handling these gels, suggests that at least some of the lens surfaces are not completely wettable by water or saline. A study by Holly and Refojo (1975) on the wettability of HEMA gels, produced some very interesting results. HEMA hydrogels with equilibrium water contents ranging from 32% - 43% were prepared by varying the amount of cross-linking agent, and used for the measurements.

Two methods were used in the wetting angle determinations:-

(a) SESSILE DROP TECHNIQUE (water in air)

(b) CAPTIVE BUBBLE TECHNIQUE (air in water)

In both techniques the advancing contact angle when for (a) the drop size OR in (b) the bubble size increases, and the receding contact angles (vice versa) were measured. The two methods produced consistent differences in the contact angles for the same samples and no explanation for this was found. However another more controversial result was that for both techniques and all samples, the advancing contact angle values were unexpectedly high. Ironically some of the HEMA gels were even less wettable than P.M.M.A. Secondly there was a large consistent difference between the advancing and receding contact angles for all the hydrogel samples. Structural surface changes were proposed as the explanation for the large HYSTERESIS of the water contact angle. A polyhema gel contains both polar, hydrophilic binding sites and hydrophobic non-polar parts of the polymer backbone (FRANKLAND 1973). The optimum arrangement from an energetics viewpoint is for the polymer chain segments to expose the hydrophobic side groups (non-polar), to the air-interface and contain the polar, hydrophilic

sites within the central aqueous phase. Thus presenting a relatively hydrophobic-air interface. However when this relatively hydrophobic surface is put in contact with water, the energetics situation is changed and initially an unfavourable high interfacial tension is created, producing unexpectedly high advancing contact angles. Thus according to HOLLY and REFOJO (1975) the polymer segments rearrange to achieve minimal interfacial energy, by allowing the gel surfaces molecular arrangement to mimic the arrangement of the gel matrix. Thus producing a much reduced, and more easily accepted value for the receding contact angles. It should be noted that since these values never reached 0^{o}, then the hydrogels can never be completely wetted by water. The results also show that the equilibrium water content of the HEMA gels used, did not affect the wettability as determined by the contact angle measurements.

This contact angle hysteresis is now an accepted facit of contact angle measurements. However the very large differences between the advancing and redecing contact angle values produced by Holly and Refojo (1975) are not easily reproduced by other investigators (LARKE et al 1973 (a) and (b)). It should be remembered that contact angle values are extremely sensitive to change according to the exact conditions under which they are performed, and

the presence or absence of an interfacial water layer can greatly influence the quantitative values (Tighe 1979).

CHAPTER 4

The Problem Reassessed

General Functions of Synthetic Polymers

The adhesion of biological substances to hydrogel contact lenses is interestingly paralleled by the problems associated with most synthetic polymers which are introduced to the body, and bathed by a variety of body fluids. Efforts to interpret the mechanisms of adhesive behaviour in biological environments have received new impetus recently from research in such normally unrelated fields as surgical adhesives, dental restoratives, prosthetic implants and marine fouling. The requirements for each different type of surface obviously varies according to the particular function which it performs. (BAIER et al 1968). The desirability of qualities demands good tissue-to-plastic adhesion for oral bandages to remain exactly in place, - thus some of the implanted prosthetic material will be assimilated into the body structure, (EIRECH 1969)

Conversely, desirable surface properties to minimise possible thrombus formation whilst maintaining compatability with the complex antibody and rejection mechanisms, are required for materials used in heart valve replacement

(ANDRADE 1973). Dental applications require adhesives capable of intimate molecular contact and good spreading to eliminate cavities and particle entrapment while maintaining extremely durable adhesive bonds (SKINNER and PHILIPS 1967).

Tissue Response to Synthetic Polymers

The preservation of these desired surface properties is of the utmost importance since, unlike contact lenses, the accessibility of most applications is at best difficult and at worst requires further surgical procedures. This may further complicate the system by introducing "wound healing responses" (ANDRADE 1973). Thus minimal or no maintainance should be required.

Regardless of how inert and stable the implant material is, it is still a static discontinuity in a dynamic, living system and will cause that system to make various responses to it. Thus a foreign body reaction, often with an accompanying "wound response" mechanism is induced, and the inert implant is often said to be "encapsulated" with connective tissue, or "rejected" by the body. The presence of an interface between the implant and the surrounding tissue, serves to permit a variety of phenomena, which may be important in the foreign body response. Some of these factors

have been speculatively discussed by KORDAN (1967). He states that rapid and, perhaps irreversible, protein adsorption processes will occur at the interface. This adsorbed protein may appear to be altered or structurally changed to its neighbouring proteins, still in solution, and thus rejection of this now "foreign protein" may occur. This is thought to be due to changed membrane potentials caused by the lack of normal physiological ions at the implant surface. KORDAN (1967) also stated that there seemed to be an inverse correlation between the extent of the gel graft, and the tissue reaction, concluding that minimal interference would be achieved by producing a system whose interfacial free energy is as close to zero as possible, and where the implant's volume is the minimum possible.

Comparison of the Main Constituents of Blood Serum and Tears in Man

The ELECTROLYTES of these two important body fluids are compared in the table below:-

FLUID	MEAN CONCENTRATION IN μgE /ml			
	Na^+	K^+	Cl^-	HCO_3^-
TEARS	145	24.1	128	26
SERUM	140	4.9	102	26

FIGURES FROM BOTELHO (1964)

It is readily observed from the preceding table that the electrolyte concentrations are similar, with the exception of the Potassium (K^+) and Chloride (cl^-) ions. The concentration of K^+ is usually three to five times higher in tears than in serum. Both Sodium (Na^+) and K^+ play an essential role in the osmotic regulation of the extracellular and intercellular spaces of body tissue. The cl^- anion also has a similar role and it's concentration is higher in tears than in serum.

The bicarbonate (HCO_3^-) and Carbonate ions are involved in the regulation of tear pH. The HCO_3^- level is very similar in tears and in serum.

Other cations such as Calcium and Magnesium are normally present in the tears in very low concentrations.

The total protein concentration in tears is approximately 1/10 of that in plasma. Albumin represents about 60% of the total protein in tears, as it does in plasma. The remainder is divided equally between globulin and lysozyme fractions in tears.

Body Fluid Reactions at the Non-Physiologic Interface

As discussed in the preceding chapter it is the

outermost atomic constitution of a material which must dictate its initial interactions with its environment. In the past decade there has been considerable interest in the effects of charge and energy of various surfaces on the initiation of blood clotting, by the adsorption of Fibrinogen (GORMAN, STONER and CATLIN (1971)). When blood first contacts a non-physiologic interface a sequence of events is initiated which can end in blood coagulation or thrombus formation (DUTTON et al (1969)). These processes, normal in the wound-healing mechanism, are considered undesirable with implanted prosthetic materials. BAIER and DUTTON (1969) determined the nature of the events occurring immediately after exposing foreign surfaces to fresh, flowing blood using a combination of three techniques.

(1) Multiple attenuated internal reflection spectroscopy (M.A.R.)

(2) Ellipsometry

(3) Critical surface tension measurement.

Their results confirmed the common assumption that the initial event was the rapid deposition of a strongly adherent proteinaceous film. Due to similarities in the wetting and spectral characteristics they suggested that Fibrinogen may be a major component of this naturally deposited layer. Since

there is a certain threshold time interval before platelet adhesion occurs, it was suggested that the fibrinogen layer was a pre-requisite of thrombus formation.

Forces existing on a bio-molecule at an interface of a foreign material with an aqueous medium will normally include:- (HOFFMAN 1974)

(1) Ionic Forces

(2) Hydrogen Bonds

(3) Polar forces (orientation and induction effects)

(4) Dispersion forces (hydrophobic bonds)

The relative dominance of the above individual forces is dependent upon the relative polar or non-polar character of the interface and how this affects the water structure at the interface. Water contacted with a NON-POLAR foreign surface should structure as it would around a hydro-carbon molecule, which does not readily mix with water. (DROST-HANSEN 1971) The hydrophobic character of the hydrocarbon molecule causes water molecules to organise around it and envelope it. A polar surface is wettable due to dipolar interactions between the polar sites on the water molecules and those on the polar surfaces (HOFFMAN 1974). Thus polar hydrogel surfaces are more compatible with body fluids than the non-polar type of polymer surface, since protein molecules in the vicinity

of the polar bio-interface would be expected to release and disorder the formerly orientated water molecules, during its adsorption onto the foreign surface.

Conversely, (BAIER and ZISMAN 1968) a protein molecule at a non-polar interface may undergo certain structural changes to expose its non-polar groups. Such an "unfolding" would normally result in an irreversible denaturation of the protein and thus initiating possible bio-incompatability mechanisms.

The Thermodynamic Interactions

The thermodynamic implications of adsorbed protein monolayers are discussed by LEVINE (1969) in a highly mathematical paper. Basically the equilibrium adsorption of a protein and its resultant conformational changes will be dependent on the thermodynamic changes ocurring during this process. Whether the overall free energy change is positive or negative determines if the adsorbtion will occur. The free energy change is expressed by the second law of Thermodynamics (HOFFMAN 1974):-

$$\Delta G = \Delta H - T\Delta S$$

where ΔG - free energy change, Cal/g mole

ΔH - enthalpy (HEAT) change, Cal/g mole

ΔS - entropy change, entropy units (Cal/g mole ^{o}K)

T - Temperature, ^{o}K

Thus theoretically it can be seen that an exothermic reaction will accompany the adsorbtion process, which is favoured by a positive "s" factor i.e. by an increased randomness or disorder factor. Conversely, adsorbtion is not favoured by a positive "H" and a negative "S" factor.

Influential Factors on Protein Accumulation Rates

MacRitchie and Alexander (1960) listed protein concentration, nature of interface, pH, ionic strength, and temperature, as being factors which could influence the rate of build-up of a denatured protein layer on polymer surfaces. They stated that pH seemed to be the major factor. At pH's away from the ISOELECTRIC POINTS, the adsorption of the proteins was thought to be retarded by repulsive forces arising from the charged surface molecules. They also found that low concentrations of sucrose produced increased

adsorption rates, and proposed the explanation that sucrose assisted the unfolding of the protein molecules either at the interface or in the bulk of the material. It has been known for some time that the addition of calcium has adhesion-promoting properties in some interfacial bio-systems. The explanation of its role is not absolutely clear, but STEINBERG (1962) proposed that Calcium ions interacted specifically with hydrated protein surfaces, thus altering the molecular conformation and the hydrophilic-phobic balance. JARVIS et al (1964) reported considerable success in the biomedical applications of incorporating diffusable surfactants in some plastics, in order to increase or decrease the adhesive qualities of their surfaces. Shafrin and Zisman (1968) demonstrated that the incorporation of Chlorine atoms in the outermost structure of "adsorbed materials" can significantly increase their critical surface tension of wetting.

These "adsorbed materials" normally refer to conventional synthetic adhesion promoters which did not quite meet the requirement for a low enough contact angle to enable organic liquids to spread spontaneously on their surfaces. (Tripathi 1977)

Lee and Kim (1974) studied the adsorption of albumin, gamma-globulin and fibrinogen from solution onto

several hydrophobic polymers, using internal reflection infra red spectroscopy, under both static and flow conditions. They found that the rate and amount of protein adsorbed was dependent upon both the nature of the polymer substrate and on the flow rate of the solution. They postulated that increasing flow rates delayed the adsorbtion plateau, due to the shear forces opposing the diffusion of protein molecules into the surface. They also suggested that a more nonthrombogenic surface should show a higher albumin adsorption rate with a higher concentration than it does with either gamma-globulin or fibrinogen.

KIM (1976) further suggested that platelets adhere to adsorbed fibrinogen and gamma globulin molecules through a complex formation between incomplete heterosaccharide moieties on the adsorbed molecules and platelet glycosyl transferases on the platelet membrane. Since albumin has no such moieties this explains its lack of attraction for platelet adhesion.

Thus it is the newly formed outermost adsorbed film which will determine the ultimate compatability of the surface with its biological environment.

The Analysis of Accumulations on Hydrogel Contact Lenses

All polymers available for the manufacture of hydrogel lenses, have chemical sites which are potentially available as anchorage sites for protein deposition. Cordrey (1974) investigated the extent to which this occurred by the technique of Ultra Violet flourescence spectroscopy. Samples of Sauflon and Polyhema were prepared in four ways:-

(1) Uncontaminated as a reference

(2) Soaked in protein solution after hydration in water

(3) Hydrated in an aqueous protein solution

(4) as in (3) and subsequently chemically cleaned.

The results showed that those samples which were prepared as method (3) were significantly more contaminated than those from condition (2). This emphasised that lenses must be fully hydrated before insertion in the patients' eyes, to help minimise the amount of deposits. The results also showed that the efficacy of the chemical cleaner was high, since it removed a substantial amount of the bound protein, particularly that from the Sauflon material.

Karageozian (1974) used the technique of amino acid analysis to investigate the protein deposits

on six human worn Bausch and Lomb Hema, contact lenses. There was an extremely high correlation in the amino acid ratios for the contact lenses and the published values for Human Tear Lysozyme, and it was concluded that this was the main constituent of hydrogel lens deposits.

Ocular Factors which Influence Accumulations

Hathaway and Lowther (1978) reported that although clinical intuition prompted a suspected relationship between tear protein levels and deposit rate on soft lenses, that in fact their study did not show any significant correlation. However they did find that the quantity of the tear film, as measured by Schirmer strips, was highly correlated with the rate of deposit formation. An even higher correlation was found to exist between the tear film break up time and deposit formation. They postulated that the drying and thinning of the tear film on the lens surface, concentrates proteins and other tear components on the lens surface, resulting in a rapid build-up of deposits.

Classification Systems for Hydrogel Accumulations

Rudko and Proby (1974) proposed a method for the classification of protein deposits on hydrophilic contact lenses. The grading scale is composed of

three major sections:-

(1) General visibility of films and deposits

(2) Characteristics of films or deposits

(3) Area covered by the films and deposits

Each of these three sections are divided into specific sub-sections which allow for a detailed description of the general state of the lens surfaces. It is a relatively simple classification system and could be carried out by most practitioners since it does not require expensive apparatus or large amounts of time. However it is very subjective and inter-practitioner variations would be predictably high. Lowther and Hilbert (1975(a)) proposed that the clinical aetiology of hydrophilic lens deposits could be determined by their specific appearance under bio-microscope or regular light microscope. Non-distilled water was named as the cause of discrete spots, appearing to have a crystalline nature. They compared these deposits with those found on hydrogel lenses which Ruben (1976) has shown are due to high tear calcium levels. He found that 10% of the analysed lenses had calcium present. Lowther and Hilbert (1975(a)) suggested that the more uniform and larger deposits are due to incomplete cleaning of the tear film constituents from the lens surfaces prior to asepticising.

The Pharmaceutical Approach to Hydrogel Accumulations

Thus slowly the realisation that the problems associated with hydrogel lenses were mainly due to those factors which were also responsible for their chief attractiveness i.e. the presence of hydrophilic binding sites within the lens matrix and on the lens surfaces. Initially responsible for vastly increased comfort levels and eventually after a few weeks/months wear, also responsible for the accumulation of environmental and tear film debris. This opened the flood gates for the introduction of many new cleaning and disinfecting systems for hydrophilic lenses. There are three common types of cleaning agents used for hydrophilic lenses. (HATHAWAY and LOWTHER 1978) Surfactants, oxidising agents and enzyme cleaners are all currently advertised within the optometric press. Each method seems to possess inherent advantages and disadvantages. The three groups are outlined below:-

The first group are SURFACTANT cleaners. These depend on their ability to lower the surface tension interface between the polymer and aqueous medium as outlined by SHIVLEY (1975). The overall purpose of the surfactant is to mobilise, emulsify and remove proteins and other accumulated material. Examples of this type are SOFTMATE T.M. Barnes-Hind Pharmaceuticals) and PLIAGEL T.M. (Flow Pharmaceuticals. These are recommended for

prophylactic use to prevent the deposits.

The second group are the OXIDATIVE cleaning systems such as REN-O-GEL T.M. (Flow Pharmaceuticals) which work best when combined with heat. Their postulated action by ERIKSON (1975) is that they modify the tertiary structure of the denatured protein. He does emphasise that although the deposited material is greatly reduced by the use of oxidising agents so the deleterious effects on the lens structure increase with continued usage. He shows these changes to be caused by oxidation of the basic HEMA polymer chain with the introduction of pH sensitive molecular groups, thus producing a "sensitisation" of the lens surfaces which after the initial cleanings, actually seemed to have an increased attraction for debris.

The third group is the enzyme digestion system e.g. as developed by Allergan Pharmaceuticals. Enzyme cleaners act by hydrolising the peptide linkages of the denatured protein on the lens surface as demonstrated by RUDKO and GREGG (1974). They investigated for possible toxicological and sensitisation effects and found that PAPAIN, the enzyme of choice, showed no ill-effects.

Hathaway and Lowther (1978) in an independent comparison of cleaners from the three previously discussed groups, concluded that the Allergan

enzyme cleaner was the most effective available at that time, but that it was not capable of removing all types of deposit formation. They found that lab-coated lenses with gamma-globulin and glycoprotein were resistant to all cleaning systems, but those coated with albumins and lysozyme were effectively removed using enzymatic cleaning with Allergan's Hydrocare T.M. Karageozian (1976) used the amino acid analyser to illustrate the efficacy of Allergan's cleaning routine. However only lenses coated with lysozyme were used. BLANCO, CURRY, BOGHOSIAN (1975) determined some physical parameters of a batch of BAUSCH and LOMB SOFLENS, before and after enzymatic cleaning procedures. The physical parameters included diameter, water content and refractive index. Both PAPAIN and FICIN were used as proteolytic enzymes, but only PAPAIN, obtained from the dried and purified latex of the CARICA PAPAYA tree, left the measured physical characteristics unchanged.

SHIVELY (1975) discussed the requirements for a hydrophilic lens cleaning and disinfection systems and proposed that PLIAGEL T.M. should be used as a prophylactic cleaner with PLIACIDE as its chemical disinfection counterpart. Additionally REN-O-GEL was suggested as a rejuvenation system for use to remove any opacified proteins. SHIVELEY

(1976) presented further details of laboratory and clinical testing of the PLIACIDE disinfection system using WEICON and BAUSCH and LOMB hydrophilic hema lenses. The results showed that only minimal changes seemed to be induced as a result of the disinfection.

Hydrophilic contact lenses present a unique situation in the contact lens field since they can actually adsorb and bind many of the chemicals used in cleaning and disinfection systems, which are present solely in a preservative role. (GUMPELMAYER 1976). This is a matter for concern since the relatively large surface area of these lenses provides a potential store for these substances which presumably with changes in environmental pH, tonicity etc, may release them in relatively high concentrations. Many preservatives were investigated by PETRICCIANI and KREZANOWSKI (1977). They concluded that the only preservative which may give rise for concern was Benzalkonium Chloride, which is normally only used in solutions for hard, rigid contact lenses.

Davies (1976) however felt it was necessary to establish a balance between the two opposing interests of legislating regarding a minimum antibacterial activity for contact lens solutions, (and therefore a minimum preservative concentration)

and keeping the level of preservatives below a potentially hazardous level within the lens matrix. OTTEN and SZABOCSIK (1976) investigated the preservative binding of Chlorhexidene and Thimerosal to BAUSCH and LOMB Soflens, where they found that although initial levels within the solutions were very low, in the order of 0.005%, that small quantities of the preservatives could be washed from the lens, long, after it had been removed from the storage solution. They proposed that tear fluid can wash the lenses and extract preservatives in the same way.

Their results showed that:-

(1) THIMEROSAL, THIMEROSAL with DISODIUM EDETATE and PROPYLPARABEN bind weakly.

(2) PHENYLMERCURIC ACETATE and PHENYLMERCUBIC NITRATE bind moderately.

(3) CHLORHEXIDENE GLUCONATE, BENZALKONIUM CHLORIDE and an ALKYL TRIETHANOL ALBUMIN CHLORIDE COMPOUND bind strongly to the lens material.

The debate surrounds the extent to which preservatives can be stored either in the lens or in the corneal tissue. PHARES (1972) investigated the use of two preservatives found in contact lens solutions.

Pharmacological studies for evaluating the lens/ solution compatability were conducted using albino rabbits. The lenses used were BAUSCH and LOMB Soflens and GRIFFENS Natural Lens. He found that Benzalkonium Chloride concentrated in toxic amounts and Thimerosal caused irritation when it had prolonged contact with the ocular tissues. He recommended the use of periodic cleaning procedures to remove absorbed preservatives from within the lens, as a safety precaution.

A disturbing report by Cooper and Constable (1977) described eight cases of infective keratitis in wearers of soft lenses for therapeutic reasons, in which four (one continual wear and three daily lens wearers) were found to be using contaminated lens solutions, demonstrates the need for stringent hygiene and the use of effective preservatives within the solutions.

It is difficult to assess the objectivity of reports regarding the efficacy of different cleaning and storage solutions since they are often published by personnel from the various pharmaceutical companies who have an obvious vested interest.

CHAPTER 5

Alternate Methodology

Initial Overview

One of the initial approaches to the problem was to closely investigate any patients who were experiencing a deterioration in comfort and visual performance when using their HYDROGEL contact lenses. This was done using a NIKON slit-lamp to view the lenses in situ, and some interesting photographs were taken using a camera attatchment to the slit-lamp. The observed deposits in the surfaces differed in degree and type. Some lenses had an overall "oily" film on the surface sometimes with discreet crystalline deposits apparently superimposed. The patients were always asked to blink firstly a few times to ensure that the deposits were firmly attached. The discrete deposits occurred principally on the anterior lens surface while the oily film could occur on either surface, but again was most likely on the front surface. Some of these "soiled" lenses were then observed under a microscope at differing magnifications from x 1.25 to x 25. Some of the lenses were spun-cast and some were lathed. It was interesting to observe that there was apparantly no greater accumulation of deposits along the clearly visible lathe marks of some lens surfaces. Due to the critical

focusing of the microscope it was possible to observe that some of the larger crystalline type of deposits actually protruded from the lens, while some of them seemed to have become imbedded into the lens matrix. No fundamental clinical impression of differing deposits on the various lenses of differing water contents, was gained from these initial observations.

Photography

Careful consideration had to be given to the photography of these hydrogel lenses, since the heat given out by the light sources accelerated the water loss from the polymers, and the lenses, very quickly, showed the characteristic shape distortion associated with these drying effects, especially with the higher water content hydrogels. Initially a clean, unworn Bausch and Lomb 38% water content hydrogel lens was used to assess the optimum values for this type of photography using a WATSON-BARNET microscope fitted with a x 10 objective and a x 10 eyepiece. A NIKON camera was attached. The film used was KODAK PLUS x PLAN. Graticules were used in the form of a transparent stage micrometer of 0.1 mm and 0.01 mm to assess the actual magnification of the system. The exposure time was varied from 1/125 to 1/15 seconds, using both full and reduced field iris, taking care to

maintain an even field illumination. The results showed that using the full iris, the exposure time of 1/60 gave the best results. Using the reduced field iris the 1/30 seconds was better than the 1/15 second, but it was felt that the optimum would be around 1/25 second.

A graticule was then introduced into the viewing system of the microscope to assess the relative sizes of the deposits and surface debris. However to follow the development of any particular deposit accurately, it was necessary to make reference points on the lens surface so that the subsequent photographs could be taken with the lens in exactly the same position. Using dehydrated buttons it was decided to make four very small marks at 90^{o} intervals around the edge of the surface. However when these buttons were subsequently hydrated and boiled a few times, surface cracks appeared around these markings very quickly. Then using some indelible marking ink the same markings were used, which were initially fairly successful, however subsequent boilings gradually erased these markings. It was felt that this system could not be used on lenses which may actually be worn by human subjects. Thus an effective, permanent non-toxic method of marking the lenses could not be determined.

Protein Specific Staining

Having decided that the deposits on the hydrogel lenses were indeed a major problem for any long term wearing prospects, the next step was to determine if a protein- specific stain had an effect on the deposits. The use of any of these stains would render the soft lens useless and thus since finances did not permit an unlimited source of prescription lenses, it was decided to use discs of polymers, cut from rods donated by C.L.M. Using a lathe, discs of 3mms thickness were cut from rods. These were hydrated for a period of 48 hours and boiled twice in physiologic saline solution. It was decided to try two commonly used protein stains. The first, BIURET, stains proteins a pale lilac colour. The second, MILLONS REAGENT, stains protein a deep-wine colour. After various experimental trials it was decided to use only the MILLONS reagent for two reasons:- (a) it was a very deep coloured stain which is preferable for photographic purposes and (b) it is virtually permanent, whereas the BIURET stain fades rapidly after about one week.

Laboratory "Soiled" Lenses and Hydrogel Buttons

When the buttons were fully hydrated, indicated by a stable hydrated weight over at least 3 days, they

were immersed in a solution of physiologic saline and lysozyme. Two concentrations of lysozyme were used: (1) Physiologic concentration which was 0.169 gm LYSOZYME/100 ml (ADLER 1965) and (2) 10 x physiologic concentration. The lenses were subjected to a varying number of cycles where one cycle constituted immersion in the lysozyme solution for eight hours and then boiled in physiologic saline for twenty minutes each day. The buttons were then stained using the Millons reagent and it was obvious that buttons which had been in lysozyme solutions stained and the "controls" which had only been hydrated and stored in physiologic saline did not stain. Since the buttons had undergone 1 - 14 "daily cycles" the interest was in any differential amount of staining. By observation, the protein stain, seemed to penetrate further into the buttons which had undergone a larger number of cycles. However it was also observed that after repeated boilings, the surface of the buttons were beginning to split and since this effectively increased the surface area of the buttons it was decided to repeat the experiment in an attempt to decrease the tearing of the surfaces due to repeated "hot and cold" cycles.

The cutting technique for the buttons had to be modified to cut buttons smaller than the original 3mm thickness (dehydrated measurement) due to the effect of heat during the cutting process and also due to mechanical limitations of the lathe.

Eventually consistent thicknesses of 1mm were achieved. These were then hydrated and subjected to the same procedures as were originally used. Their hydrated thickness was approximately 3mms and they did not suffer from the surface cracks of the thicker buttons. The stain still looked as though it penetrated beyond the surfaces of the buttons.

Freeze-Sectioning of Hydrogel Buttons

In order to determine the extent to which this penetration occurred, the buttons were sliced using a PEARSE SLEE CRYOSTAT.

This process had been feasible with the thicker buttons, but proved a very difficult, delicate procedure with the thinner buttons, since they tended to crack more during the freezing process. The shorter sections, caused by the reduced thickness, tended to curl up at the edges making it extremely difficult to achieve a full flat section from which the penetration could be measured. However it was sufficiently successful to determine that penetration of the polymer surfaces had occurred within 2 or 3 cycles. Thus it was tentatively suggested that the problem being dealt with could not be considered solely as a surface phenomenon, at least if boiling methods of asepticising were used with higher water content hydrogels.

Comparison of "Lab-Soiled" Buttons and Human Worn Lenses

Visual inspection of the pre-stained button surfaces (both with and without magnification) did not reveal similar deposits to those obtained with human worn lenses. Generally the surfaces of the buttons looked cloudy, as would be expected with denaturised protein - but no discrete deposits were observed. A possible reason for this was thought to be the lack of "drying" effects on the surfaces as would be experienced with a human worn contact lens, on the front surface between blinks. Thus a model was constructed, in which the lens or button, was held in a small, supporting basket with many small holes and was dipped in and out of a solution at a variable frequency. The range of frequency was 2-30 cycles per minute. The ratio within each cycle of the lens being in and out of solution could be changed by the introduction of a delay mechanism. Initially the solution used was the lysozyme again in physiologic and x 10 physiologic concentrations. However since the deposits on the lenses still did not resemble those on human worn lenses, it was decided to try albumin and globulin solutions, both separately and mixing all three together. The concentrations for these lab-made solutions can be found in the Apendix. Results showed that it was possible to

obtain the overall oily film and granular type of deposits with an accompanying reduction in the transmission properties of the polymer, only when all 3 proteins were mixed together to form the laboratory-made solution. Protein staining did occur into the polymer matrix, however the very large "mulberry" type of discrete deposits were not demonstrated using these "lab" methods. An attempt to determine the substances of which these deposits consisted, led to the sending of two heavily coated lenses to a Biochemical Analytical Service at Birmingham University for Amino Acid analysis. The lenses were sacrificed, but since they were apparantly uncleanable and grossly soiled lenses it was not considered a great loss. The results are shown in the Appendix together with the standard figures for lysozyme, which was the protein that the amino acid pattern most resembled.

Electrophoretic Techniques

Attention was then turned towards methods for tear protein analysis. Electrophoresis seemed the only realistic method for the very small volumes involved. The extensive literature on electrophoresis suggested that polyacrylamide gel electrophoresis would be the most promising support medium. Since the gel

pore sizes can be adjusted to each researchers' particular requirements by the varying of several parameters such as acrylamide to bisacrylamide ratio and overall percentages, buffer pH's etc. Many combinations were subsequently tried.

In order that all the proteins run towards the same electrode then the pH of the buffer solutions must be either more acidic than the isoelectric point of the most acidic protein or more alkaline than the isoelectric point of the most alkaline protein. This means using a buffer of pH less than 5.0 or more than 11.0. Since theoretically a low pH encourages surface adherence of proteins, a high pH buffer would seem to be preferable. Thus the buffer listed below was tried first.

pH = 12.0

36 ml of 5.0 M SODIUM CHLORIDE

15.2 ml of 1.0 M GLYCINE

12.4 ml of 2.0 N SODIUM HYDROXIDE

Dilute to 2 litres

This gave reasonable results for the albumin and globulin groups, however the lysozyme band was very faint and distorted, indicating that there had been a lot of heat given out during the

electrophoresis and certainly the running tubes felt very hot immediately after the electrophoresis run. Other high pH buffers did not show any marked improvement in the results. Thus it was decided to try an acid buffer listed below:-

pH 4.0

2.4 gm of ACETIC ACID GLACIAL

17.25 gm of 1.0M GLYCINE

Distilled water to one litre

The results seemed much better with this acid buffer the bands were sharper with little or no heat distortion and the lysozyme band was well defined From a conventional electrophoresis viewpoint, the best results are usually obtained with reservoir buffers around neutrality, however with the range of isoelectric points present in tear proteins (from 5.0 - 11.0), to obtain all the bands, this would mean:-

(a) Two runs for each sample since the electrode connections would have to be reversed for the second run.

(b) A fairly complicated procedure in which extra long tubes were used, and a gel polymerised in the lower half of the tube, with the sample

applied to the top of this first gel and then another gel polymerised on top of the sample within the top half of the tube. This would allow the basic and acidic proteins to migrate in opposite directions.

Method (b) was tried but polymerisation of the top gel was never complete and the gel never achieved its full viscosity. This was thought to be due to "impurities" from the sample which prevented complete polymerisation. Thus no retrograde runs were possible. Method (a) was not used since it would mean using two samples of tears for each complete analysis and this was not feasible since the tear volumes collected were being kept to a minimum. It was then decided to return to the acid reservoir buffer of pH 4.0, in order to estimate the optimum acrylamide gel constituents.

In polyacrylamide gels the main monomer is acrylamide and this is used in conjunction with the comonomer, methylene bis acrylamide. As previously described the concentrations of the monomers can be varied to suit individual requirements. Apart from attaining the correct "pore" size of gel the amount of monomers was being kept to a practicable minimum since bisacrylamide and acrylamide have a very high absorbtion rate in the ultra-violet (U.V.)

range of the spectrum and it was hoped at this stage, to scan the gels directly with U.V. This procedure of direct scanning with U.V. is very useful since it is very quick and convenient, obviously it means that the gels don't have to be stained and destained and transferred to different tubes.

The most common concentration of acrylamide gels is around 7.5% to 5% but up to 30% gels have been used. The concentration referred to is that of the final concentration in the monomer-buffer-catalyst mixture, and the stock solutions are consequently of much higher concentrations. It was decided to make some 7% and 5% acrylamide gels each with varying bisacrylamide contents of 1%, 0.5%, 0.4% and 0.3%. It should be noted that as the acrylamide and bisacrylamide content decreases so the time taken for polymerisation increases and the completed polymers become more fragile. All the gels made in this way proved to be sufficiently durable. For all these gels one volume of monomer solution was mixed with two volumes of ammonium persuphate (the catalyst) and one volume of gel buffer. Various gel buffers were used, having different pH values and the most effective is listed in the Appendix.

The lowest practicable ratio of Bis:Acryl for a 5% acrylamide gel was found to be 1:30. Thus for 25ml of stock (Monomer) solution the amounts involved were:-

5 gm ACRYLAMIDE
0.17 gm BISACRYLAMIDE
(Mixed in Distilled Water to 25ml)

Several trial runs with both human tears and lab-made protein solutions as samples, on the gel, showed that the aim of using U.V. to detect and quantify the proteins was just not feasible with such small volumes (around 5µl) of these samples. The background absorbtion of the gels themselves, was as much, if not more, than that from the proteins. Gels which had been run with samples, and stopped when the tracer dye was about 10mms from the end, were then scanned using a Joyce-Loebl Densitometer with an U.V. source. These traces can be seen in the appendix, and obviously no proteins could be distinguished from the transmission or reflection characteristics of the gel. When these same gels were then treated with a protein sensitive stain, the bands showed up easily and distinctly.

Protein Sensitive Stains and Densitometry

Thus reluctantly it was decided that the gels would have to be scanned with visible light to produce a transmission trace. Acrylamide gels have excellent transmission properties in the visible spectrum. Various protein sensitive stains were tried such as Naphthalene Black, Coomassie Brilliant Blue R250, and Ponceau S. Coomassie Blue R250 was found to be the most reproducible and easy to use, showing protein as a deep blue colour. It does require destaining which can be carried out by repeated changing of the destaining solution, or electrolytically. Coomassie Blue G250 stains protein a pale green colour and does not require destaining since it is not absorbed by the gel itself i.e. no background staining occurs. However, again perhaps because of the small quantities of protein involved, the staining of the protein was very faint and would not produce reasonable traces from the densitometer. Joyce-Loebl also recently introduced a Chromoscan Filter specifically for use with Coomassie Blue R250 and thus it was decided to order this filter and use Coomassie Brilliant Blue R250, as the optimum stain for the protein fractions.

Sodium Dodecyl Sulfate (S.D.S.) as an Electrophoretic Additive

Since Ultra Violet was no longer being used it was then decided to try incorporating S.D.S. into the gels, as now its disadvantage as an "impurity" to U.V. was not relevant. At this time, S.D.S. polyacrylamide gels were producing marked improvements especially in the analysis of membrane proteins (WEBER et al 1972).

The incorporation of S.D.S. both with the sample as applied to the top of the gel, and into the gel polymer itself, produced a significant improvement in both the resolution and reproducibility of the bands.

The final recipes for the gels, buffers, staining and destaining solutions are given in the Appendix.

CHAPTER 6

Electrophoresis and Densitometry

CONVENTIONAL AND SODIUM DODECYLSULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS

The general principle on which all electrophoretic techniques are based, is that when an electric field is applied across a charged particle or group, then these particles or groups will move towards the anode or cathode depending upon their own individual charge. The term was first used by Michaelis (1909) to describe the movement of colloid ions in an electric field. Most early electrophoretic separations were carried out using a "free solution" of ions, known as "moving boundary electrophoresis" and highly developed by Tiselius (1950). However the inherent problems with this technique such as high cost of apparatus, difficulties in isolating separate zones and that of fixing the zones after the charged field has been removed, led to the introduction of several types of stabilising media, in which the separative techniques were performed. (BLOCK et al 1955). This type of electrophoresis is known as Zone electrophoresis, since the ions after separation remain as discrete areas or zones on the supporting medium, and thus facilitate several alternative methods of detection.

The simplest and earliest form of zone electrophoresis was carried out using paper as the stabilising

medium (MARTIN and SYNGE 1945). In an attempt to increase the resolution of these separative techniques new media such as starch (Smithies 1955) agar (Consden et al (1946)), and cellulose acetate membrane (Kohn 1957), were introduced. Polyacrylamide gel was suggested by Raymond and Wong (1960) and Raymond and Weintraub (1959) as an improvement over starch gel Raymond (1962), and is used either as a horizontal or vertical slab, or in the "disc electrophoresis" of Ornstein and Davis (1962). The resolving power of methods for the separation of proteins was increased greatly by the introduction of these media. These polyacrylamide gels retard the migration of the proteins, and there is little doubt that this improved resolving power is due to the associated molecular sieving effects. Thus the rate of migration of an ion or charged group will be influenced by the applied current, the shape and the size and charge of the migratory unit. HJERTEN (1965). Thus it follows that units which differ in any of these factors, can be separated by electrophoresis. Several investigators have found that a sharpening of the separation bands occured when a chemically different buffer, with a different PH value, was used in the stabilising medium to that used as the electrode buffers. This is sometimes known as a discontinuous buffer

system. ORNSTEIN (1964). Some confusion arose as to why the technique of "acrylamide disc electrophoresis" was so named. It is because the bands or zones stack up as a series of concentrated discs at the beginning of the electrophoresis procedure and not because of any discontinuity of buffers, or because the proteins are located as a series of discs at the end of the experiment (SMITH 1976). Acrylamide disc electrophoresis possesses a number of advantages over the other electrophoretic techniques for the analysis of protein samples (RAYMOND and WEINSTRAUB 1959). Amongst these advantages are ease of preparation, greater resolving power, simpler and much quicker running times, thus requiring less man hours per experiment. Polyacrylamide is also stable and chemically inert over a wide range of chemical and physical conditions. However the most significant advantage is that the gels can be polymerised in differing concentrations, thus producing a variety of pore sizes. An increase in acrylamide concentration which results in a reduced water content, produces a decrease in pore size. Thus the gel characteristics can be constructed for a specific set of experimental circumstances, using this molecular sieving effect (HJERTEN 1965).

Proteins can be classified as ZWITTERIONS or AMPHOLYTES i.e. they will behave as acids or bases depending on the pH of their surrounding medium. Obviously somewhere between being a cation or an anion there will be a characteristic pH for each

protein at which these opposing charges are equally balanced and this is known as its ISOELECTRIC POINT (pI), where the protein does not migrate to the anode or cathode (SUTTIE 1972). Thus there are many conditions which can be altered to produce the optimum set of experimental circumstances for each investigator's particular requirements.

In the 1960's Acrylamide gel electrophoresis was extensively used as a technique for the separation and characterisation of proteins as a resultant of two factors (a) size, and (b) charge. In the very early 1970's it was thought advantageous to eliminate charge effects and thereby obtain a direct estimate of the size of protein molecules (REYNOLDS and TANFORD 1970 (a) and (b)). By introducing the anionic detergent sodium dodecylsulfate (S.D.S.), into the technique, charge effects were thought to be eliminated and separation was primarily due to molecular weights. (SHAPIRO et al 1967). While the principles remain unchanged, there do exist certain differences when S.D.S. is used in polyacrylamide gel electrophoresis. One of the most significant being that only micrograms of sample protein are required (PITT-RIVERS and IMPIOMBATO 1968). The S.D.S. binds to the proteins approximately in the ratio of one S.D.S. molecule to two amino acid residues, for S.D.S. concentrations above 0.02%

(REYNOLDS and TANFORD 1970 (a) and (b)). It is therefore important that during sample preparation the weight ratio of S.D.S. to protein is approximately 4:1. These protein/S.D.S. complexes, as studied by hydro-dynamic and optical techniques, would seem to behave as rodlike particles in which their length varies with the molecular weight of the polypeptide chain (FISH et al (1970)). These complexes contain a high degree of secondary structure, and should not be visualised as rigid rod like structures. Although the S.D.S. does help unfurl and regiment some of the structural bonds, the presence of disulphide bonds and chemical cross-linking within the protein, does limit the amount of S.D.S. binding, and thus produces decreased electrophoretic mobility, and a consequent deviation from expected molecular weight values (REYNOLDS and TANFORD 1970 (a) and (b)). The disulphide bonds limit the degree of unfolding of the polypeptide chains and this leads to decreased S.D.S. binding (GRIFFITH 1972) with consequent lower mobilities. Chemical cross-linking means that the proteins are not joined end to end, but are linked through internal (side-chain) residues which are randomly positioned along the polypeptide chain. Thus the deviations from the expected electrophoretic mobilities for proteins of that molecular weight are a function of specific bondings within the complexes which

produce spatial geometries outwith the conventional helical structures, normally attributed to proteins (SWANK and MUNKRES 1971). In practice however these deviations are not normally found to be as large as predicted and can be easily surmounted by the use of a Ferguson plot (FERGUSON 1964; SHELTON 1971). This is achieved by running the protein samples in different gels of varied acrylamide content and plotting the Rf values (mobility relative to dye marker), against the percentage acrylamide values. Anomalous proteins will have a different Rf_o value, i.e. obtained when the lines are extrapolated to zero acrylamide concentration (HAYASHI, 1974). Usually the high degree of S.D.S. binding, negates the intrinsic charge of an individual protein, however in practice it is found that some proteins still deviate from expected results which cannot be explained in terms of chemical cross-links or disulphide bonds, thus another possible explanation of these deviations is that very highly charged polypeptides bind decreased amounts of S.D.S. and consequently give lower than the predicted mobilities. GRIFFITH (1972) WADA and SMELL (1972) suggested varying the percentage acrylamide until an optimum level was found. Very low molecular weight polypeptide chains (less than 15,000 daltons) present a particular problem, since their short lengths begin to approximate their diameters and they deviate from the "rod-like" model of the heavier proteins. The very low molecular weight complexes may even

migrate with the marker dyes (WILLIAMS and GRATZER 1971). However careful control of the bisacrylamide content of the gels can solve this problem (DUNKER and RUECKERT 1969), since generally an increase in the cross-linking of the gel will produce a decrease in the pore size, thus providing some resistance to even the smaller molecular weight components.

Thus S.D.S. polyacrylamide disc electrophoresis is considered, at present, to be the most sensitive analytical tool for the separation of polypeptide fractions from very small sample volumes (NEVILLE, 1971). The reproducibility of a polypeptide relative to a marker has been given an accuracy of ± 2% (SMITH 1976). Absolute migration distances obviously vary more depending on batch to batch variation in gels and sample applications.

Detection Methods used after Electrophoresis

During every run using polyacrylamide gel column electrophoresis a tracking dye is used. Suitable dyes are bromo-phenol blue or methyl green depending on the pH of the gels and buffers. After the allotted time for the run, the marker dye is situated towards the lower end of the tube, - usually about five millimeters from the end. There are several methods which can now be used for the detection and measurement of the separated

proteins within the gels.

Proteins selectively absorb ultra-violet radiation, due mainly to the presence of the aromatic amino acids, TRYPTOPHAN, TYROSINE and PHENYLALANINE residues. Direct measurement of peptide bond absorbance in the far ultra-biolet (205 - 210 nm) is effectively prevented by the high ultra violet absorbance of acrylamide gel in that range (SMITH 1976). Therefore an ultra-violet source with a wavelength of between 260 - 280 nm must be used, which is unfortunate since protein absorption is both higher and more uniform at 210 nm (MURPHY and KIES 1960). Proteins also vary with respect to the concentrations of aromatic amino acids which absorb at 260 - 280 nm and therefore vary with regard to their molar absorbances (WATKIN and MILLER 1970). However this absorbance is constant for any particular protein at a given pH value. If the gel is polymerised inside a round quartz tube, it is possible to scan the gels directly, without removal from the tubes. The quantitative measurement of protein can then be assessed by the trace from the densitometer and compared with that for a protein standard solution. This is a very valuable method due to the speed and ease with which it can be performed (WATKIN and MILLER 1970). However sample amounts of protein usually have to be fairly large, although certain recent modifications

have been reported where it was possible to scan samples with as little as 5µl, of human serum (WATKIN and MILLER 1970).

INOUYE (1971) and WILLIAMS and GRATZER (1971) reported that protein samples mixed with DANSYL CHLORIDE prior to electrophoresis allows the bands to be visualised directly by their fluorescence under ultra violet light. Care should be taken as excessive dansylation can affect protein mobility. Another method of detection (AMES 1974) is by radioactively labelling the proteins before electrophoresis and using autoradiography and/or quantitated by scintillation counting of sectioned gels. The dried-paper backed gels are exposed to x-ray film and then transmission of different bands on the film can be measured. Gels can then be sectioned and the radioactivity of each slice measured using a conventional liquid scintillation counter (MAIZEL 1971). Probably the most common method for detection of the bands, within the gel, is to use a protein sensitive stain.Various staining solutions are available, one of the most recently introduced is coomassie brilliant blue R250 (Payne 1973), which is considered to be a much more sensitive and reproducible stain than those previously used, such as PONCEAU S, AMIDO BLACK and NAPTHALENE BLACK. The gels were completely immersed in the stain for

a standard time interval and then de-stained, to visualise the protein-bound stained bands. The gels can then be scanned by a conventional densitometer with a source in the visible part of the spectrum (PARISH and MARCHALONIS 1970). By using standard protein solutions both quantitative and qualitiative assessments of protein can be made. Differential uptake rates of coomassie Blue by different proteins have been reported (PARISH and MARCHALONIS 1970), but these effects are generally considered minimal, and Coomassie Blue would seem to be less of an offender in this respect than other protein sensitive stains (VESTERBERG 1971). Complexes of proteins with this dyd have been shown to follow the LAMBERT-BEER law up to higher concentrations than those with AMIDO BLACK. (GROTH et al 1963). Moreover, protein staining with Coomassie Blue R250 is extremely sensitive being able to detect protein concentrations down to 0.5 - 2.0 $\mu g/cm^2$. An interesting technique, recently developed by DIEZEL (1972) uses a derivative of Coomassie Blue R250, and is names Coomassie Blue G250. The usefulness of G250 arises from the fact that it is much less soluble in tricholoacetic acid (T.C.A. 12.5%), than R250 and therefore forms a colloid. (Diezel 1972)

When used as a staining agent it selectively binds to the protein zones and prevents the dye's penetration into the network of the gel, thus avoiding a stained background and obviously dismissing the need for the de-staining procedure (DIEZEL 1972). Generally larger amounts of sample protein are required, since the depth of colour in the bands is much less obvious than with R250, thus making quantitative scanning procedures less accurate (RIGHETTI and DRYSDALE, 1974; REISNER 1975).

It can be seen from the preceding discussions that the general technique of electrophoresis had advanced considerably over the past few decades. The analysis of biological protein solutions has developed from fairly crude methods, requiring large columes of sample (in order of Mls), to the very sensitive techniques of the present day which can operate with only a few µls. of sample solution. The biological fluid of interest here, is that of the Pre-Ocular Tear Film (P.O.T.F.) Initially work on the P.O.T.F. was done using filter paper electrophorecis. One of the major draw-backs of this technique was the length of time required for each run e.g. Erickson et al - 6 hours, Krause - 8 hours, and McEwen and Kimura - 16 hours. The volume of tears required was very large and produced relatively poor resolution of three major protein groups - Lysozyme, Albumin, and the grouped Globulins.

In 1949 Smollens et al reported 4 protein components in pooled tears analysed by liquid microelectrophoresis. Later CASELLI and SCHUMACHER (1954), reported 5 protein components in their filter paper electrophoretic analysis of tears. BRUNISH (1957) reported between 5 and 6 components depending on the number of bands in the globulin group. McEwen and Kimura (1955) and McEwen et al (1958) reported 5 components. It was thus accepted that there were 3 main protein groups. At the pH values used, mostly around neutrality or slightly basic (pH 7.0 - 8.0), it was established that one group always moved towards the cathode and the remaining groups to the anode. This positively charged group was identified as lysozyme, which has an isoelectric point of 10.5 - 11.0. BRUNISH (1957) reported the presence of 2 lysozymes of different mobilities in pooled tear samples from several subjects. A "slow moving" lysozyme was found by ERICKSON et al (1956) from several species of animals. Apparently lysozyme may possess different electrophoretic mobilities. (TALLAN and STEIN 1953). However only one lysozyme component is ever found in a single individual at any one time (McEWEN et al 1955). The fastest moving component to the anode was identified as ALBUMIN (McEwen et al 1957). This albumin component in tears has a higher mobility than serum albumin and is thus called "tear albumin". The globulin group correspond approximately with

the serum globulins (GAMMA, BETA and ALPHA 2). However they did not correspond exactly due to the fact that they stained with the MUCO-POLYSACCHARIDE procedures of FEENEY and McEWEN (1956). McEwen et al (1958) also established than when the lacrimal gland had been surgically removed then LYSOZYME and TEAR ALBUMIN disappear from the subjects' tear patters, thus indicating the source of their production.

The use of cellulose acetate membrane (C.A.M.) strips overcomes the time factor, as a complete run can be performed in about two hours. Usually 3 fractions plus lysozyme are separated. FRANCOIS and RANAEY (1960) used Agar gel for tear fluid analysis, however it was found that lysozyme did not migrate normally in this medium. It is usually precipitated due to an interaction between it and the gel. It was only with the fairly recent introduction of Polyacrylamide gel electrophoresis, that the resolution of tear samples into as many as 10 bands (averaging 7 or 8 bands) by FARRAL (1973) and 12 - 14 by CALLANDAR (1973) has been achieved. Farral's method for collection of tear samples was by inserting a schirmer strip into the lower canthus from which a 5mm disc was cut and inserted into the neck of the gel tube using forceps. Thus some of the sample tears were probably produced by reflex stimulation due to the foreign body sensation of

the strip, and the sample volume could not be assessed accurately due to it being absorbed onto, and then removed from the paper disc during the electrophoretic procedure. Callandar (1973) took 2 x 2 µl samples of tears from the outer canthus of both eyes of each subject using a micro pipette. One sample for each eye was stored, frozen for proetin determination at a later date, using AGAROSE GEL to identify the protein bands. The second 2 µl sample (one from each eye) was analysed immediately by acrylamide gel electrophoresis. The running tubes for the gels were very much thinner than conventional tubes. They were 1.8mms (internal bore). This was to enable 2 µl of tears (half of a 4 µl POOLED sample from R and L eye) to produce realistic results. One gel from each pair was stained with Coomassie Blue R250, and the other with P.A.S. stain. He found that 14 bands stained with Brilliant Blue and only 7 stained with P.A.S., showing that only 7 are glycoprotein complexes. A buffer of pH 8.5 was used. All but one of the fractions migrated towards the anode and obviously the cationic fraction would then be lost in the upper buffer reservoir. Therefore another 2 µl sample was applied to a fresh tube and another run was carried out using reversed polparity on the electrodes; for only half the time (5 mins) used for normal polarity. Thus lysozyme with its highly basic Io does create problems with electrophoretic

analysis of tear fluid.

There are many variables within the electrophoretic technique. The precise requirements of each individual experimental process can be met by carefully adjusting some or all of these variables until the desired sensitivity is achieved.

CHAPTER 7

Experimental Design

EXPERIMENTAL DESIGN

Before any rigid design of the proposed contact lens studies could commence the presence of absence of any time dependent variation in tear proteins was established. Therefore the first study to be carried out was to investigate any possible diurnal variation in either the total or individual tear protein fractions.

Selection of Subjects and Diurnal Group Design

Ten male subjects between the ages of eighteen and forty years old were monitored, for one day, at two hourly intervals between 8.00 a.m. and 8.00 p.m., thus constituting a "waking hours" study. Ten subjects were chosen since it was felt that the group size should be the same as for the envisaged contact lens studies, and this number of subjects in each group seemed to be the maximum that one investigator could competently supervise. Male subjects were chosen, since it was necessary to eliminate the possibility of menstrual influences on the tear proteins.

The age limits were imposed partly by the availability of subjects, but also for clinical reasons. The upper age limit excluded presbyopes and the lower limit was to exclude the rapidly fluctuating

prescriptions of the young myope. No abnormal restrictions such as dietary, were imposed upon the subjects. However any systemic or ocular pathology obviously eliminated several prospective subjects. The subjects were asked not to swim or use any eyedrops, and to consciously avoid any ocular irritants especially on the data collection days.

Collection of Tear Samples

For the diurnal study one 5µl sample was collected from each subjects right eye at regular, two hourly intervals. A microcapillary tube was used, placed gently at the outer canthus, while the subject looked steadily towards his left, to avoid any possible corneal involvement (Plate I)

The normal blink rate was maintained, and all forms of ocular irritation and therefore reflex lacrimation were avoided. This sample volume of 5µl , was chosen as a compromise between being about the average maximum normally obtainable without stimulation, over a reasonable period of time, about ten minutes, and being about the minimum volume from which reasonably consistent and repeatable analytical results could be obtained.

Initially it had been planned to take tears from some left eyes and some right eyes. Since sympathetic lacrimation can occur between the eyes it is not practical to take a sample from each eye of the same subject within a short time interval. Another reason for only using right eyes, was that since the microcaps are made of glass, it is a potentially hazardous technique and thus a very firm and steady hand is required to hold the microcap at the tear/lid junction. This was only considered sufficiently safe with the investigator's right hand and thus the subjects' right eye.

Hydrogel Contact Lens Groups

After the Diurnal study was completed it was decided to design three completely separate twelve week studies, to investigate any relationship between tear protein levels and three different hydrogel contact lenses.

These lenses were:-

		Water Content at 18°C
(a)	Bausch and Lomb soflens	(38%)
(b)	Sauflon 85	(82%)
(c)	Sauflon 70	(70%)

This then encompassed:

(a) a low water content - Hema, daily wear lens.

(b) a high water content co-polymer of methyl methacrylase and vinyl pyrrolidone, extended wear lens, and

(c) a relatively high water content co-polymer of methyl methacrylate and vinyl pyrrolidone daily wear lens.

The intention was to produce a representative cross section of lenses currently available to the contact lens practioner.

All the subjects were myopic with an upper limit of one dioptre on the degree of permissable astigmatism.

The groups were designed exactly as the diurnal group.

A control group of ten, male non-contact lens wearers with the same age limits was also monitored.

Each of the three experimental groups were fitted with their hydrogel lenses using conventional and recommended fitting techniques. For the group with extended wear lenses, fitting procedures included at least two overnight wearing trials to enable

(a) the subject to fully realise the requirements of the study and

(b) the researcher to estimate the ocular tolerance to extended wear lenses.

All subjects were fully briefed on the handling of their lenses before the trials began. The subjects in the daily wear lens studies, had to be competent in both insertion and removal of the lenses. The subjects for the extended wear lenses were shown and practised only the removal of the lenses for two reasons:-

(a) if the lens had to be removed for reasons of discomfort then it was felt that an ocular assessment should be made before re-insertion of the lens.

(b) it was felt that a danger of over frequent insertion and removal may exist for reasons of subjects convenience, rather than of discomfort and therefore the investigator would not be able to judge how permanent had been the wear of these lenses.

All subjects were informed that no "water sports" could be carried out during the wearing of these hydrogel lenses.

The cleaning procedure for each of the groups was as follows:

(a) for the Bausch and Lomb group.

Since both cleaning by chemical and asepticising methods are recommended for these lenses, it was decided that each subject would clean one lens by boiling in a Bausch and Lomb aseptor unit, and the other would be chemically cleaned using the solutions designed for this purpose by Optrex. Thus half the group, chosen randomly, cleaned the right lens using the aseptor unit, and the left lens with the solutions. The other half of the group reversed this process.

The contents of the Optrex solutions are given below:-

a) <u>Soft Lens Rinse</u>

Contains % w/v	
Chlorhexidene Gluconate	0.0025
Thiomersal B.P.	0.001
Disodium Edetate B.P.	0.001

b) Soft Lens Soak

Contains % w/v

Chlorhexidene Gluconate	0.005
Thiomersal B.P.	0.002
Disodium Edetate B.P.	0.001

In an Isotonic Buffered solution.

c) Soft Lens Clean

Contains a non-ionic surfactant in an Isotonic, Buffered solution preserved with % w/v

Chlorhexidene Gluconate	0.005
Thiomersal B.P.	0.002
Disodium Edetate B.P.	0.001

(b) Sauflon 70

Only one method of cleaning these lenses is recommended by the manufacturers and this is to use the solutions produced by Sauflon Pharmaceuticals. Thus this group used the recommended storage and cleaning solutions.

The contents of these cleaning solutions are given below:-

a) Steri-Sal

Sodium Chloride B.P.	0.9%
Disodium Edetate	0.10%
Thiomersal	0.002%
Chlorhexidene	0.001%

In a buffered aqueous polymer complex base.

b) Steri-Solv

Sodium Chloride B.P.	2.5%
Hypromellose	0.41%
Disodium Edetate	0.10%
Thiomersal	0.004%

In a buffered aqueous polymer complex base.

(c) Sauflon 85

The extended wear group of subjects did not use any cleaning procedures for their lenses. This was carried out by the investigator at regular four weekly intervals, throughout the study. The recommended "ALCAM" cleaner was again used.

It was impressed upon all subjects that extreme cleanliness and hygiene was required at all times when relating to the lenses.

Data Collection and Subject Instruction

The number of appointments which eachsubject made for data collection, throughout study, was the same for all groups ie. for three contact lens wearing groups and one control group.

They were as follows:-

DAY 1 – Wearing of Lenses commenced

DAY 2

ONE WEEK

TWO WEEKS

THREE WEEKS

FOUR WEEKS

EIGHT WEEKS

TWELVE WEEKS

All subjects were asked to report for these visits wearing the contact lenses, if possible. Firstly the tear sample was collected and then the eyes and lenses were assessed in the normal clinical routine.

1) History and any difficulties experienced by the subject relating to the lenses.

2) Retinoscopy and subjective assessment with the lenses in situ on the eye

3) Slit lamp examination of the eyes and lenses

Removal of lenses

4) Inspection of the lenses (cleaning if required)

5) Slit lamp inspection of the eyes without the contact lenses.

Anterior segment photography and staining of the corneae were sometimes performed.

It was impressed upon the subjects that they must report at the specified data collection times, and must also report as soon as possible if for any reason they experienced occular or visual discomfort, or were in any way concerned regarding their eyes or lenses. A twenty four hour on-call service was provided to the subjects in case of any emergencies.

The daily wear subjects were asked to wear their lenses for a maximum of three hours on the first day, and increase this by a daily maximum of three hours, until all day wear was achieved.

The extended wear subjects were asked not to remove their lenses unless any discomfort occurred. They must then report to the investigator as soon as possible.

Before the studies commenced, tear samples were collected from the subjects to establish the base line data for each subject. When the tear samples were collected in the microcaps they were then

sealed immediately at both ends, using cristaseal, and placed in the fridge at zero ^{o}C. The samples were never stored for more than a few hours and never frozen before the gel column electrophoresis was carried out.

All subjects were given a written description of the intended experimental procedures and asked to sign a declaration form agreeing to act as a volunteer experimental subject. This written description and declaration form are shown in the appendix.

CHAPTER 8

Results of the "Waking Hours" Study

DIURNAL VARIATIONS

There is known to exist a circadian relationship between time and several body functions. Specifically such a relationship exists between intra-ocular pressure and corneal thickness which relates to tear tonicity and possibly tear protein levels (WILEY 1975).

It was felt necessary to investigate for any waking hours variation in tear proteins before continuing with the contact lens studies, since this would effect the experimental design for the data collection times of the contact lens wearing groups.

Waking Hours Study

The results are given for ten male subjects monitored over a twelve hour period, between 8.00 am to 8.00 pm The mean and standard deviations are given for the total protein value in each 5µl sample, and also for each of the four sub-groups of proteins.

These sub-groups are numbered 1 - 4 and correspond to their positions beginning from the top end of the gels. On average a total of nine bands could be clearly distinguished on each gel and these

correspond to the quantified groups as follows:-

Table 8:1

GROUP	BAND NOS	FRACTION
1	1	UNKNOWN (see later)
2	2 and 3	TEAR AND SERUM ALBUMIN
3	4, 5, 6 and 7	α_1, α_2, β AND GAMMA GLOBULIN
4	8 and 9	LYSOZYME

The fractions as listed in the Table 8:1 are in order of increasing mobility (i.e. group 4 migrate most quickly during the electrophoresis analysis), for the particular conditions used in this electrophoresis, as specified in the experimental design chapter.

Results

The values for each subjects total protein value at every data point, was plotted against time. These graphs are shown in the test.

FIGURE 8:2

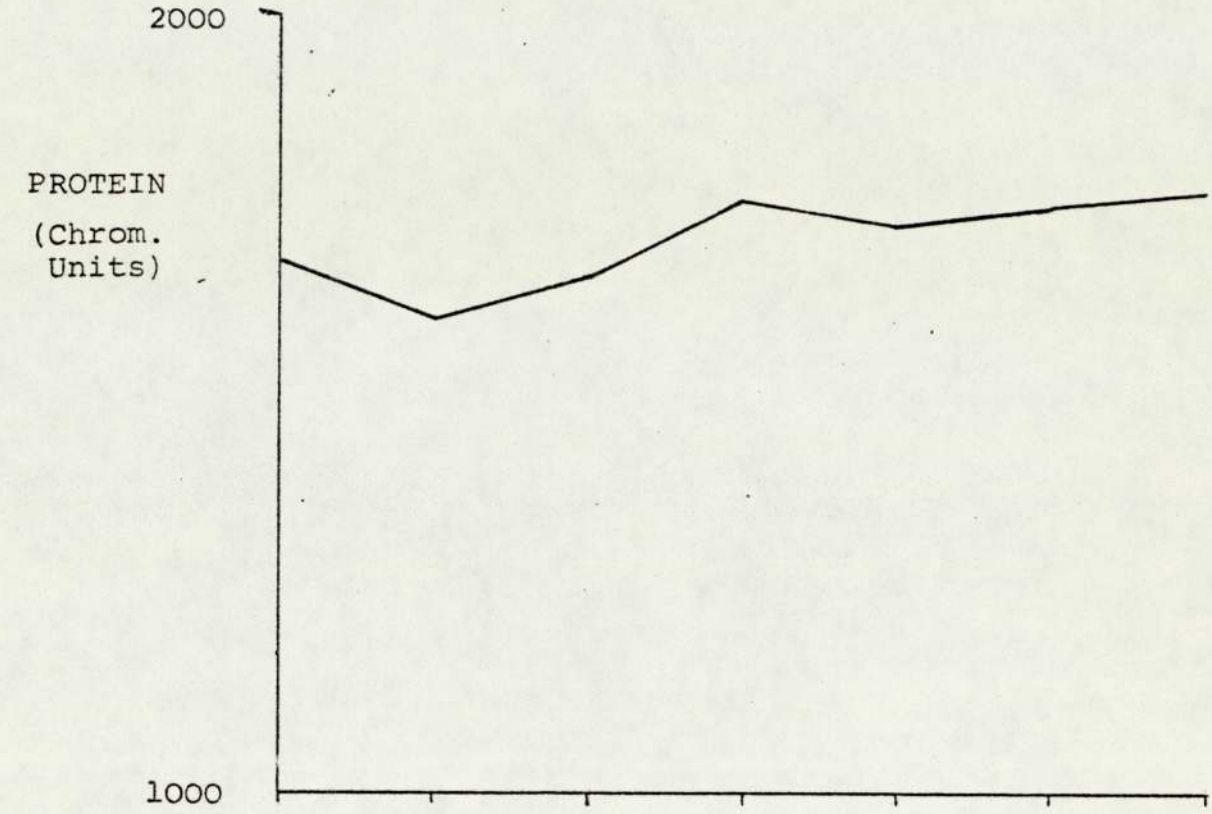


FIGURE 8:1

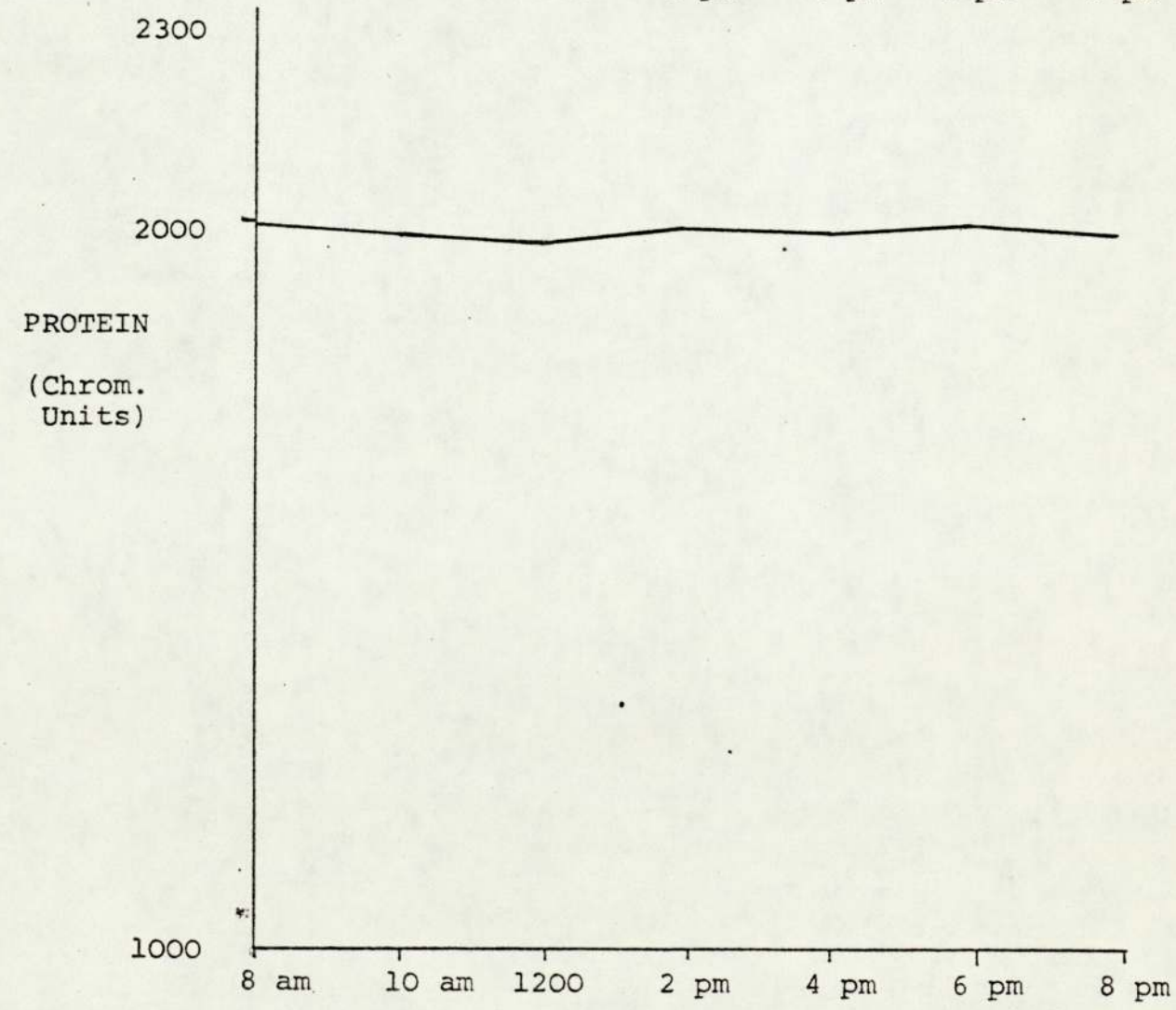


FIGURE 8:4

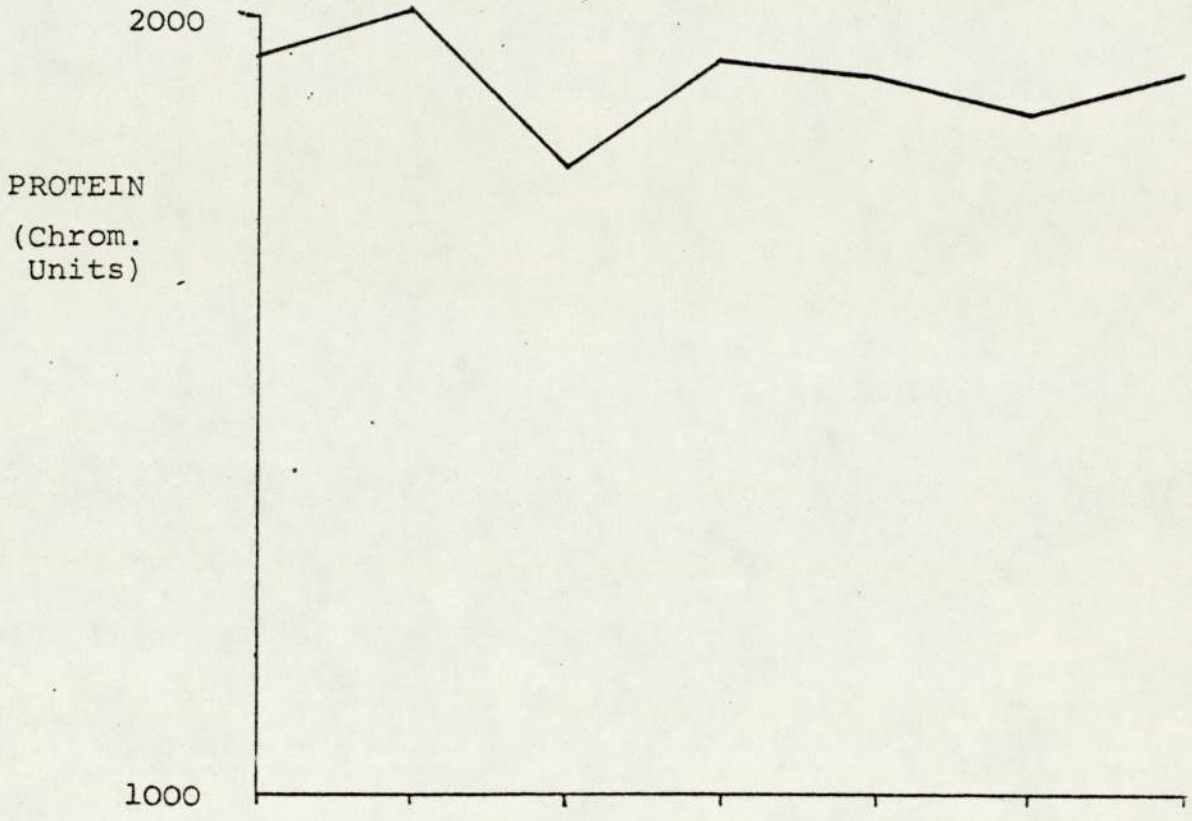


FIGURE 8:3

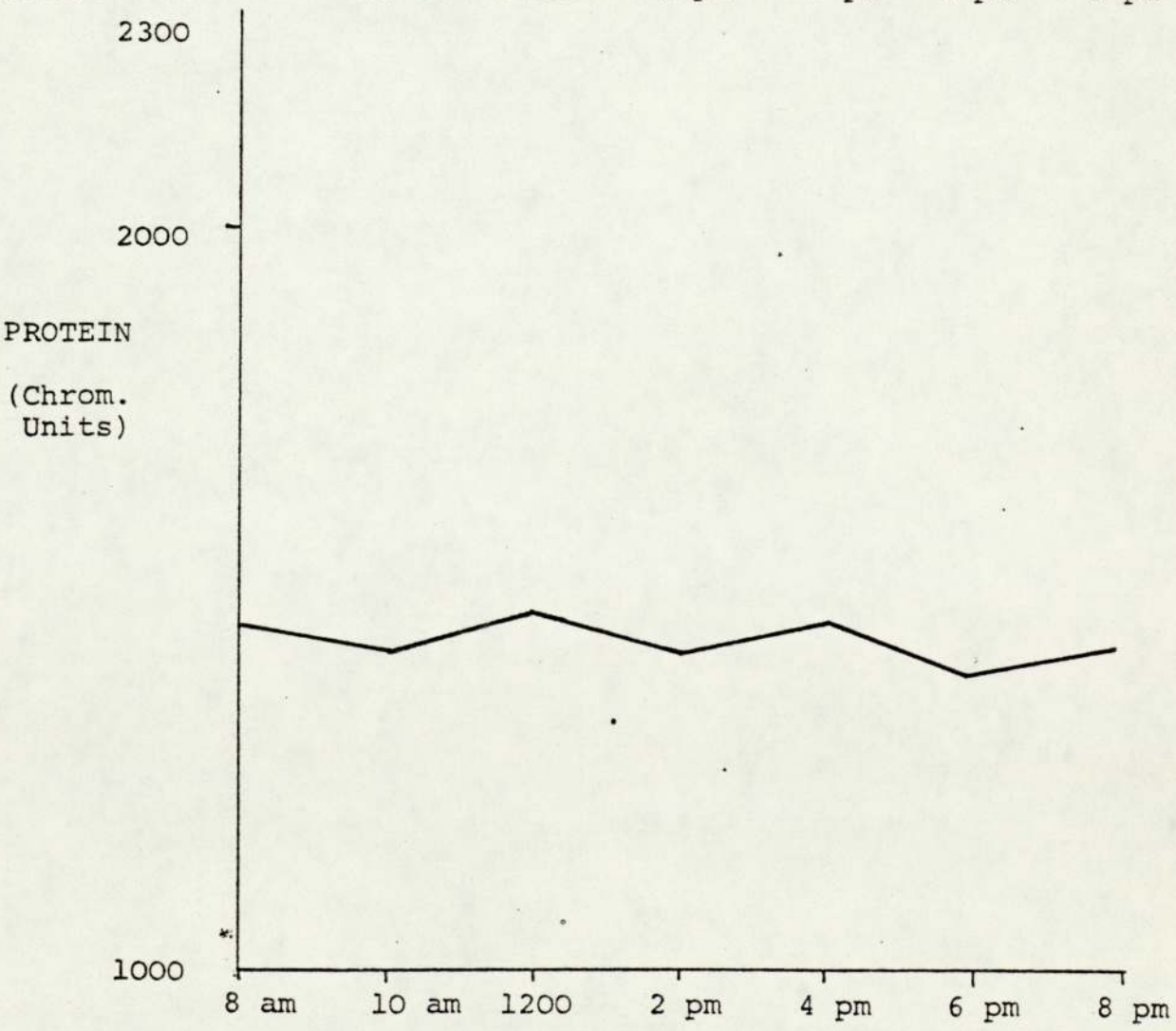


FIGURE 8:6

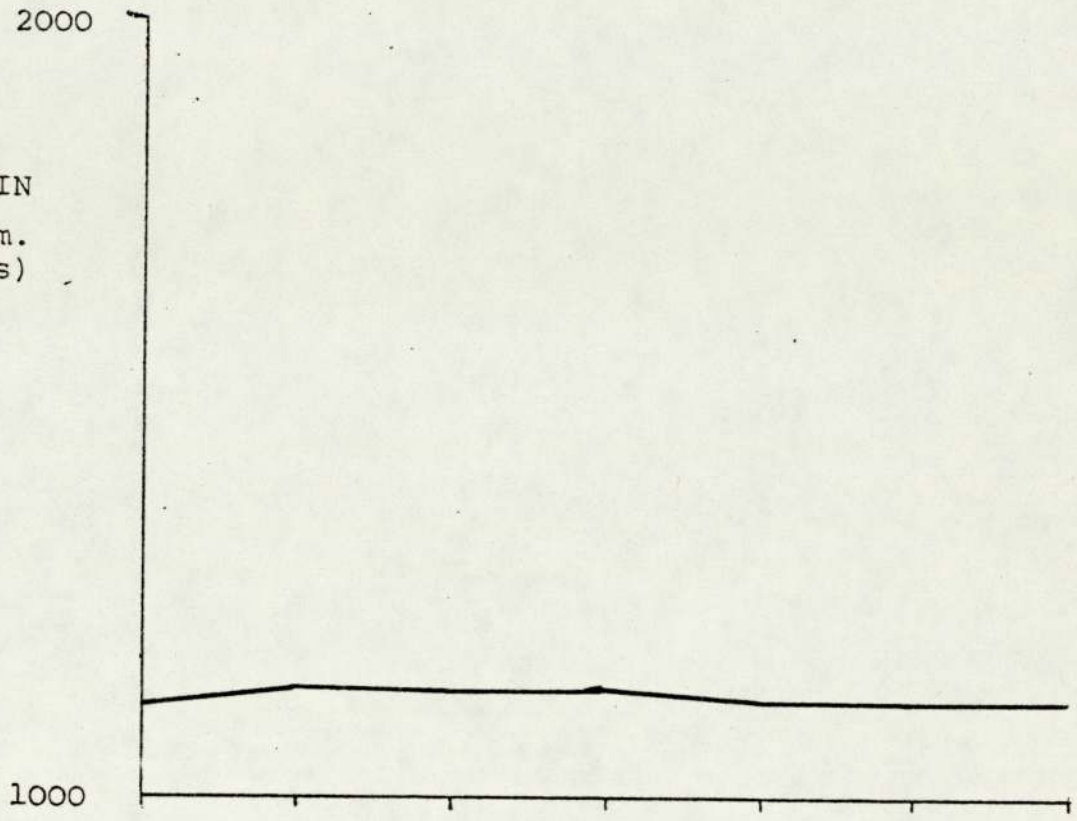


FIGURE 8:5

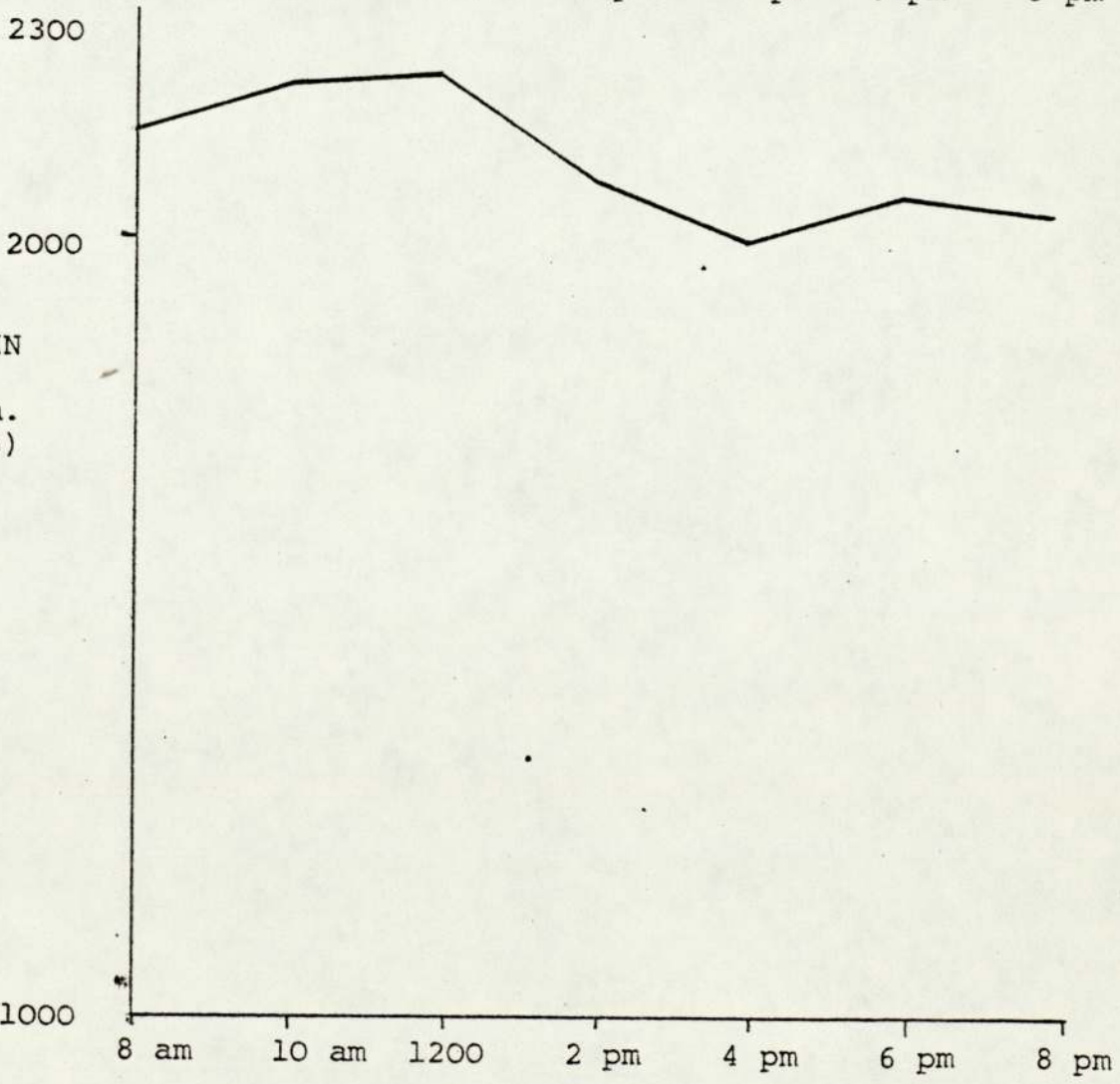


FIGURE 8:8

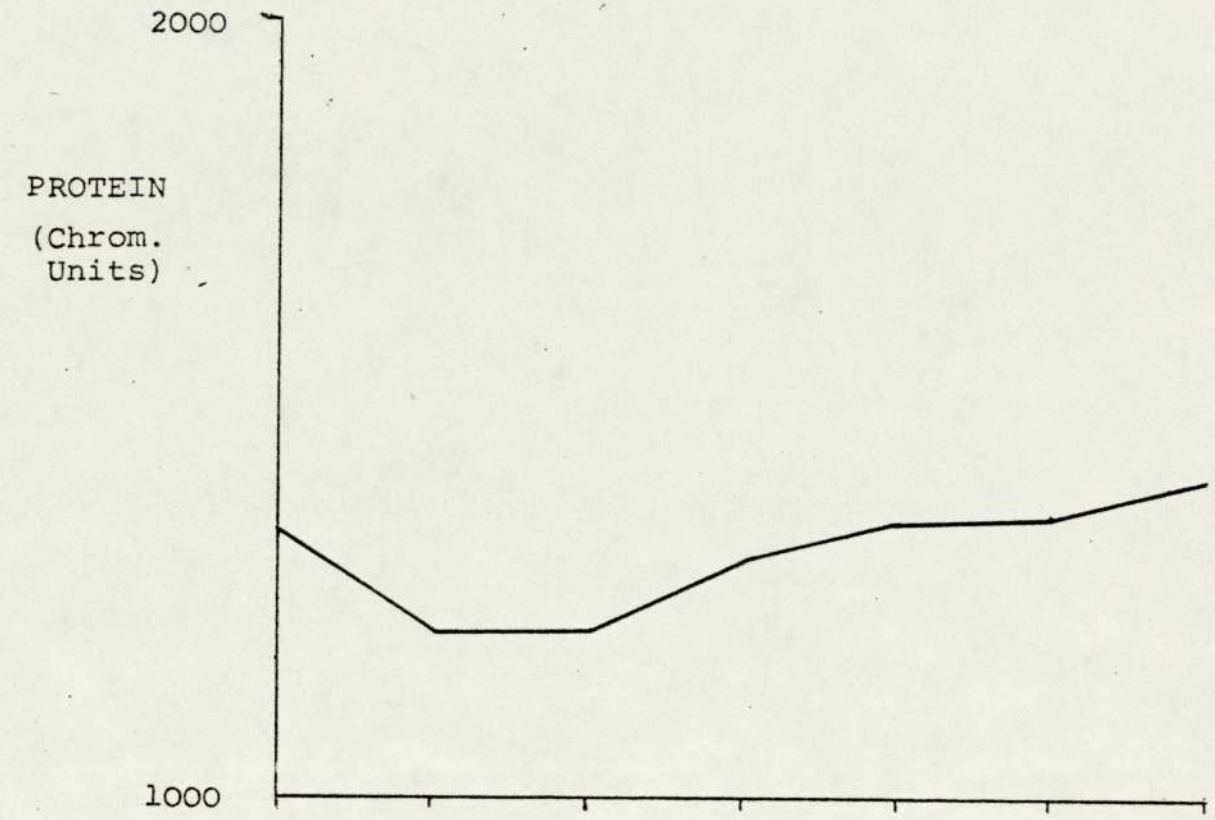


FIGURE 8:7

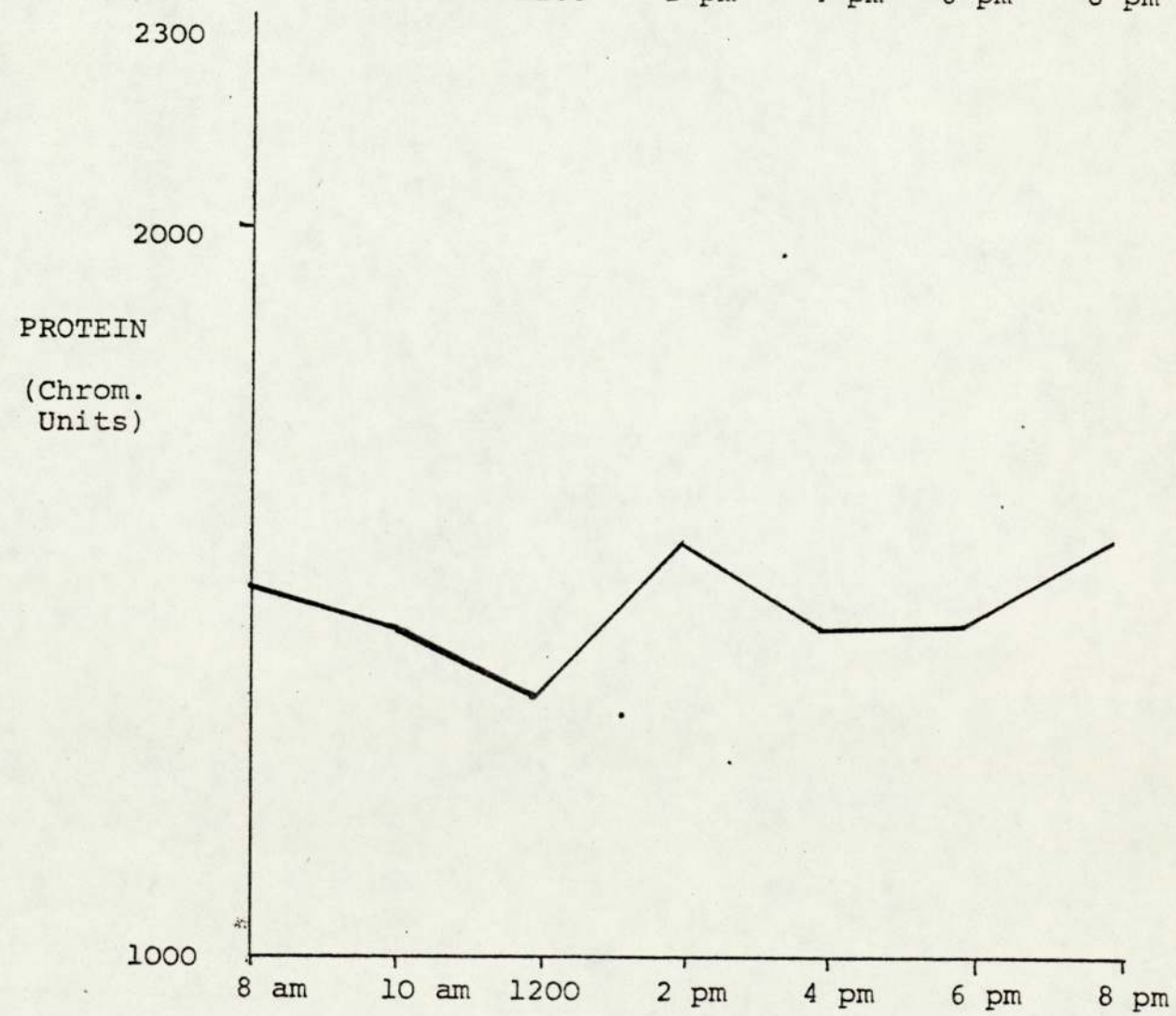


FIGURE 8:10

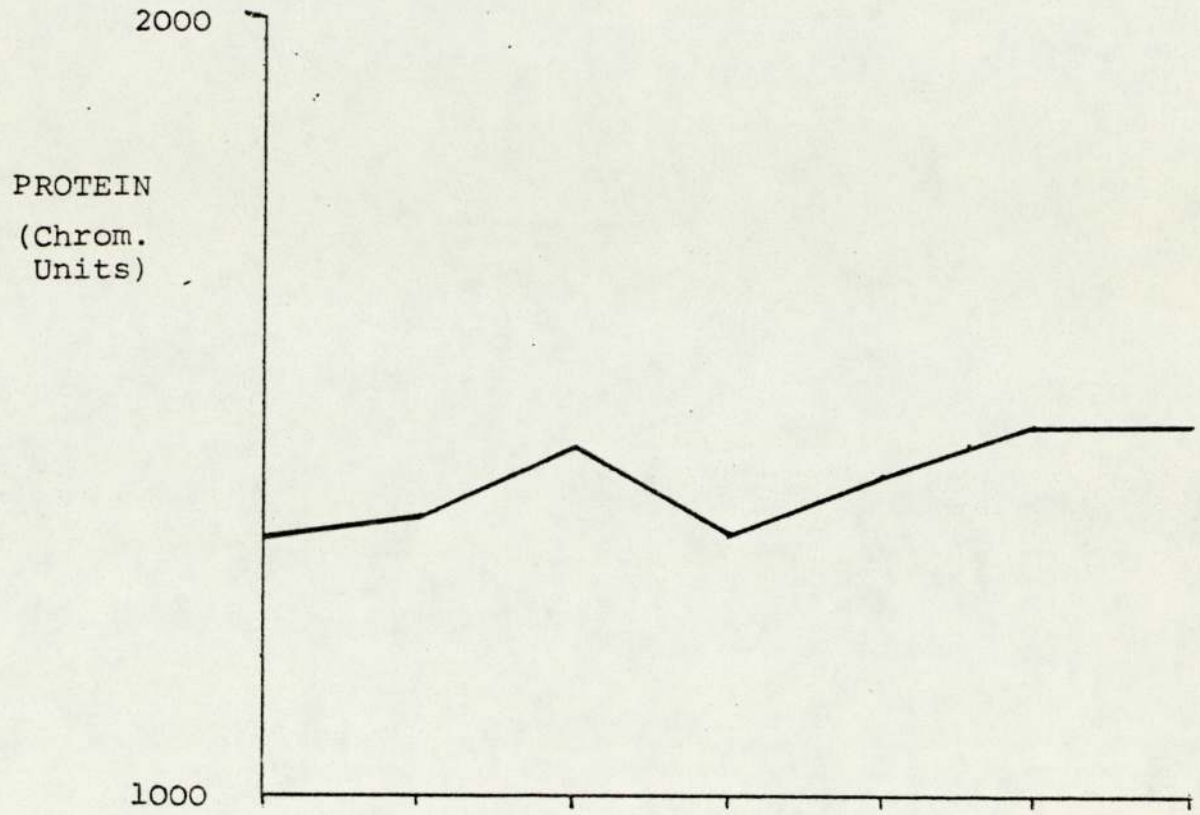
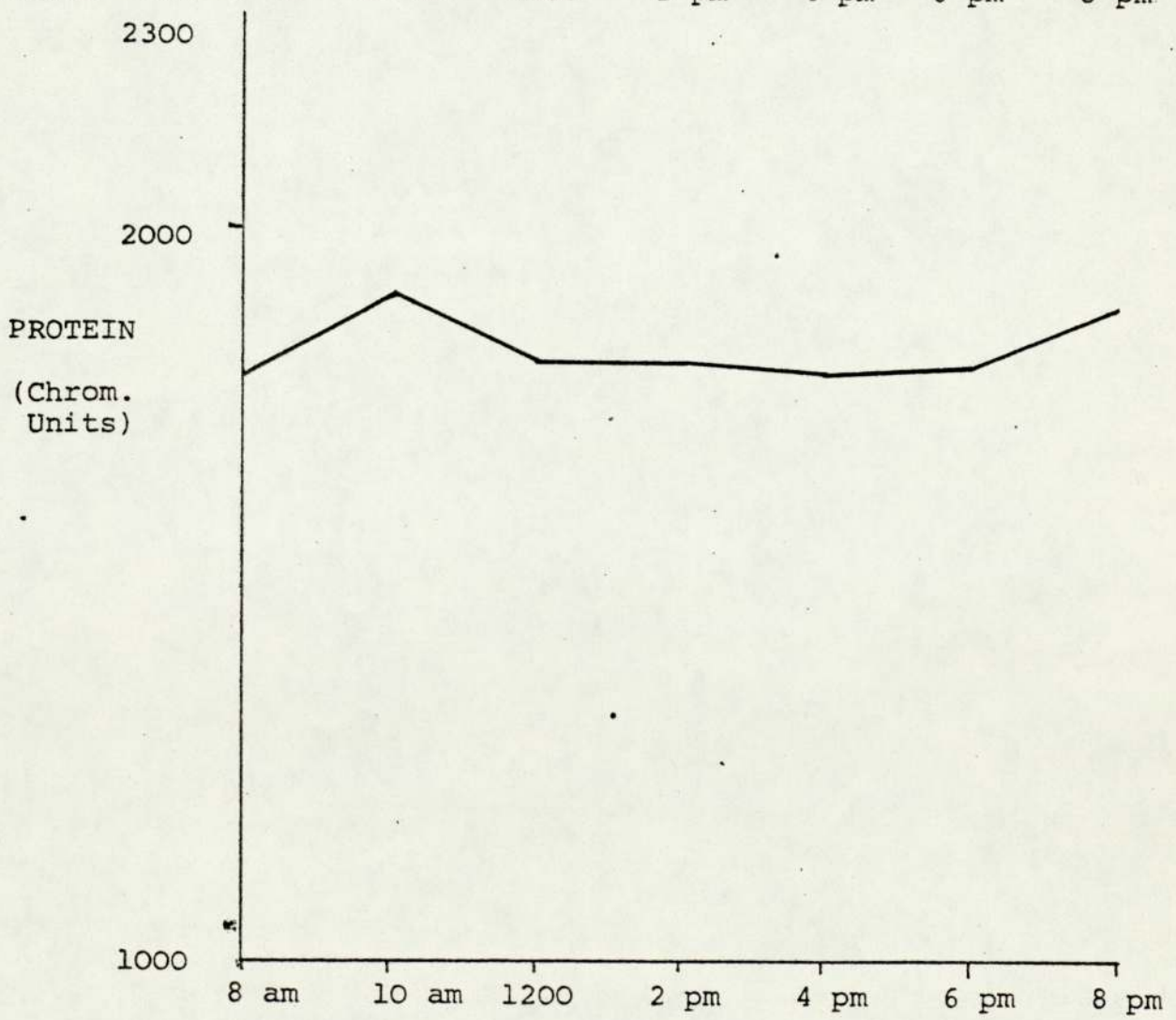


FIGURE 8:9



The slope of the regression line of X on Y was calculated, where X represented the time axis, in the equation:-

$$\text{SLOPE (X on Y)} = \frac{\Sigma xy}{\Sigma y^2}$$

These results are listed below:-

Table 8:2

SUBJECT NO	SLOPE (Y on X)
1	1.99×10^{-3}
2	2.38 "
3	2.75 "
4	2.07 "
5	1.84 "
6	3.16 "
7	2.70 "
8	3.07 "
9	2.15 "
10	2.88 "

As can be seen from the graphs and the figures in Table 8:2, the regression lines are almost flat, thus the data did not show any consistent trends in a "waking hours" variation.

In order to use a more powerful statistical analytical method, it was decided to calculate the Pearson r (correlation coefficient) and the Fisher test for any significance levels within the data. The raw score method was used:-

$$\text{(Pearson r)} \quad r = \frac{\frac{\Sigma XY}{n} - \overline{XY}}{S_x S_y}$$

where X represents time values

Y represents Protein values

S_x - standard deviation of X

S_y - standard deviation of Y

Using the preceding formula the value for Pearson's r was calculated for each of the ten subjects. These sample r's were then transformed to the statistic Zr as described by Fisher, to yield a sampling distribution more closely approximating the normal curve. This transformation from r's to Zr's is greatly simplified by the use of standard statistical tables which give the corresponding values. Z is then calculated using the formula:-

$$Z = \frac{zr - Zr}{\sqrt{\frac{1}{n-3}}}$$

where zr = the transformed value of the sample r

Zr = the transformed value of the population correlation coefficient specified under Ho.

Ho - (Null-Hypothesis) The population correlation coefficient from which the sample was drawn equals 0.00 ($p = 0.00$)

Hl - (Alternative Hypothesis) The population correlation coefficient from which the sample was drawn does not equal 0.00 ($p \neq 0.00$)

α = (Significance Level) 0.05, two-tailed test.

Critical Region : $z_{0.05} \geq 1.96$

$z_{0.05} \leq -1.96$

The values for each subjects r and z are given on the following table.

Table 8:3

Pearson r and Fisher z Values for the "Waking Hours" Study

SUBJECT NO	r	z
1	0.58	1.326
2	0.032	0.060
3	-0.33	0.69
4	-0.24	0.49
5	-0.66	1.58
6	-0.50	1.10
7	0.31	0.642
8	0.60	1.38
9	0.24	0.49
10	0.70	1.73

Since none of the computed values fell outwith the value of z greater than 95% from the mean, Ho cannot be rejected. Thus all the values could be taken from a population in which the correlation coefficient is 0.00.

Thus it was concluded that no significant "waking hours" trend variation was present in the tear protein values and the data collection for the remaining contact lens studies was arranged accordingly.

CHAPTER 9

Results for the Control Group and the Tear Protein Profiles in Hydrogel lens wear

Each tear sample having undergone the electrophoresis and densitometric procedures produced a quantifiable trace on the chromoscan paper as shown in the appendix, figures 1 and 2 appendix 9.

As previously described in the experimental design chapter, a figure was obtained for the total amount of protein in each tear sample, and a figure for each of the four protein sub-groups, (designated groups I - IV). The results given in this chapter are in directly-read chromoscan units, but can be converted to µgm of protein using a conversion factor of:-

10 Chromoscan units $\equiv$ 0.2642 µgm protein

The results are analysed using five values (i.e. total protein and each of the four sub-group protein values) for each of the four groups of volunteers (Ten subjects in each group).

The group are designated as follows:-

1) Control subjects
2) Group A - subjects fitted with the Bausch and Lomb Soflens lenses.

3) Group B - subjects fitted with Sauflon 85 lenses

4) Group C - subjects fitted with Sauflon 70 lenses

All the results were analysed using the technique of analysis of variance, (A.O.V.) to assess any degree of change in the tear protein levels, produced by three different types of hydrogel contact lenses.

Table 9:1

Relevant A.O.V. Statistics for the:-

Total Protein Values

SOURCE OF VARIATION	SUM SQUARES	DEGREES OF FREEDOM	VARIANCE ESTIMATE	F	SIGNIFICANCE LEVEL
BETWEEN GROUPS	3122821	3	1040940	38.5	0.01
WITHIN GROUPS	756524	28	27019		
TOTAL	3879345	31			

Table 9:2

Sub-Group I Protein Values

SOURCE OF VARIATION	SUM SQUARES	DEGREES OF FREEDOM	VARIANCE ESTIMATE	F	SIGNIFICANCE LEVEL
BETWEEN GROUPS	93746	3	31249	11.83	0.01
WITHIN GROUPS	73954	28	2641		
TOTAL	16770	31			

Table 9:3

Sub-Group II Protein Values

SOURCE OF VARIATION	SUM SQUARES	DEGREES OF FREEDOM	VARIANCE ESTIMATE	F	SIGNIFICANCE LEVEL
BETWEEN GROUPS	379561	3	126520	32	0.01
WITHIN GROUPS	109200	28	3900		
TOTAL	488761	31			

Table 9:4

Sub-Group III Protein Values

SOURCE OF VARIATION	SUM SQUARES	DEGREES OF FREEDOM	VARIANCE ESTIMATE	F	SIGNIFICANCE LEVEL
BETWEEN GROUPS	787425	3	262475	64	0.01
WITHIN GROUPS	115161	28	4113		
TOTAL	902586	31			

Table 9:5

Sub-Group IV Protein Values

SOURCE OF VARIATION	SUM SQUARES	DEGREES OF FREEDOM	VARIANCE ESTIMATE	F	SIGNIFICANCE LEVEL
BETWEEN GROUPS	21601	3	7200	5.9	0.01
WITHIN GROUPS	33928	28	1212		
TOTAL	55529	31			

An inspection of the preceding five tables shows that each of the five F- ratios are significant at the 0.01 level.

An "a posteriori", test for making pairwise comparisons among means was then applied to the data. This particular test was developed by Tukey (1953) and named the H.S.D. (honestly significant difference) test.

A difference between two means is significant, at a given α - level, if it equals or exceeds H.S.D., which is:

$$\text{H.S.D.} = q_{\alpha} \sqrt{\frac{s^2_w}{n}}$$

in which,

s^2_w = the within-group variance estimate

n = number of values in each condition

q_{α} = tabled value for a given α - level

K = number of means between and including the two being compared

Table 9:6

Differences Among Means:-

Total Protein

	$\bar{X}_1$ (CONTROLS)	$\bar{X}_2$ (GROUP A)	$\bar{X}_3$ (GROUP B)	$\bar{X}_4$ (GROUP C)
$\bar{X}_1$ = 2302	-	837	659	456
$\bar{X}_2$ = 1465	-	-	178	381
$\bar{X}_3$ = 1643	-	-	-	203
$\bar{X}_4$ = 1846	-	-	-	-

Table 9:7

Sub-Group I

	$\bar{X}_1$	$\bar{X}_2$	$\bar{X}_3$	$\bar{X}_4$
$\bar{X}_1$ = 433	-	121	101	8
$\bar{X}_2$ = 312	-	-	20	113
$\bar{X}_3$ = 332	-	-	-	93
$\bar{X}_4$ = 425	-	-	-	-

Table 9:8

Sub-Group II

	$\bar{X}_1$	$\bar{X}_2$	$\bar{X}_3$	$\bar{X}_4$
$\bar{X}_1$ = 677	-	279	237	225
$\bar{X}_2$ = 398	-	-	42	54
$\bar{X}_3$ = 440	-	-	-	12
$\bar{X}_4$ = 452	-	-	-	-

Table 9:9

Sub-Group III

	$\bar{X}_1$	$\bar{X}_2$	$\bar{X}_3$	$\bar{X}_4$
$\bar{X}_1$ = 831	-	425	259	134
$\bar{X}_2$ = 406	-	-	166	291
$\bar{X}_3$ = 572	-	-	-	125
$\bar{X}_4$ = 697	-	-	-	-

Table 9:10

Sub-Group IV

	$\bar{X}_1$	$\bar{X}_2$	$\bar{X}_3$	$\bar{X}_4$
$\bar{X}_1$ = 350	-	92	41	73
$\bar{X}_2$ = 442	-	-	133	165
$\bar{X}_3$ = 309	-	-	-	32
$\bar{X}_4$ = 277	-	-	-	-

There are four means in each group for pairwise comparisons.

The means should be ranked and the two most extreme means compared first. Then K would be at a maximum of four, and at a minimum when the second and third means were compared when K = 2.

For this data three possible values of K are 2, 3 and 4, along with two possible values for q_α (0.05 and 0.01 significance levels).

The following table gives the q_α values for these possible permutations with one constant df_w = 28.

Table 9:11

	VALUE OF K (No of MEANS)		
	2	3	4
q (0.5 level) =	2.89	3.49	3.85
q (0.1 level) =	3.89	4.45	4.80

Thus the values for H.S.D. at the 5% and 1% levels can be calculated.

These are shown in the two following tables:-

Table 9:12

GROUP	$\sqrt{\frac{\hat{S}^2_w}{n}}$	H.S.D. (0.01)		
		K = 2	K = 3	K = 4
TOTAL PROTEIN	58	225.6	258.1	278.4
SUB-GROUP I	18	70	80.1	86.4
SUB-GROUP II	22	85.6	97.9	105.6
SUB-GROUP III	22.7	88.3	101	109
SUB-GROUP IV	12.3	47.8	54.7	59

Table 9:13

GROUP	$\sqrt{\frac{S_w^2}{n}}$	H.S.D. (0.05)		
		K = 2	K = 3	K = 4
TOTAL PROTEIN	58	176.6	202.4	223.3
SUB-GROUP I	18	52	62.8	69.3
SUB-GROUP II	22	63.6	76.8	84.7
SUB-GROUP III	22.7	65.6	79.2	87.4
SUB-GROUP IV	12.3	35.5	42.9	47.3

The following conclusions can be drawn from relating these H.S.D. values to the differences among means for the five groups of data.

Table 9:14

TOTAL PROTEIN GROUP	SIGNIFICANCE LEVEL
$\bar{X}_1$ VS $\bar{X}_2$	0.01
$\bar{X}_1$ VS $\bar{X}_3$	0.01
$\bar{X}_1$ VS $\bar{X}_4$	0.01
$\bar{X}_2$ VS $\bar{X}_3$	0.05
$\bar{X}_2$ VS $\bar{X}_4$	0.01
$\bar{X}_3$ VS $\bar{X}_4$	0.05

Table 9:15

Sub-Group I

$\bar{X}_1$	VS	$\bar{X}_2$	0.01
$\bar{X}_1$	VS	$\bar{X}_3$	0.01
$\bar{X}_1$	VS	$\bar{X}_4$	N.S.*
$\bar{X}_2$	VS	$\bar{X}_3$	N.S.*
$\bar{X}_2$	VS	$\bar{X}_4$	0.01
$\bar{X}_3$	VS	$\bar{X}_4$	0.01

Table 9:16

Sub Group II

$\bar{X}_1$	VS	$\bar{X}_2$	0.01
$\bar{X}_1$	VS	$\bar{X}_3$	0.01
$\bar{X}_1$	VS	$\bar{X}_4$	0.01
$\bar{X}_2$	VS	$\bar{X}_3$	N.S.*
$\bar{X}_2$	VS	$\bar{X}_4$	N.S.
$\bar{X}_3$	VS	$\bar{X}_4$	N.S.*

Table 9:17

Sub-Group III

$\bar{X}_1$	VS	$\bar{X}_2$	0.01
$\bar{X}_1$	VS	$\bar{X}_3$	0.01
$\bar{X}_1$	VS	$\bar{X}_4$	0.01
$\bar{X}_2$	VS	$\bar{X}_3$	0.01
$\bar{X}_2$	VS	$\bar{X}_4$	0.01
$\bar{X}_3$	VS	$\bar{X}_4$	0.01

Table 9:18

Sub-Group IV

$\bar{X}_1$	VS	$\bar{X}_2$	0.01
$\bar{X}_1$	VS	$\bar{X}_3$	0.05
$\bar{X}_1$	VS	$\bar{X}_4$	0.01
$\bar{X}_2$	VS	$\bar{X}_3$	0.01
$\bar{X}_2$	VS	$\bar{X}_4$	0.01
$\bar{X}_3$	VS	$\bar{X}_4$	N.S.*

(*N.S. - Not significant)

CHAPTER 10

Discussion and Conclusions

THE CLINICAL INTERPRETATION OF THE RESULTS

A. In Terms of Protein Groups

A worthwhile appraisal is to consider the results in a clinical perspective, by restating them in the following manner:-

For the "Total Protein" Values Tables 9:6, 9:14

1) All three types of contact lens caused a statistically significant decrease in the overall amount of protein in a 5 µl tear sample compared with the values for the control group.

2) Each of the three types of lenses caused differing extents of decrease. The Bausch and Lomb lenses produced the largest decrease and the Sauflon 70 the smallest decrease in the protein level.

3) The levels for the Bausch and Lomb group were statistically significantly lower than for the other two lens groups. At the 0.05 level for the Sauflon 85 and at the 0.01 level for the Sauflon 70 group.

For Group I Proteins (Tables 9:7, 9:15)

1) Only the Sauflon 85 and the Bausch and Lomb group were significantly reduced from the Control group. The Sauflon 70 group did not produce any significant difference.

2) The values for the Sauflon 70 and the Control group, were so similar that the Sauflon 85 and the Bausch and Lomb groups were almost as far removed from the Sauflon 70 as they were from the Controls. (Significant at the 0.01 level).

3) The values for the Sauflon 85 and the Bausch and Lomb group were very similar, and no significant difference between them was elicited.

For Group II Proteins (Tables 9:8, 9:16)

1) All the values for the contact lens groups were significantly different (0.01), from the Control group.

2) None of the contact lens groups were significantly different from each other.

3) The Bausch and Lomb group showed the greatest reduction, and the Sauflon 70 showed the least reduction in the overall values.

For Group III Proteins (Tables 9:9, 9:17)

1) All the contact lens groups were significantly different (0.01), from the Control group.

2) All three contact lens groups were also significantly different (0.01), from each other.

3) The Bausch and Lomb group showed the greatest reduction, and the Sauflon 70 showed the least reduction in the overall values.

For Group IV Proteins (Tables 9:10, 9:18)

1) All the contact lens group produced significant differences compared with the Control group.

Bausch and Lomb, and Sauflon 70 at the 0.01 level
Sauflon 85 at the 0.05 level.

2) The Bausch and Lomb group produced a RISE in the level, and the other two lens groups produced a decrease. The Bausch and Lomb group values were significantly different from the other two groups, at the 0.01 level.

3) The Sauflon 85 and Sauflon 70 levels were not significantly different.

FIGURE 10:1 Controls (Total Protein)

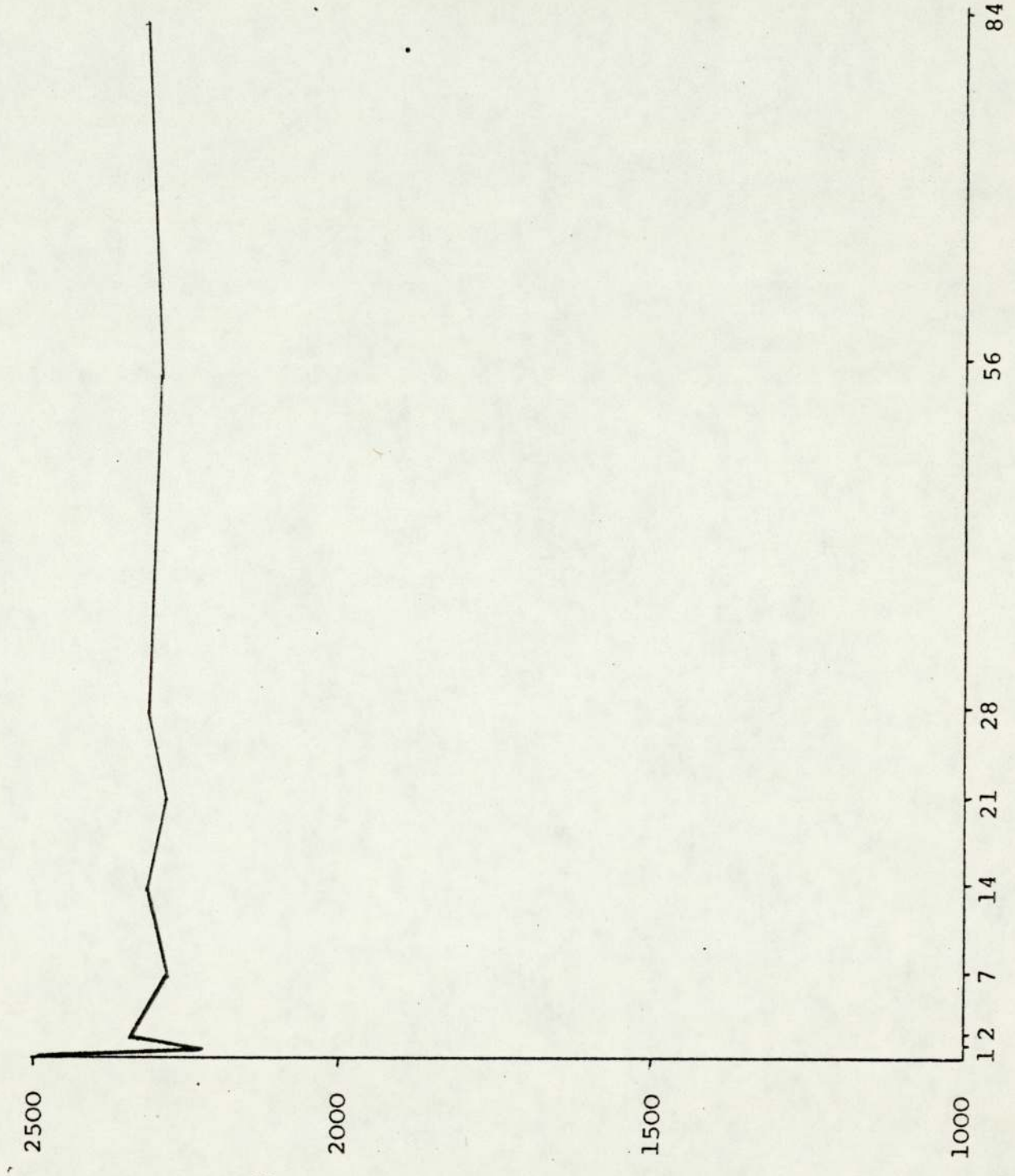


FIGURE 10:5 Controls - Group 1

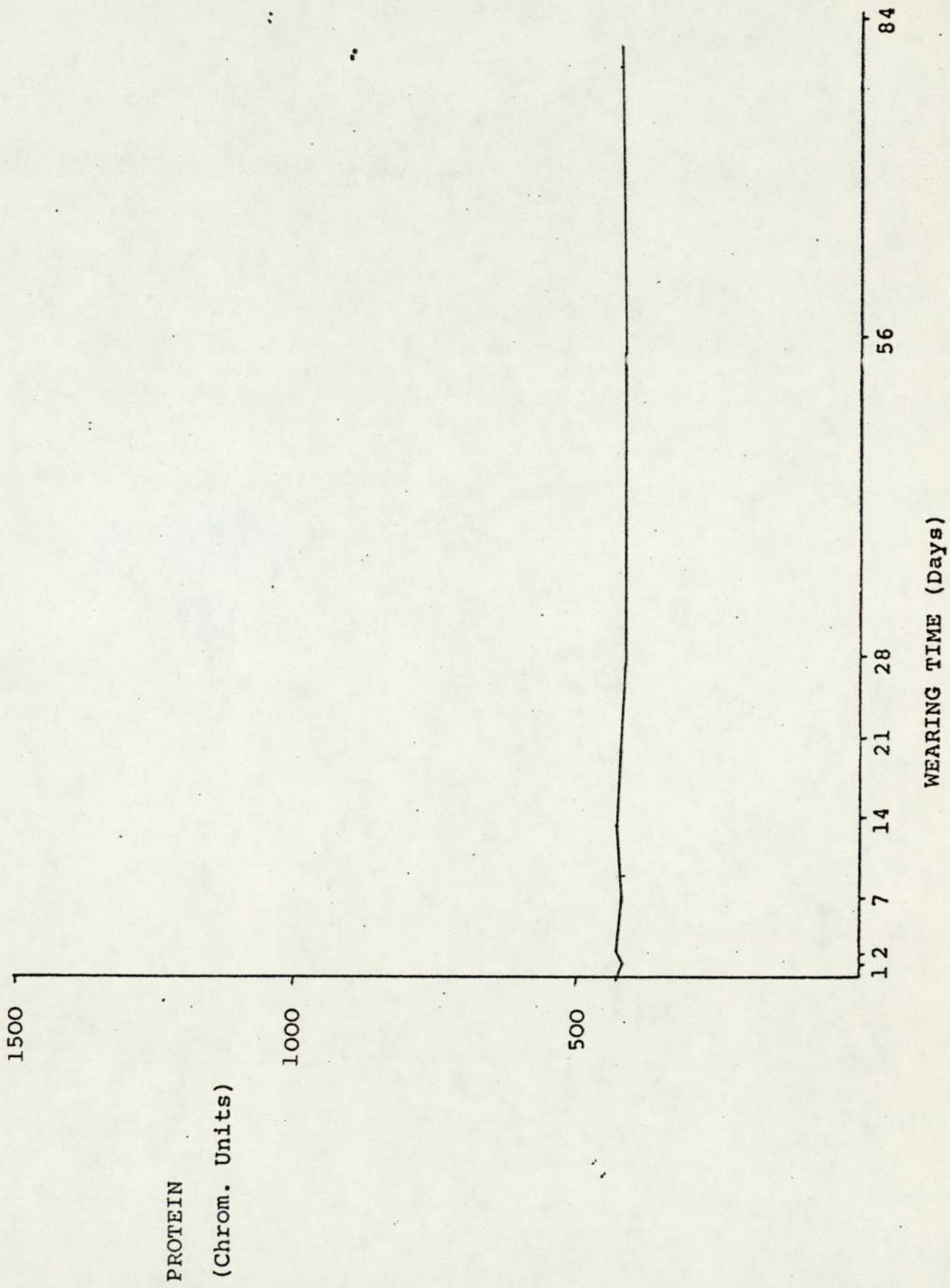


FIGURE 10:6 Controls - Group II

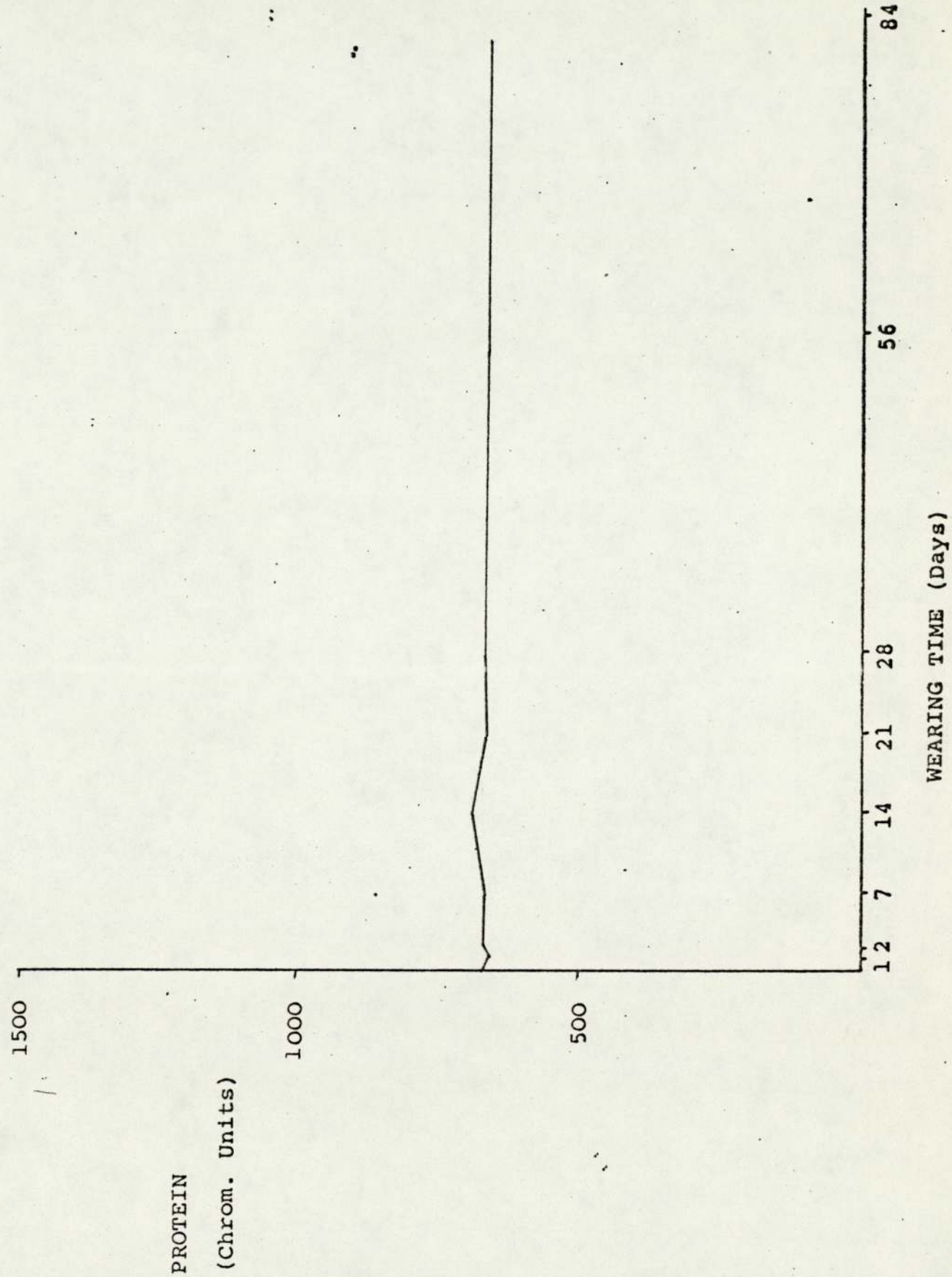


FIGURE 10:7 Controls - Group III

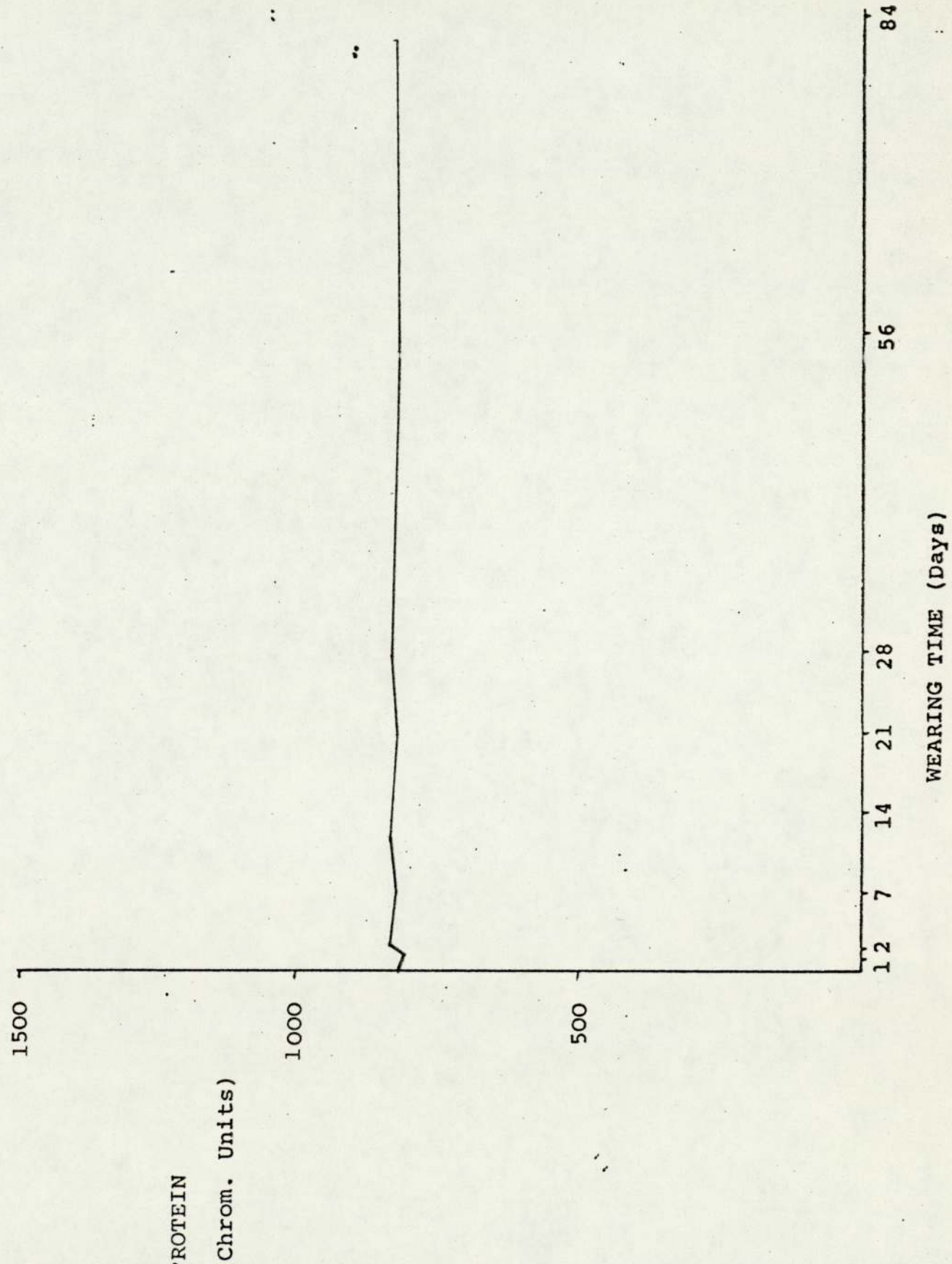
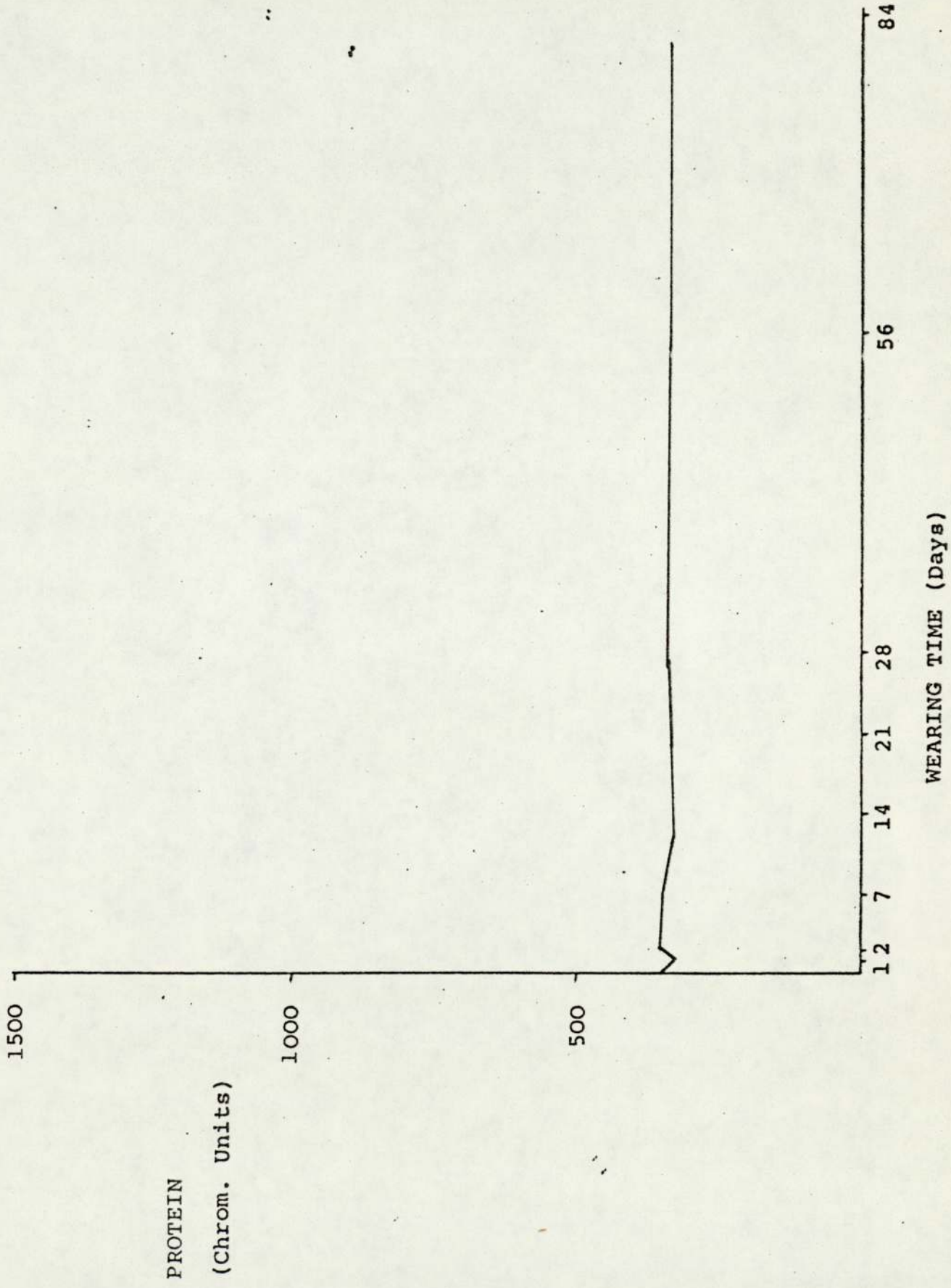


FIGURE 10:8 Controls - Group IV



B. In Terms of Subject Groups

The trends within each of the four groups of subjects are now discussed with some help from the graphical illustrations of the results, plotted against the data collection intervals.

Control Group (Figures 10:1, 5, 6, 7, 8)

From the preceding graphs, listed above, it can be seen that the overall results for the control group show very little variation in either the total or the sub-group values of protein. The mean range of the results for this group over a twelve week period was 2240 to 2352 chrom units. Using the conversion factor as stated in Chapter 9, this is equivalent to a range of 59.18 to 62.14 µgm of protein/5 µl of tear fluid. This would give an approximate average value of 12 µgm/µl.

The values for the individual groups are given below:-

Group	µgm/5µl of Tear Fluid
I	11.44
II	17.91
III	21.96
IV	9.26

These figures would approximate the following percentage values:-

Sub-Group	% of Total Protein
I	19
II	30
III	36
IV	15

It is interesting to compare the values obtained in this study to those given by Botelho (1964), and Francois and Rabaey (1960), as stated in Chapter 2 of this thesis. Both these authors only specify values for three protein sub-groups. It is probable that their values given for the albumin group, combine the figures given here for groups I and II, since Group I fractions follow just behind the Group II (Albumin) fractions during the electrophoretic analytical method.

If this combination of groups I and II is made, then the figures from this study would most resemble those given for an unstimulated lacrimal flow by Botelho (1964). The values from this study being slightly lower for the albumin group and slightly higher for the globulin group.

FIGURE 10:2 Group A Total Protein

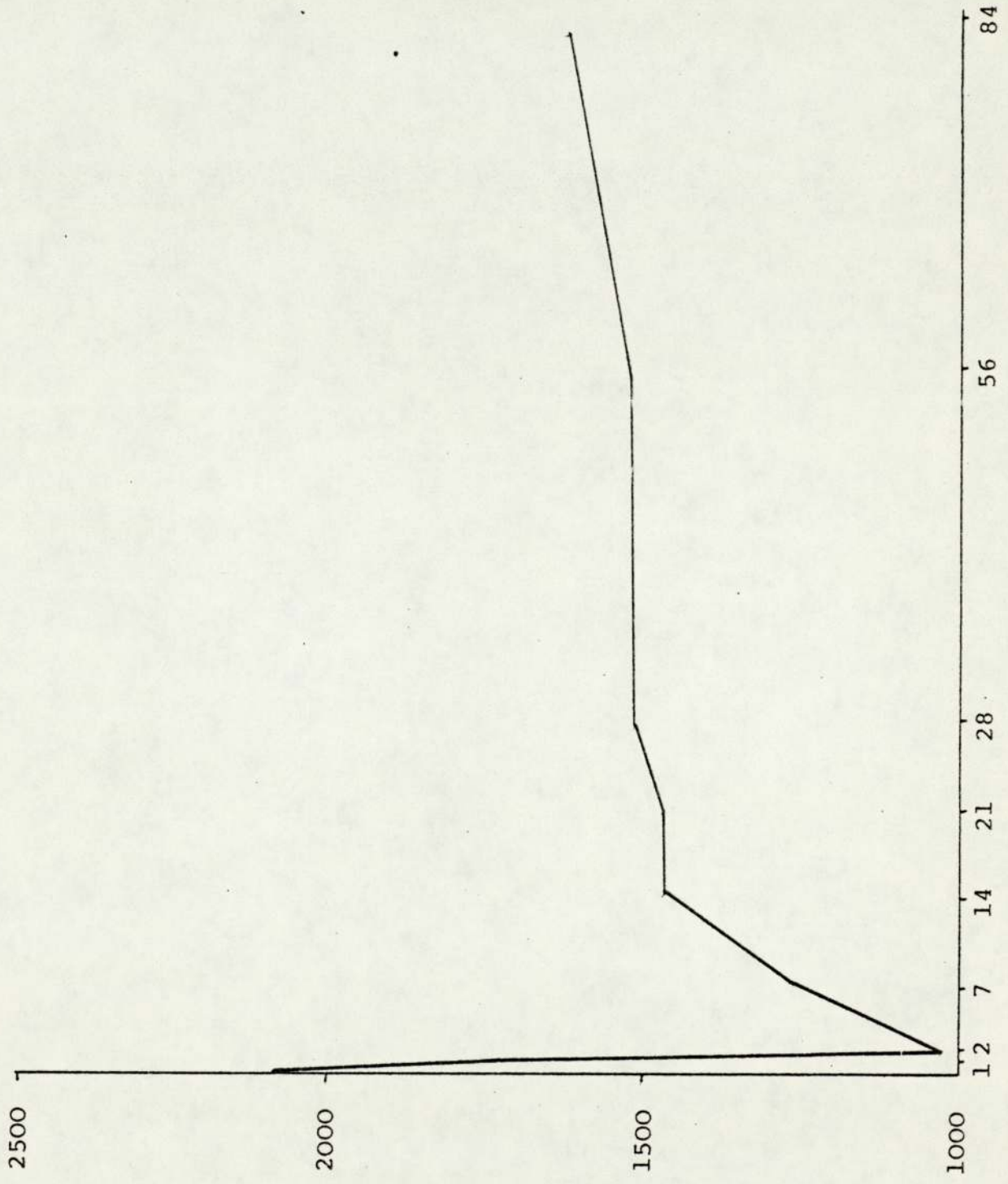


FIGURE 10:9 Bausch & Lomb Lenses - Group I

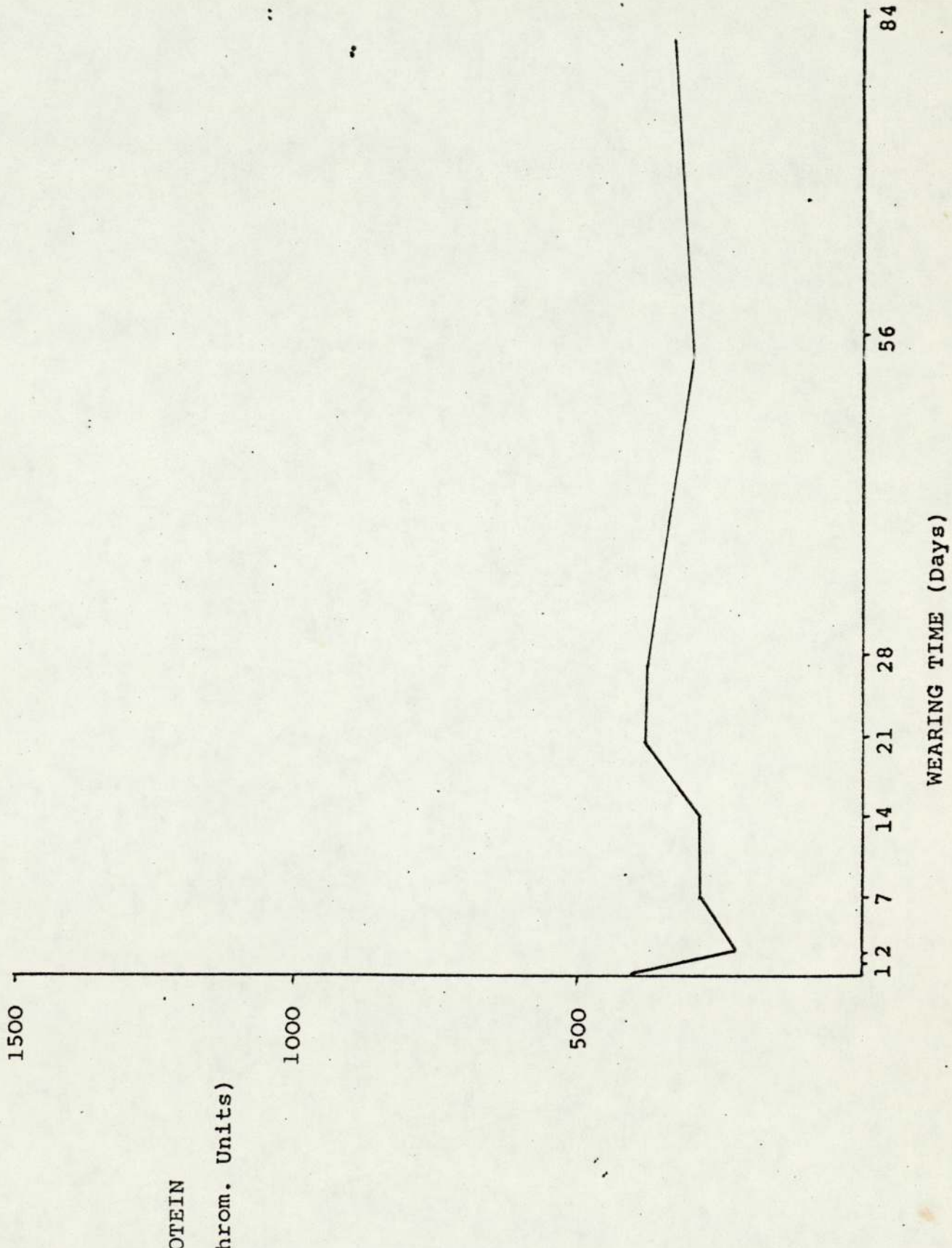


FIGURE 10:10 Bausch & Lomb Lenses - Group II

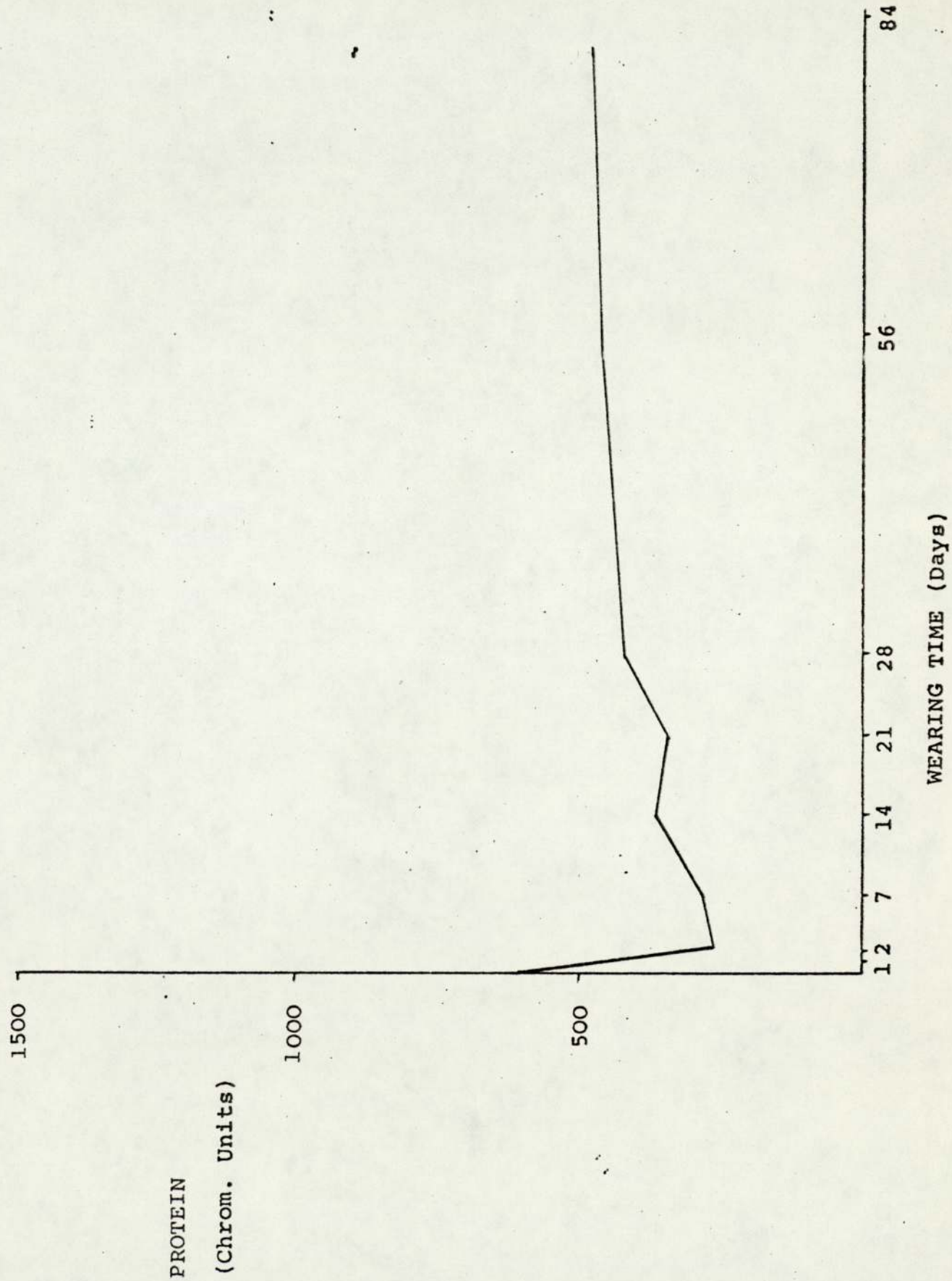


FIGURE 10:11 Bausch & Lomb Lenses - Group III

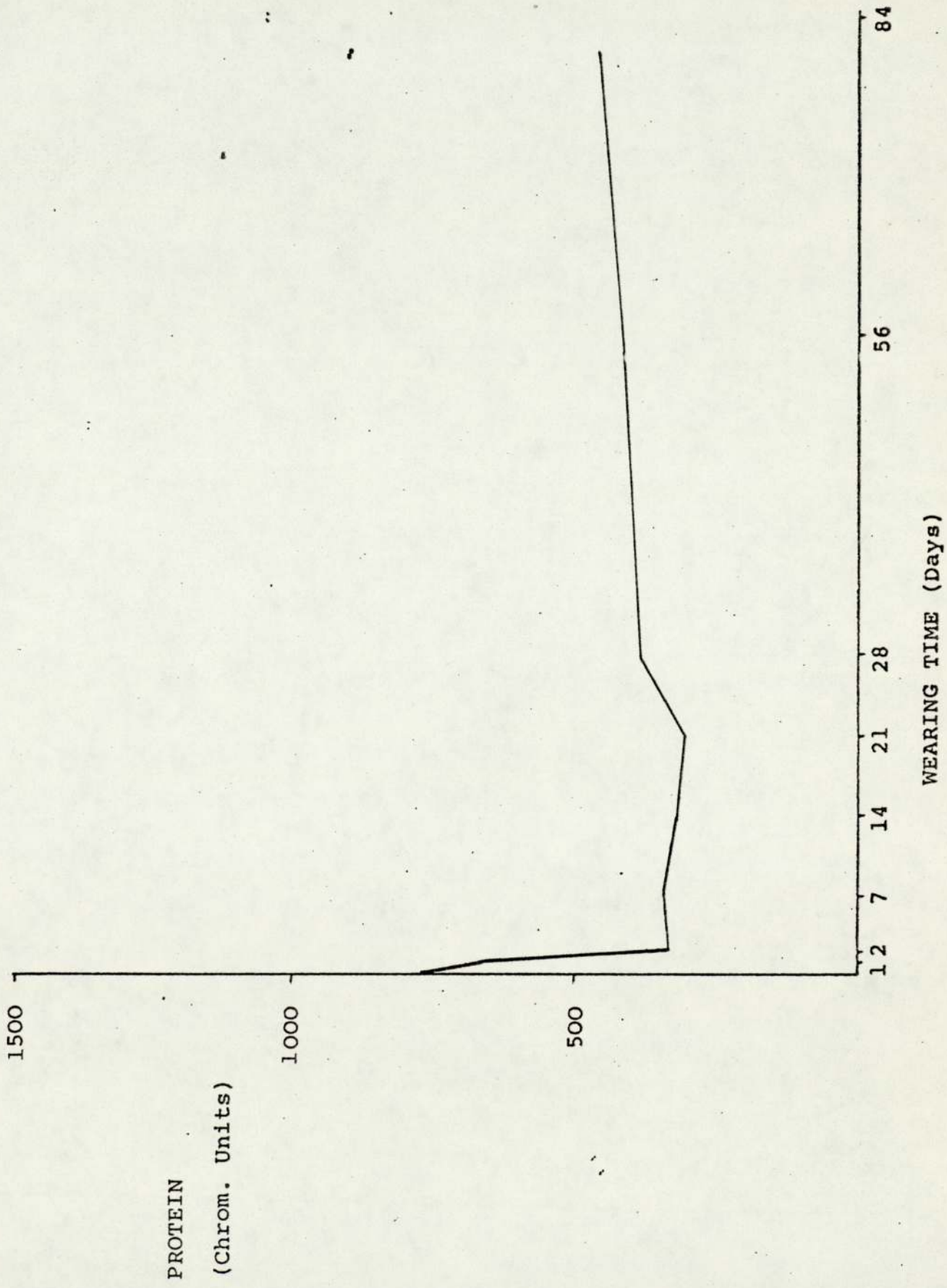
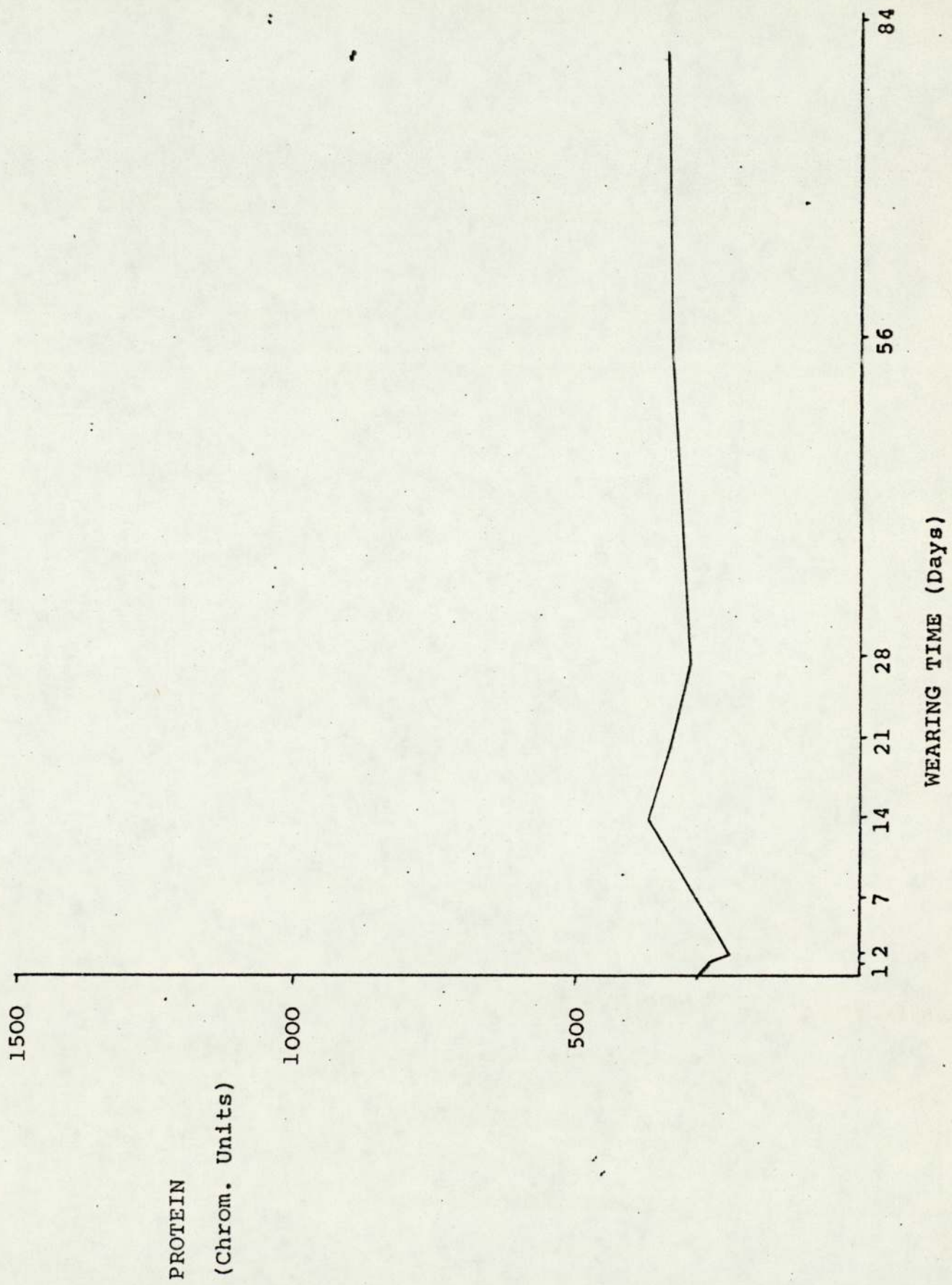


FIGURE 10:12 Bausch & Lomb Lenses - Group IV



Group A (Bausch & Lomb Lenses)

Figures (10:2, 9, 10, 11, 12)

The individual groups I, II and III closely follow the trends expressed in the values for the total protein. None of these three groups reached the pre-fitting protein levels, during the twelve weeks of contact lens wear. Group IV does show different trends compared with the other three groups. The protein levels in group IV show an initial small decrease over the first two days, and by one week the level is almost equal to the pre-fitting value. This continues to rise, reaching a maximum at two weeks and then falls gradually until four weeks, where it settles around the pre-fitting level for the remainder of the study.

FIGURE 10:3 Group B Total Protein

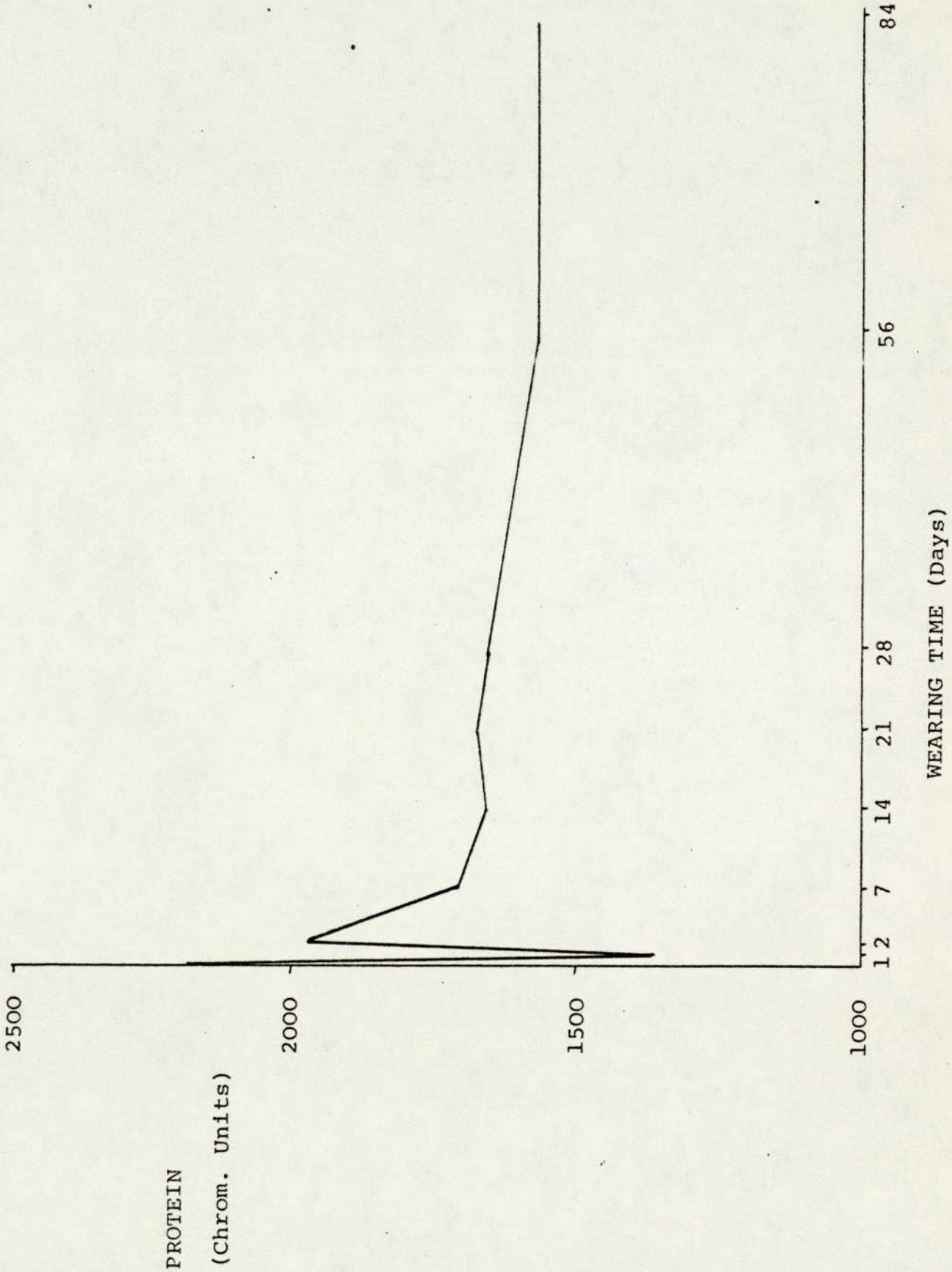


FIGURE 10:13 Sauflon 85 Lenses - Group I

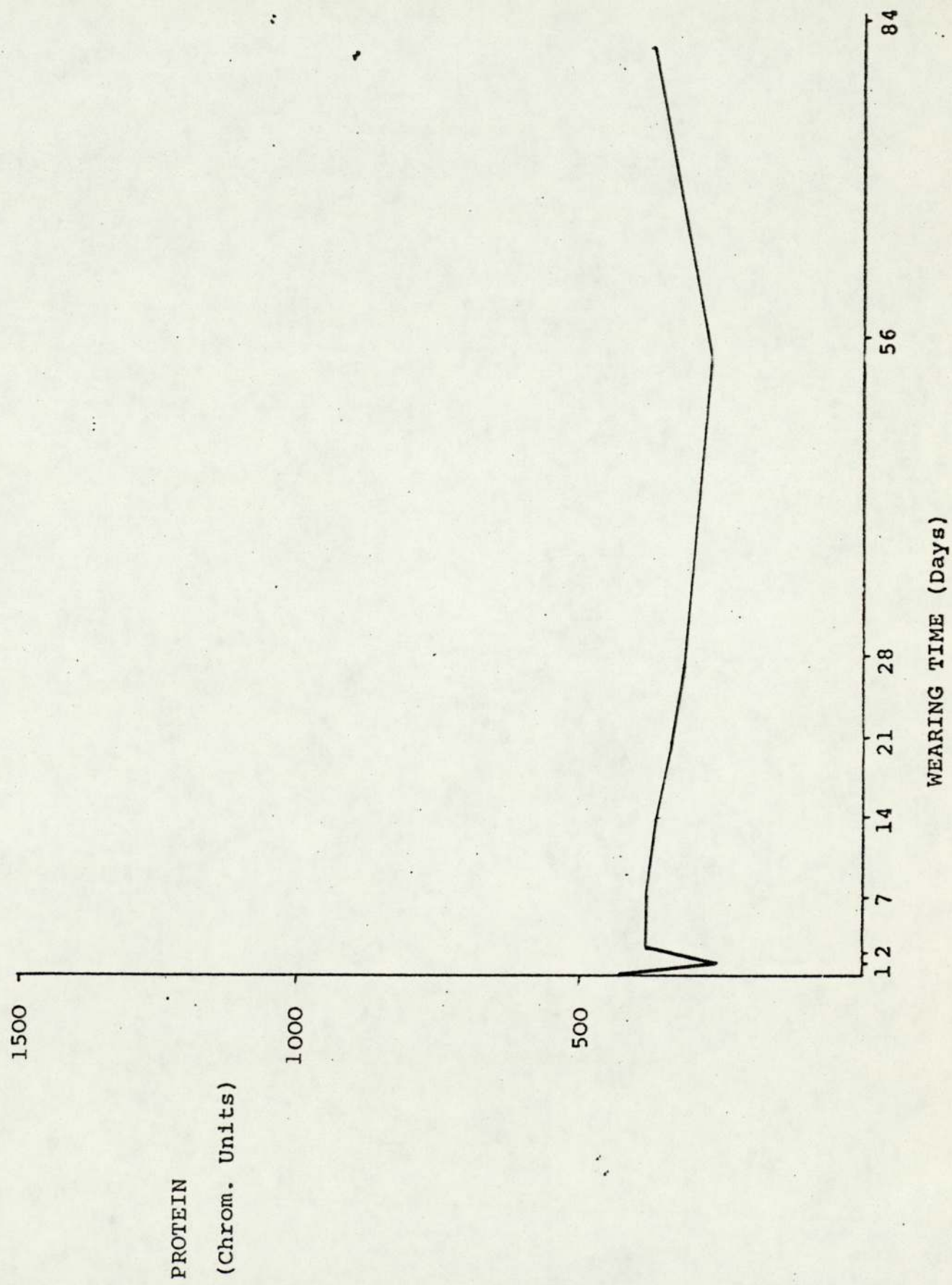


FIGURE 10:14 Sauflon 85 Lenses - Group II

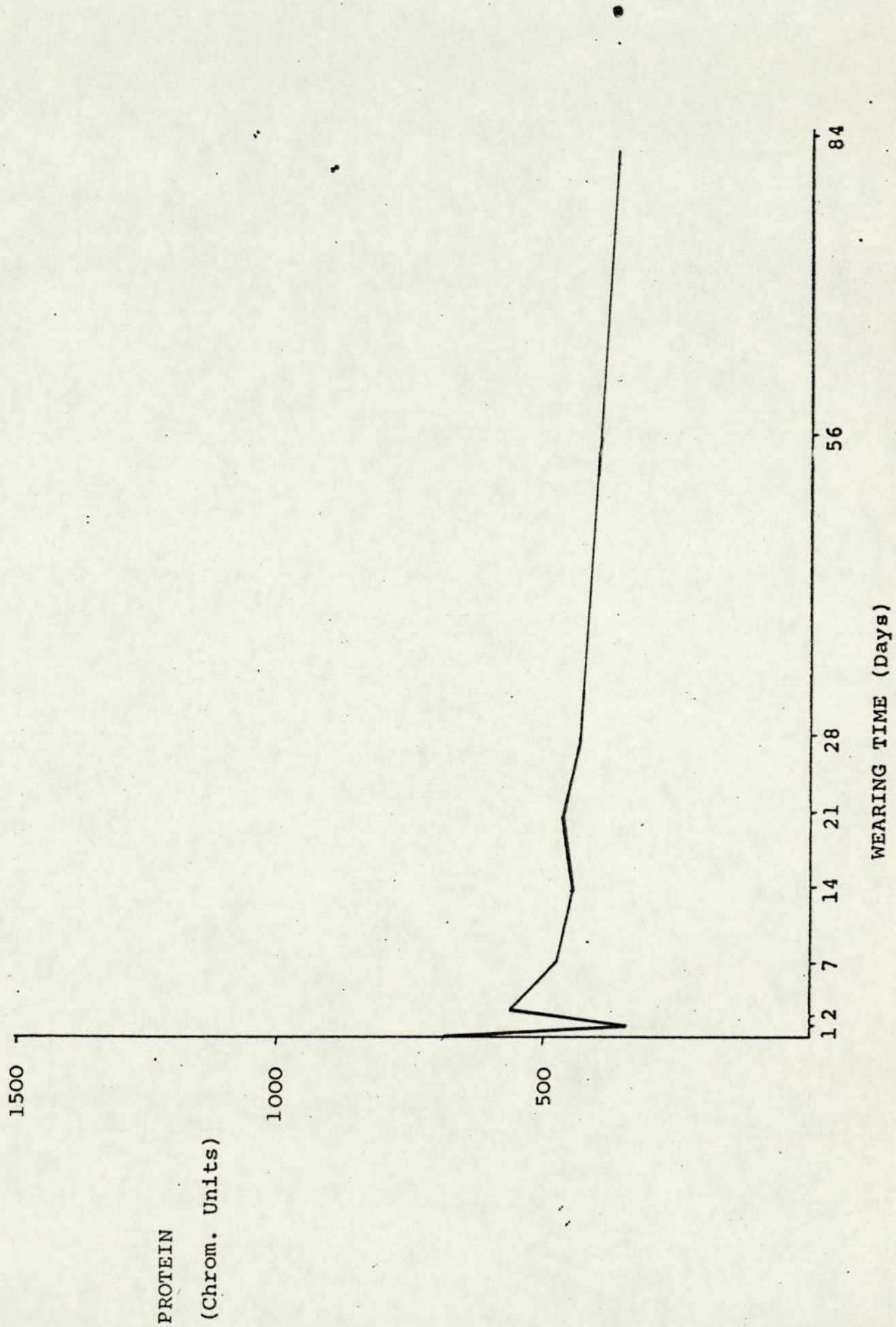


FIGURE 10:15 Sauflon 85 Lenses - Group III

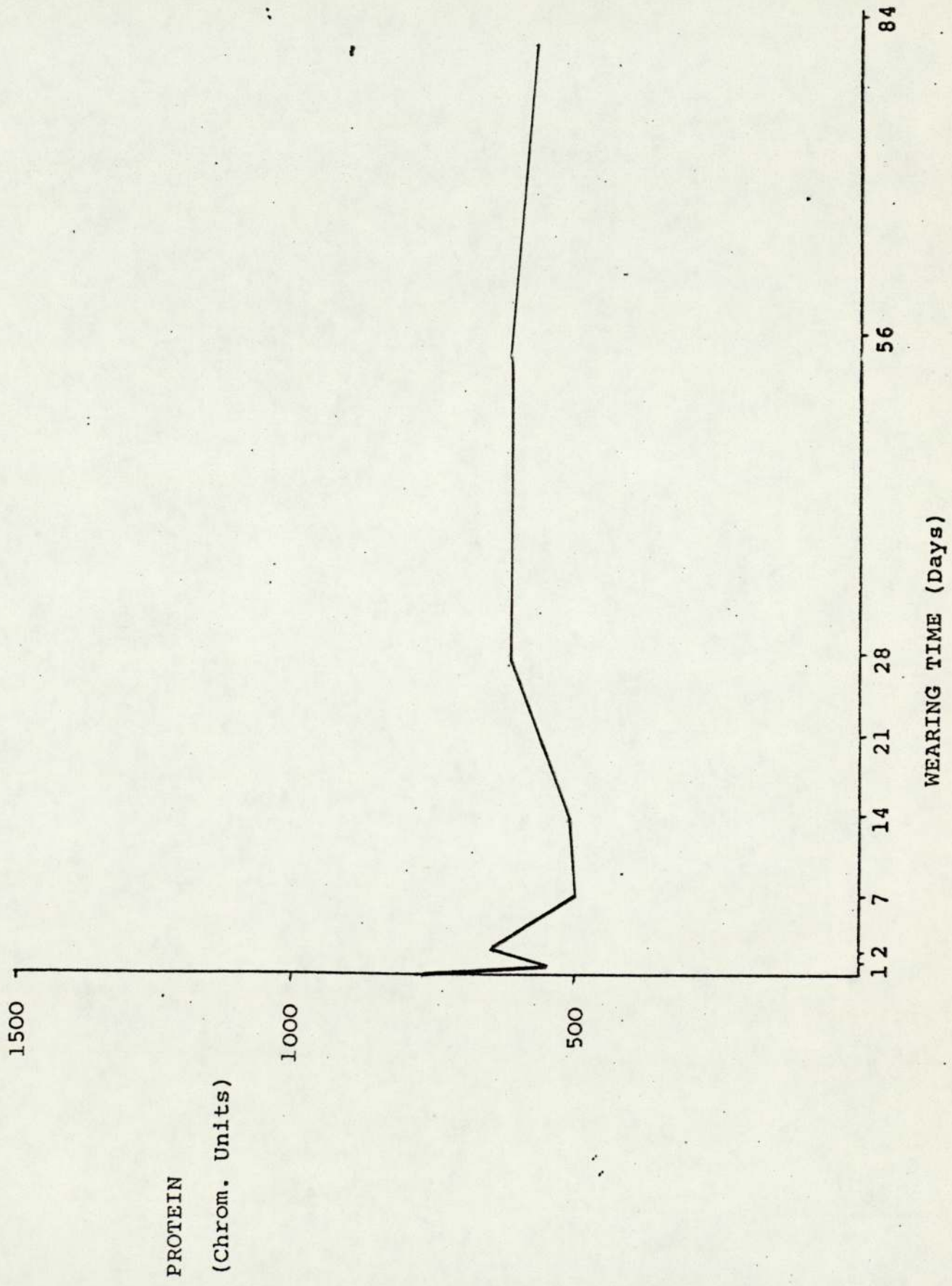
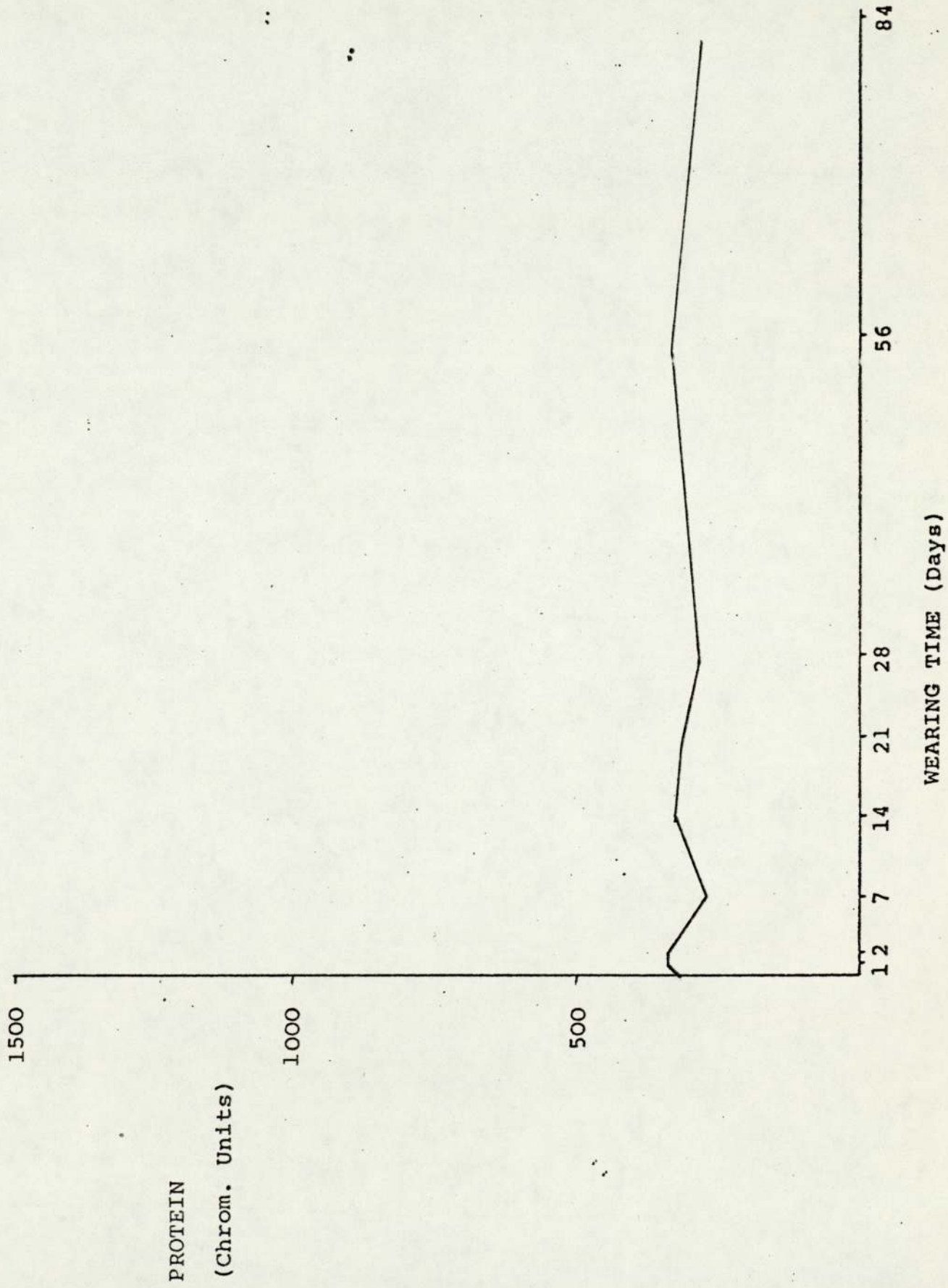


FIGURE 10:16 Sauflon 85 Lenses - Group IV



Group B (Sauflon 85)

Figures (10:3, 13, 14, 15, 16)

Groups I, II and III follow the trends as outlined by the total protein values, showing a very large decrease on the first day of lens wear. This rises rapidly on the second day to a level just below the pre-fitting value. After one week of wear this level has decreased and a new lower, more stable value is maintained for the remainder of the twelve weeks.

Two interesting deviations from this trend are found in Group III, and more markedly in Group IV. Group III reduces after two days of wear to a minimum level which is maintained for the following two weeks, and then slowly rises to establish a more stable value for the final eight weeks of the study.

Group IV tends to maintain its pre-fitting level, with a tendency towards a small overall rise in the level over the full twelve weeks of lens wear. Interestingly, the values for day one and two are slightly higher than the pre-fitting levels.

FIGURE 10:4 Group C Total Protein

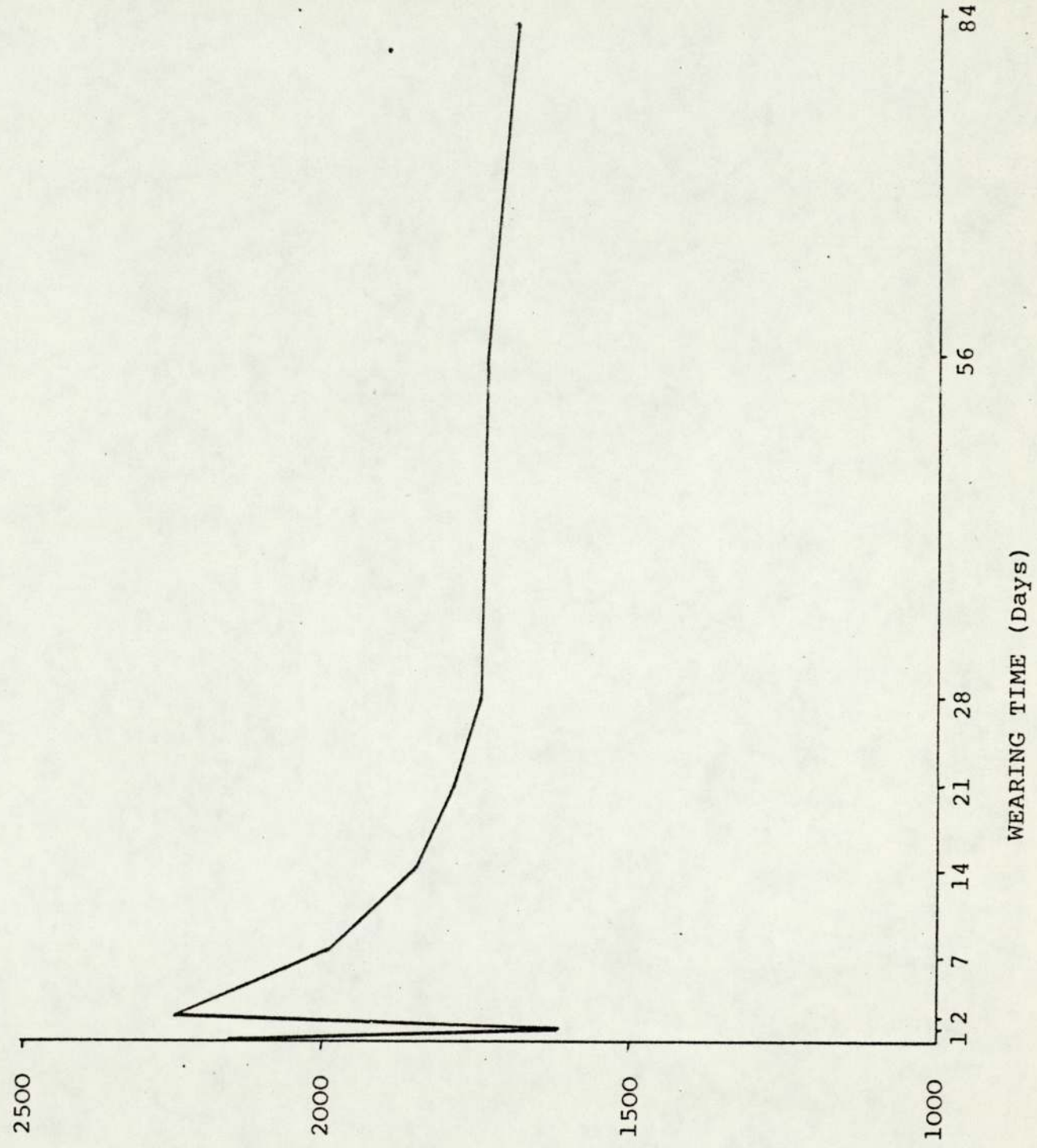


FIGURE 10:17 Sauflon 70 Lenses - Group I

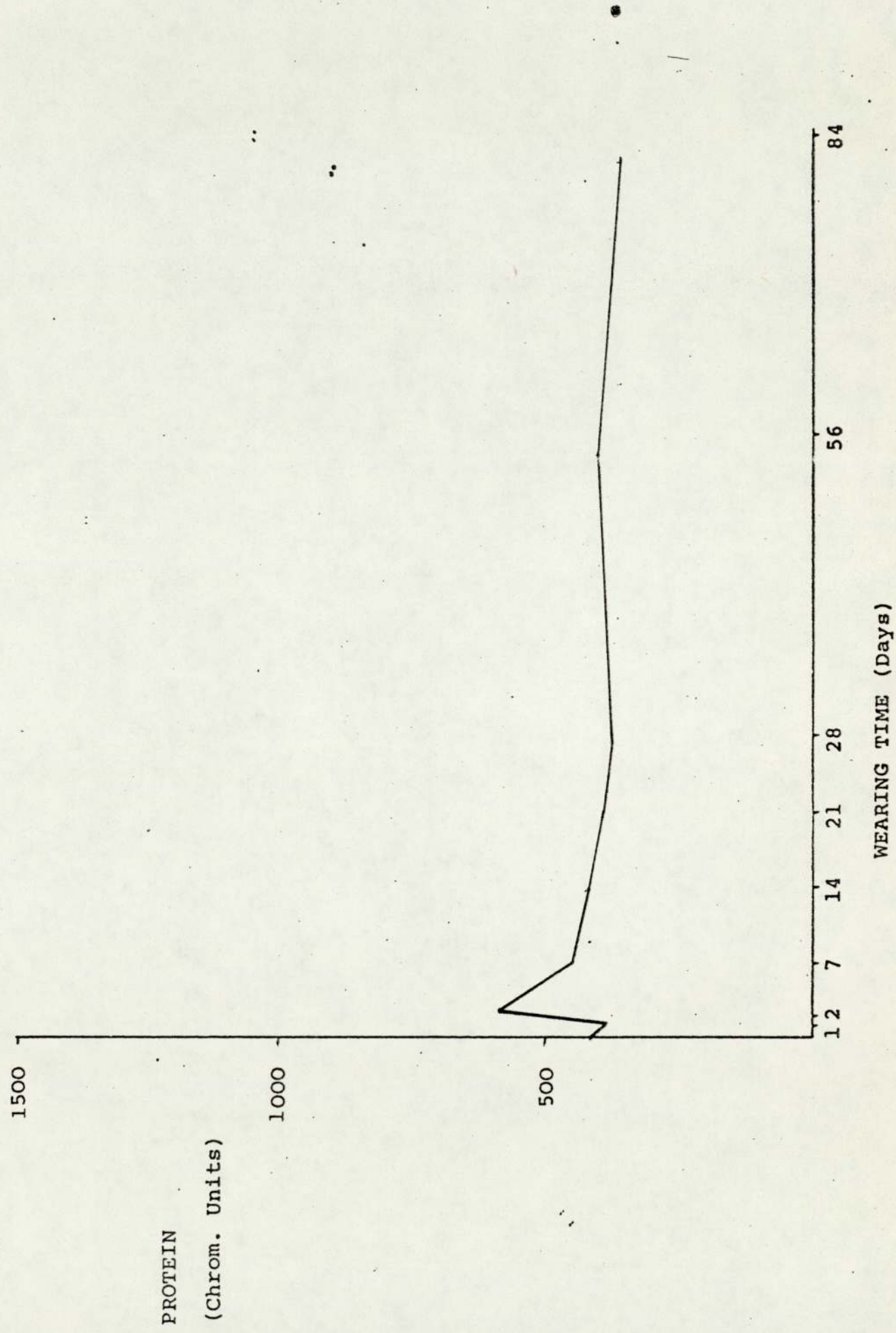


FIGURE 10;18 Sauflon 70 Lenses - Group II

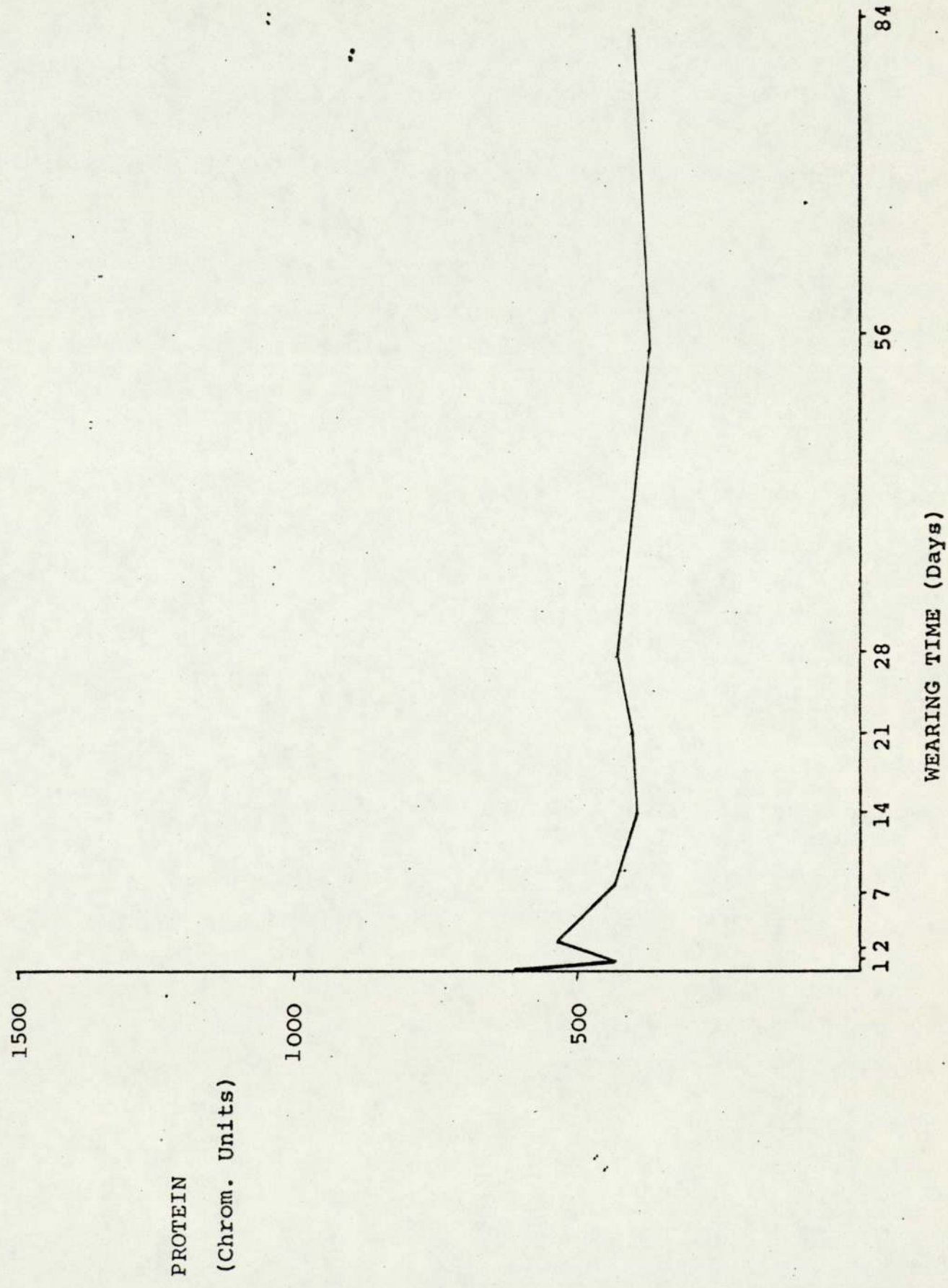


FIGURE 10:19 Sauflon 70 Lenses - Group III

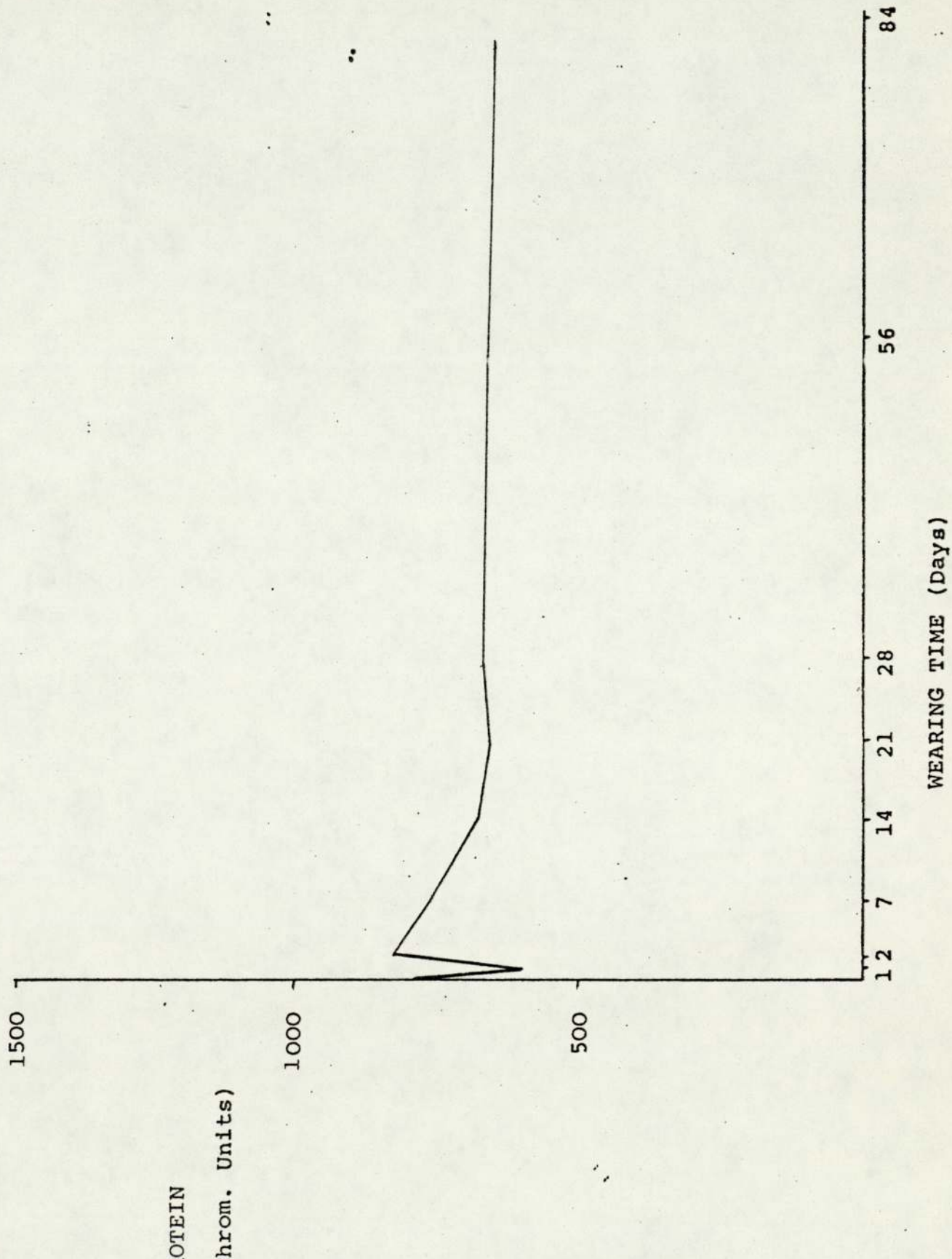
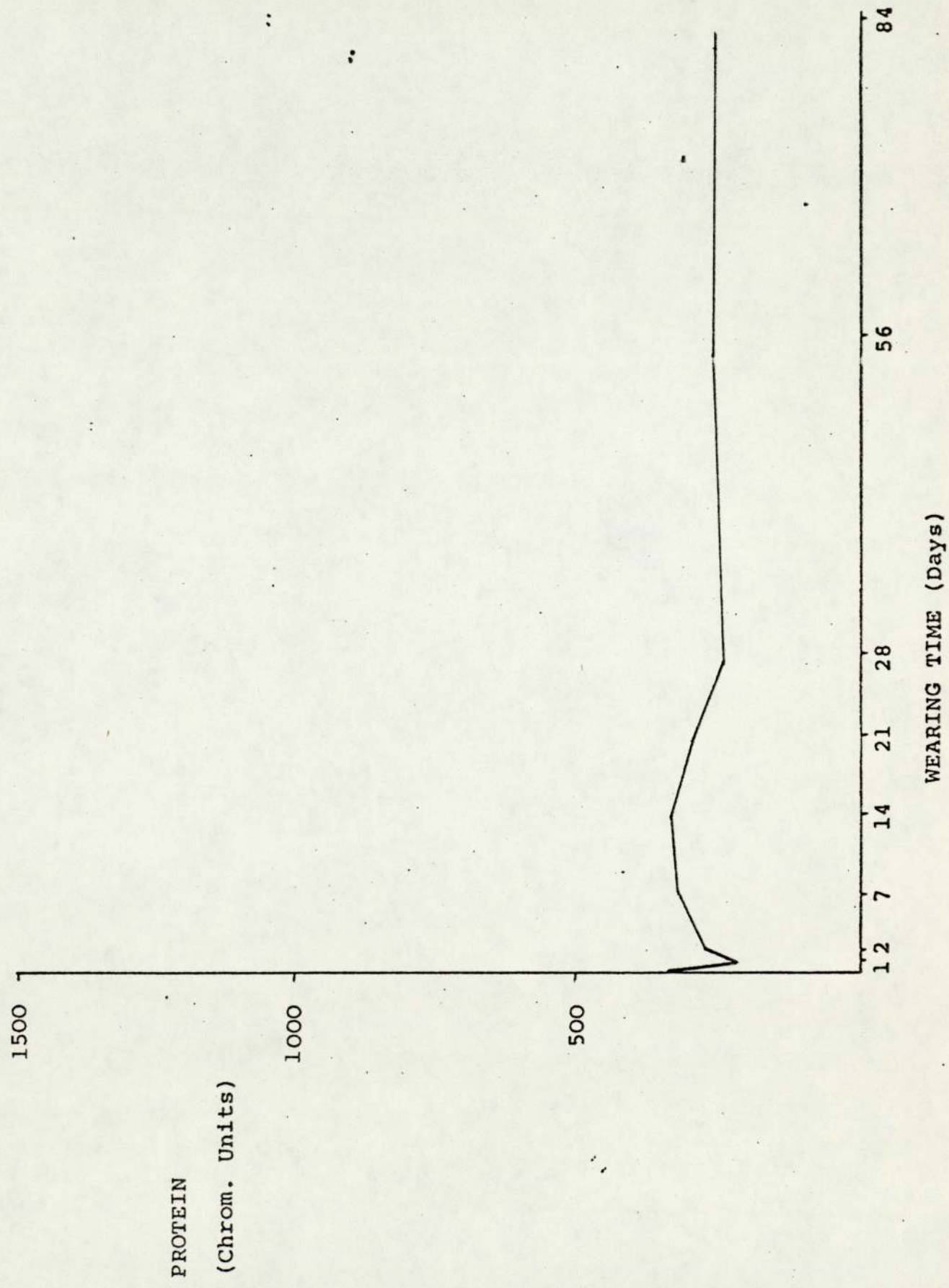


FIGURE 10:20 Sauflon 70 Lenses Group IV



Group C (Sauflon 70)

Figures (10:4, 17, 18, 19, 20)

This group shows similar trends to Group B, except that after the initial sharp decrease in the total protein values for day one, the rise on day two, is even larger than that found in Group B. Indeed, it is so great that it is slightly higher than the pre-fitting level. This is the only value for the amount of Total Protein, in any of the three contact lens groups, which exceeds the pre-fitting levels. After one week the total protein level had fallen and continued to fall gradually until around four weeks it reached a stable value for the remainder of the study. This value was considerably lower than the pre-fitting levels.

Groups I, II and III, show very similar trends to those of the total protein values. Again group IV showed different trends. After a small decrease on day one, there was a small rise on day two, which continued and established values, higher than the pre-fitting levels for the first two weeks. This then fell slightly to establish a more stable level, just below the pre-fitting values for the remaining eight weeks of the study.

General Discussion of all the Results

The normal rate of tear production in non-stimulated circumstances is 1.2 µl/min, according to Fatt (1978). Although hydrogel lenses are considerably more comfortable and biotolerable, than their hard lens counterparts, they still do produce a certain degree of "foreign body" sensation. Consequently an increase in the lacrimal production rate, at least over the initial adaptation, is almost unavoidable.

This expected increase in lacrimation could be anticipated to produce a more dilute tear solution. The results showed that all three types of lenses in this study, produced the expected reduction in the amount of protein contained in a standard 5 µl sample, as compared with base-line data, taken before contact lens wear commenced.

It is interesting to discuss the comparative results for the three different types of lenses. The Bausch and Lomb lenses produced the largest reduction in protein, and the Sauflon 70 gave the least drop in protein. A possible mechanism to explain these relative reductions is that from a clinical viewpoint, the Bausch and Lomb lenses were fitted with a slightly greater lag, or

amount of movement (i.e. relatively flatter), than the Sauflon lenses. This is mainly due to the Bausch and Lomb having a lower water content, and consequently producing lower oxygen, transmissibility values than both the Sauflon lenses. Due to this minimal lag on blinking, it is probable that the initial irritative value of the Sauflon lenses was lower than the Bausch and Lomb, and thus produced less excessive lacrimation.

Both the Sauflon lenses were fitted with approximately the same lag, if anything the Sauflon 85 was initially fitted slightly flatter than the 70, since it appeared to tighten more readily than the Sauflon 70. However, since the Sauflon 85 were not removed from the eye in periods of sleep and the Sauflon 70 were, it is not surprising that slightly greater irritative values for the extended wear lenses, were suggested by the more dilute tear solutions of the Sauflon 85 subjects.

The figures for the total protein contained in the tear samples for the Bausch and Lomb subjects, show that the pre-fitting levels were not regained within the twelve weeks of lens wear. This would suggest that a slight hypersecretion is maintained during this period of lens wear.

The Sauflon lenses only produced a marked decrease in total protein on the first day of wear. The levels for the second days, on both the 85 and 70 studies, were very close the the pre-fitting levels. Interestingly, the figures for Sauflon 70 are slightly higher than the pre-fitting levels, only for the second day of lens wear. These values eventually settle just below the pre-fitting levels.

If the degree of hypersecretion was correlated inversely with biocomfort levels, then these results would confirm the clinical impression that the adaptation to the Sauflon lenses was quicker than that for the Bausch and Lomb lenses.

However there are other contributory factors such as the possibility of absorbtion of protein molecules by the two higher water content Sauflon lenses. The pore-size figures for new Bausch and Lomb lenses, make it extremely unlikely that even the smallest protein molecules could penetrate its polymer matrix.

Thus an interesting possible hypothesis emerges to explain the rise in lysozyme levels produced in the Bausch and Lomb group, accompanied by the overall decrease obtained for the Sauflon lenses.

Since lysozyme is the smallest of the proteins monitored in this study, and could be absorbed by both the Sauflon lenses, and not by the Bausch and Lomb lenses. It is thus possible that all three types of lenses provoked at least an initial rise in the lysozyme levels, but that due to absorbtion into the Sauflon lenses, the increase in lysozyme was not evident in the higher water content polymers.

Since lysozyme has been suggested as one of the major contributory factors in the spoilation of hydrogel lenses (Karageozian 1976), it is notable that this is the only protein group which, from the analysis does not follow the trends of the "total protein" values, for any of the three lens groups which were fitted.

It is possible that the initial response of the eye, in hydrogel contact lens wear, is to increase the production rate of the tear fluid, and consequently decrease the total amount of protein, contained in a standard volume of this now, more dilute solution. However, since the lacrimal gland is the main source of this excessive tear volume, and also the source of lysozyme, there is a corresponding increase in the relative

amount of lysozyme in this hypersecretion. This effect would be masked in the higher water content hydrogels, until an equilibrium was established between the hypersecretion and the absorbtion of lysozyme into the lens polymer matrix.

The other protein groups, having different sites of production, may not be significantly affected by lens wear, although their relative proportions within the tear fluid do appear to decrease, due to the hypersecretion of tear fluid.

Group IV consisted of 2 fractions from the electrophoretic procedures, and have been found to correlate with the mobility of lysozyme. However since none of the tear samples are pooled, only one mobility of lysozyme would be expected in each sample. Since it stained using coomassie blue, it was assumed to be a protein complex but would have to be small, possibly smaller than lysozyme. Tiffany and Bron (1978) have demonstrated the presence of Non-lysozymal anti-bacterial factor in tears and they state that it moves quicker than lysozyme and has a smaller molecular weight (5000 - 7,500 compared with 14,500 for lysozyme).

Of all the protein sub-groups which were analysed, the most uniform response, for all types of hydrogels was from the albumins (Group II), where

all levels decreased on wear of the lenses, but there was no significant difference amongst the different types of hydrogels.

An interesting comparison is the response from the globulins (Group III), and lysozyme (Group IV), for the three different hydrogels. The Bausch and Lomb group which produced a rise in the lsyozyme levels, also produced the largest decrease in the globulins. The Sauflon lenses produced a small decrease in the lysozyme level and a larger overall decrease in the globulin level. Due to the large molecular diameters of the globulin group of proteins, it is most unlikely that any absorbtion into the polymers could occur. The values obtained for the globulin levels are most probably a true reflection of the actual secretion levels for this group of proteins.

The Group I proteins were not found to correspond in their electrophoretic mobilities to any of the normal protein "standards", known to be present in the lacrimal secretions. Visually they were found to be the most predictable and consistent fractions, during all the electrophoretic procedures. They consisted of two very thin closely spaced, but well defined bands which only penetrated 5-6 mms onto the top end of the polyacrylamide gels. The spacing was so small that sometimes the densitometer trace did not resolve two thin, but one slightly

thicker band. Therefore their total value was taken to form the results for Group I.

Throughout the thesis they have been named as one of the four protein sub-groups, because they consistently stained with the Coomassie Blue protein stain. However when they were not found to correspond to any single group of proteins it was then proposed that they may correspond to a stable protein complex. A protein complex with another biomolecule obviously produces a relatively large overall molecular diameter, When the relative sizes of the pore diameter of the gel was compared with the diameters of various protein complexes, the most likely complex would be one in combination with a lipid.

According to Burley (1971) the mechanism of lipid-protein interactions is one of the most important and difficult problems within current studies of biological chemistry. He defines a lipo protein as a complex, soluble in aqueous media, containing neutral lipid phospholipid and protein whose particles migrate as discrete units and resist separation into lipid and protein by physical methods. In solution lipoproteins appear as globular particles intermediate in size between large proteins and small viruses. This would explain their inability to migrate more than a few

mms, into the polyacrylamide gels, since if they did not break down their large units the relatively smaller pore size of the gel would physically prevent further movement. Several of the standard lipid stains e.g. Oil Red O, were used on gels which had already been stained with Coomassie Blue, however none of the bands stained, due to the gels being based on organic solvents which turned opaque with the lipid stains.

To investigate this further would have required either using different constituents for the gels or completely changing the type of media used for the electrophoresis e.g. Agarose gel. Since this was a side-issue which had not been taken into the original experimental design, it could not be pursued any further. However it is felt, that since the adhesion of lipoprotein type of complexes from the tears to the surfaces of hydrogels, is more than an interesting possibility, it forms a valuable area for further research.

More intricate patterns describing the variation of lacrimal proteins with hydrogel lens wear would emerge if the technique of electrophoresis could be further improved to give:-

(a) Repeatable results using even smaller volumes of lacrimal gluid,

(b) An even greater resolution of the individual protein fractions.

If condition (a) could be achieved then it may be possible to obtain two smaller volume samples from the same eye at the same time and thus assess the repeatability of the results.

If condition (b) was achieved then the variation of e.g. the very important immunoglobulins could be monitored. This would prove an extremely valuable area of research not only in the field of optometry, but in the wider sense of monitoring "foreign body" response in terms of body fluid composition, to the introduction of a hydrogel.

The partial fulfilment of condition (b) could probably have been achieved in the present electrophoretic method. However since the maximum resolution was already being obtained from the available densitometer, there seemed little point in improving the qualitative results when constraint was exerted from quantitative assessments of individual protein fractions.

Other suggestions for further work, would be to repeat this type of study over a longer time period using more subjects in each group.

The longer time period, perhaps twleve months would be more likely to produce the type of problems associated with a reduction in visual efficiency and discomfort. The lacrimal protein responses would be of interest throughout these periods, since no subjective or objective signs emerged in the present study. It should be emphasised that the lack of any discomfort, was perhaps caused by the very stringent cleaning routines given to each of the subjects. Within this type of study the levels of subject awareness of care and hygiene could be predictably greater than in the average contact lens practice.

This study did not include any female subjects since the presence of any menstrual variation in the lacrimal fluid has not been established. An obvious study could thus be to determine if any such variation exists and then design a hydrogel contact lens study around the results, using groups of female subjects.

A Brief Synopsis of the Conclusions

1) All three types of hydrogel lenses used in this study, (Bausch and Lomb Soflens, Sauflon 70 and Sauflon 85), were associated with a reduction in the overall protein content of the lacrimal tear fluid.

2) A hypersecretion of the lacrimal tear fluid was the proposed explanation for this decrease in the protein content. Initially, over the first few days of adaptation to lens wear, this excessive secretion was most marked. Since the protein levels never quite regained the pre-fitting values, this hypersecretion appeared to stabilise at a new value for the latter part of each study, usually from four to twelve weeks.

3) Under the specified electrophoretic conditions a minimum of nine fractions were consistently distinguished. The two slowest of these fractions, were classed as Group I for the analysis, and since no protein standards were found to correlate with these mobilities, they were proposed to be a conjugated protein complex, such as a lipoprotein. Group II, contained two fractions which correspond to specific tear albumin and serum albumin. Group III, contained a minimum of four fractions. Sometimes more bands could be visually distinguished but not resolved by the densitometer. These

correspond to alpha 1, alpha 2, beta and gamma globulin.
Group IV contained two closely spaced fractions the slowest of which corresponded to lysozyme. The other fraction, initially thought to be a facter moving lysozyme, was proposed to be a protein complex of lysozyme with another enzyme such as non-lysozymal anti-bacterial factor.

4) Group IV was the only one of these four sub-groups which did not follow the time-linked trends of the overall total protein values. The Bausch and Lomb group showed an overall small rise, and the Sauflon lenses showed an overall small decrease in the concentration of this sub-group. Since, theoretically lysozyme could be absorbed into the Sauflon polymers, but not into the Bausch and Lomb polymer, it was proposed that all three lenses could have provoked an increase in the lysozyme concentration but that absorbtive mechanisms had masked the effects for the Sauflon groups.

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I am greatly indebted to my supervisor Dr J R Larke (Department of Ophthalmic Optics). My sincere thanks are also due to Dr B Tighe (Department of Chemistry) and Dr Lewis (Department of Pharmacy).

To the volunteer subjects, students and staff of the Department of Ophthalmic Optics, I owe a very large debt, since without their vigilant and conscientious help the research would not have been possible.

In particular, I must include by friend and colleague Dr Christopher Wigham for his technical guidance and morale support, throughout the course of this research.

I am grateful to the Department of Ophthalmic Optics for the financial support of this work and to Contact Lens Manufacturing Limited, for the supply of various types of Polymers and Contact Lenses.

My thanks are also due to Ms Darina Ward for her unfailing patience and helpfulness during the typing of this thesis.

To Mr Ian Murray my eternal gratitude, for the years of patience and understanding.

APPENDIX

APPENDIX 1

Gel Preparation

To make 12 gels

Take:- 7.5 ml Acryl + Bis Mixture

7.5 ml Buffer

15 ml Ammonium Persulphate

The three solutions were mixed together taking care to add the ammonium persulphate last, as it is the catalyst. Care should be taken not to cause aeriation during the mixing of these solutions and after mixing for only about 1 minute, the solution should be left to settle for a couple of minutes before filling the tubes. The tubes are prepared with meticulous cleanliness as even the smallest impurity can cause delayed polymerisation and introduce variables into the procedure. The quartz tubes which have an internal bore of 5mms and external diameter of 7mms are 75mms in length. These are arranged in a stand which holds them securely vertical, and are sealed at the lower end with nescofilm. Using a large syringe with a needle attached, the monomer mixture is drawn up into the syringe and then each of the tubes is filled to 5mms from the top end, by slowly injecting the solution down the side of the tube. Again to avoid bubbles

and frothing (which can occur easily, mainly due to the S.D.S. content of the gels buffer). This mixture is then covered very carefully by a few drops of distilled water, which functions to overlay the gel solution so that it polymerises with a flat surface devoid of a meniscus distortion. Polymerisation was well under way within 30 mins, as evidenced by the presence of a Schlieren line between the gel, the water layer at the top of the gel. However the gels were never used within 2 hours of mixing, to be completely confident that polymerisation was completed.

When the gels were prepared they were never stored for more than a few hours, as marked gel shrinkage and distortion was found to occur even on overnight storage. Prior to electrophoresis the gels were removed from the stand and the lower wrapping of nescofilm was carefully removed, the top layer of water was discarded from the top of the gel, and the gel was fitted into the rubber grommets of the electrophoresis stand. A maximum of 12 tubes could be used and the 12 holes were equidistant from each other, and the electrodes.

The buffer was added to the lower electrode compartment. The samples were then removed from

the fridge, (where short-term storage was permitted), and the ends carefully broken off the micro-caps, a teat was fitted to one end and the sample was carefully ejected from the micro-cap onto the top of the gel surface, quickly, 5µl of S.D.S. solution was added to each gel on top of the sample and then finally covered with about 20µl of sucrose solution containing a small amount of methyl green, which was the tracer dye. The electrode buffer was then very carefully layered into the top compartment using a total volume of 1 litre for anode and cathode buffer compartments. The lid was then placed on the electrophoresis unit and the clock started when the voltage was adjusted to 100 volts from the constant power pack. This was allowed to run for a total of 1 hour 15 minutes.

After the allotted time the power was switched off and the gels were gently "rimmed" from their tubes into small individually coded glass bottles. This "rimming" was carefully carried out with a long, thin needle to separate the gel from the walls of the tube by injecting a layer of water slowly onto the adhering surface. "Rimming", must be carried out carefully in order not to scar or break the gel, turning the needle so that it passes right around the wall and simultaneously slowly down the gel, allowing time for the lubricant

to pass down with the needle. Since the needle would not reach the end of the tube the same process was repeated from the lower end of the tube.

When the gels were safely in the bottles, sufficient staining solution was poured into the bottle to cover the gel. The gels were stained for a period of 20 mins and then destained using several changes of destaining solution, usually over a period of a few days, to leave the distinctly stained protein bands within a clear gel background. Thus the gels were ready for scanning. Since the gels had swollen slightly during this staining and destaining process, new tubes of 7mms internal bore and 9mms external diameter were used to contain the gels for densitometric scanning. One at a time, the gels were placed into one of these wider tubes and inserted into the densitometer. The densitometer was arranged to transmit light through the Coomassie Blue filter before it reached the gel, which moved transversely across a 0.5mm wide, 7mm long beam of light, across which the gel moved, producing a transmission reading on a pen recorder which had a variable gain control. Thus a quantitative analysis of each gel was obtained, giving both an overall value for the total proteins and individual values for each protein band. The gels were always scanned from upper end first.

This scanning had to be done within a few days of staining since an unusual effect was noticed during long term storage. Theoretically, it is usually quoted that Coomassie Blue is a permanent stain. However the coloured stain was found to fade gradually and eventually disappear over a matter of weeks, if the gels were stored at a window. This did not happen if the gels were stored in a darkened cupboard. However it should that the cupboard was also cooler and it may be a combination of heat and direct sunlight, which cleared the gels. This effect has not been previously noted, to the author's knowledge. It is interesting to note that the cleared gels could be restained with Coomassie Blue, but that destaining was considerably more difficult than it had been originally.

APPENDIX 2A

Final Recipes for Making Acrylamide Gels

(a) 5 gm Acrylamide

0.085gm Bisacrylamide

Mix in 25ml distilled water

(b) 0.20 g Ammonium Persulphate in

100 ml distilled water

(Prepare fresh each week, keep cold in a dark bottle)

(c) Add 0.1 % w/v Sodium Dodecyl Sulfate

1 N KOH	48ml
Acetic Acid Glacial	17.2 ml
Temed	4.0 ml
Distilled water to 100 ml	

To make Gel:-

1 vol (a)

2 vol (b) Mix

1 vol (c)

APPENDIX 2B

Reservoir Buffer

(a) Final Recipe (pH 4.0)

Acetic Acid Glacial	2.4 g
Glycine	17.25g

Distilled water to 1 litre

APPENDIX 2C

1) Staining Solution

Make up 110 ml of a 5:1:5 (vol/vol/vol) solution of Methanol:Acetic Acid:Distilled water

i.e. 50 ml Methanol
10 ml Acetic Acid
50 ml Distilled Water

Add 0.25% (weight/volume) Coomassie Brilliant Blue R250

i.e. for the above vol of 110 ml
add 0.275 g Coomassie Blue

2) Destaining Solution

Mix 10 ml Methanol
+
15 ml Acetic Acid

Make up to 200 ml with distilled water

23 SEP 1976

Amino acid analysis, sample 8.00N

APPENDIX 3

	μ. qm. per sample.
asp	81.1
thre	25.2
ser	26.1
glu	55.2
pro	19.9
gly	30.2
ala	41.4
cystine	14.0
val	30.5
met	8.8
ileu	22.5
leu	41.7
tyr	36.8
phe	18.4
his	5.5
lys	21.1
arg	76.3
Total	554.7

J. E. Fr.

DATA SHEET

- 1 DEC 1976

Contact lens.

APPENDIX 4

residues per mole.

	(assuming lysozyme)	(lysozyme)
asp	17.5	18
thr	6.7	5
ser	8.9	6
glu	9.5	9
pro	6.0	2
gly	26.5	12
ala	23.4	14
cystine	0.8	4
val	8.9	8
met	1.5	2
ileu	5.0	5
leu	9.3	7
tyr	3.9	6
phe	2.4	2
his	0.8	1
lys	3.7	5
arg	8.9	14

J. E. Fox

APPENDIX 5

Raw Data for "Waking Hours" Study

SUBJECT NUMBER	SUB-GROUPS 1	2	3	4	TOTAL PROTEIN
1	18	32	35	32	47
	393	605	700	320	2018
2	19	36	37	28	46
	262	543	595	260	1677
3	27	29	35	24	39
	260	477	508	206	1448
4	15	48	40	31	64
	337	596	687	308	1928
5	26	54	43	40	75
	424	646	771	290	2131
6	23	41	31	38	79
	195	374	476	192	1232
7	24	46	39	35	73
	313	425	514	317	1487
8	12	43	41	37	68
	234	381	454	225	1320
9	22	29	37	21	42
	353	548	669	281	1851
10	29	38	33	16	61
	213	454	506	223	1410

APPENDIX 6

Raw Data Giving the Mean Total Protein Values for each of the Eight Data Collection Points

CONTROL GROUP X_1	X^2_1	GROUP A X_2	X^2_2	GROUP B X_3	X^2_3	GROUP C X_4	X^2_4
2240	5017600	1739	3024121	1371	1879641	1624	2637376
2352	5531904	1092	1192464	1918	3679724	2257	5094049
2291	5248681	1261	1590121	1713	2934369	2018	4072324
2322	5391684	1485	2205225	1663	2765569	1863	3470769
2290	5244100	1465	2146225	1677	2812329	1795	3222025
2315	5359225	1509	2277081	1659	2752281	1757	3087049
2295	5267025	1527	2331729	1569	2461761	1749	3059001
2311	5340721	1640	2689600	1574	2477476	1701	2893401
ΣX^2 = 42400940			17456566		21763150		27535994
ΣX = 18416			11718		13144		14764
$\bar{X}_1$		$\bar{X}_2$		$\bar{X}_3$		$\bar{X}_4$	
2302		146475		1643		1845.5	

ΣX_{TOT} = 58042

ΣX^2_{TOT} = 109156650

N = 4x8 = 32

APPENDIX 7

Truncated Data for A of V of Sub-Groups

CONTROL		GROUP A		GROUP B		GROUP C	
X_1	X^2_1	X_2	X^2_2	X_3	X^2_3	X_4	X^2_4
(1)							
3464	1500114	2496	800522	2656	899938	3403	1481387
MEAN = 433		312		332		425	
(2)							
5416	3667954	3180	1316452	3518	1582232	3620	1658334
MEAN = 677		398		440		452	
(3)							
6649	5496933	3248	1411940	5475	2634091	5575	3918441
MEAN = 831		406		572		697	
(4)							
2804	983188	2528	816982	2472	767674	2219	627077
MEAN = 350		442		309		277	

GROUP

	ΣX_{TOT}	ΣX^2_{TOT}
(1)	12019	4681961
(2)	15734	8224972
(3)	20047	13461405
(4)	10023	3194921

$\underline{N} = 4X8 = \underline{32}$

APPENDIX 8

UNIVERSITY OF ASTON IN BIRMINGHAM

DEPARTMENT OF OPHTHALMIC OPTICS

SOFT LENS RESEARCH

DECLARATION to be signed by experimental patients on initial registration.

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

I hereby agree to act as a volunteer experimental subject.

Signed . . . . . .

Date . . . . . .

NAME
(Block Capitals) . . . . . .

ADDRESS . . . . . .

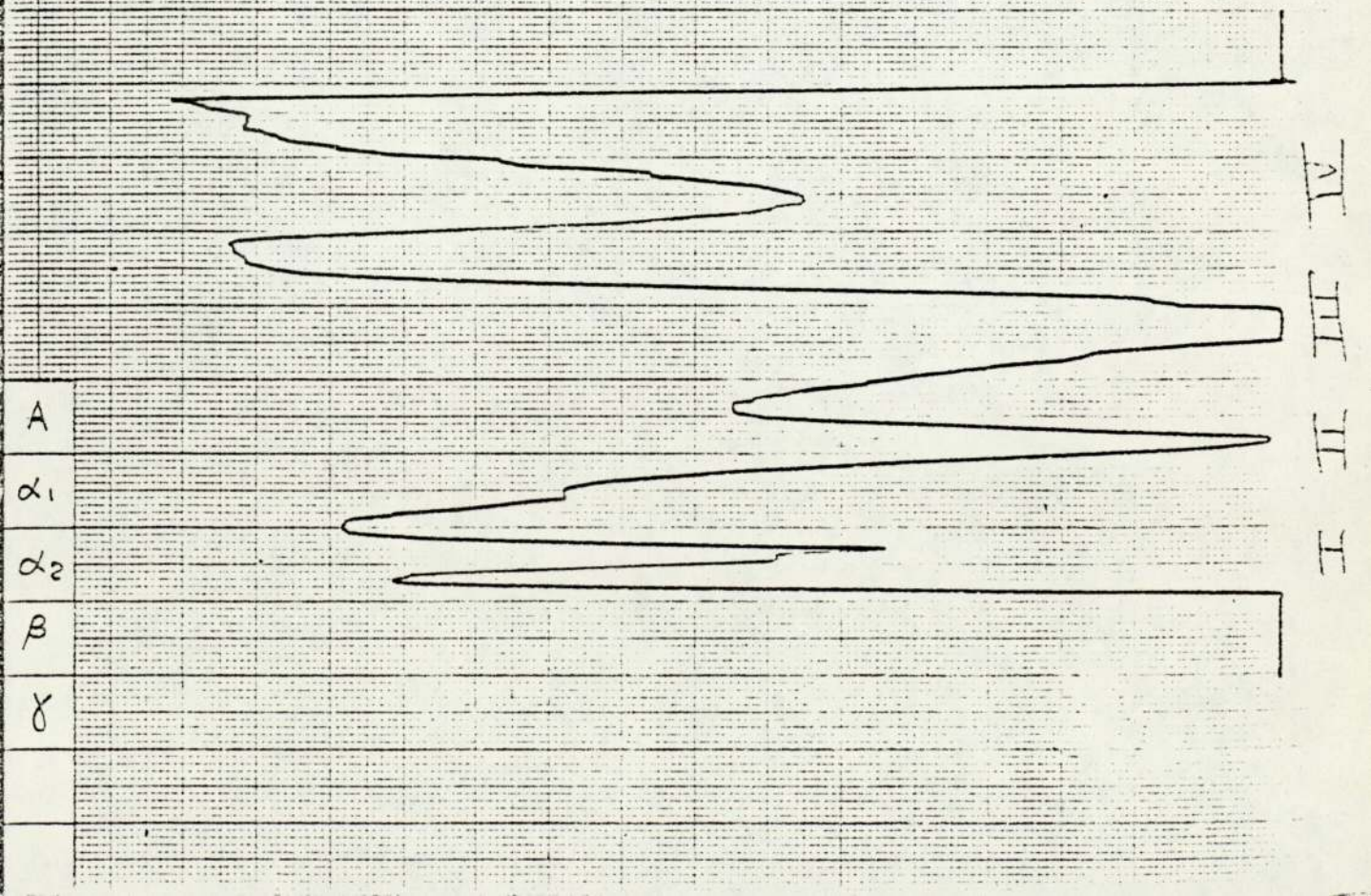
. . . . . .

. . . . . .

. . . . . .

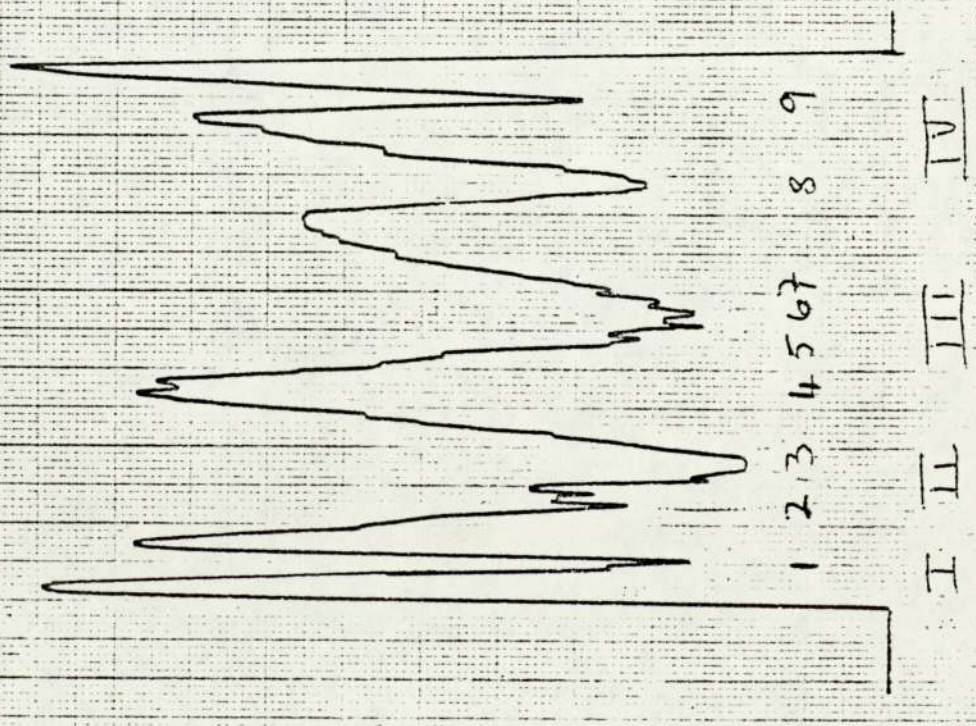
APPENDIX 9

FIG I A Typical Densitometer Trace of Acrylamide Gel before the Inclusion of S.D.S.



APPENDIX 9

FIG 2 A Typical Densitometer Trace of Acrylamide Gel after the Inclusion of S.D.S.



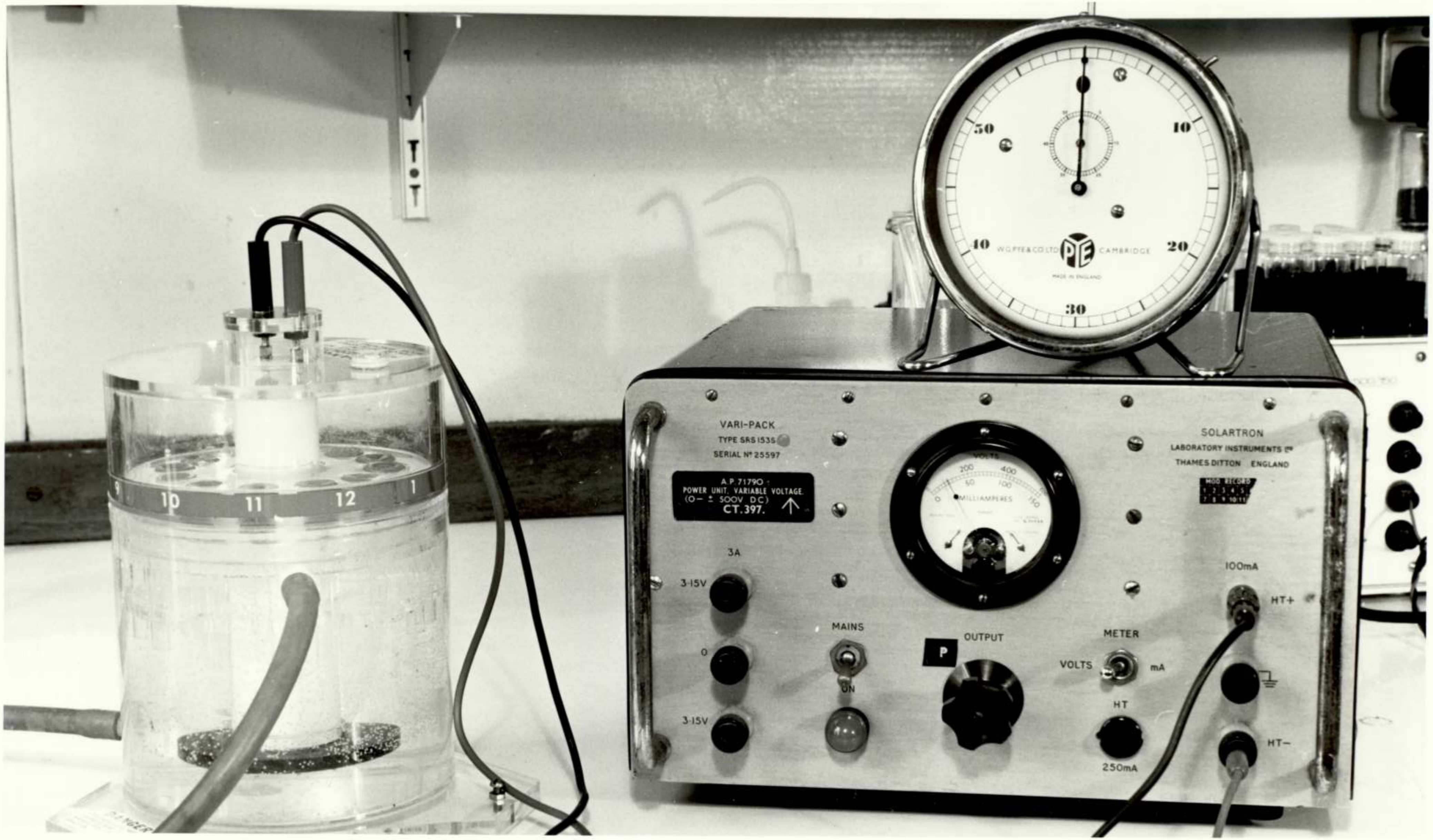
PHOTOGRAPHS

PLATE 1

The collection of a tear sample from the outer canthus, into a microcapillary tube

PLATE 2

The electrophoresis equipment and stabilised voltage supply



VARI-PACK
TYPE SRS 1535
SERIAL Nº 25597
A.P. 71790
POWER UNIT. VARIABLE VOLTAGE.
(0 – ± 500V DC)
CT.397.
SOLARTRON
LABORATORY INSTRUMENTS L^TD
THAMES DITTON ENGLAND
MILLIAMPERES
VOLTS
3A
3-15V
0
3-15V
MAINS
ON
OUTPUT
P
METER
VOLTS
mA
HT
250mA
100mA
HT+
HT–
W.G.PYE & CO. LTD
PYE
CAMBRIDGE
MADE IN ENGLAND

PLATE 3

"Gel Rimming". The removal of a polyacrylamide gel from the tube in which it was polymerised. This is performed after the electrophoresis in order to stain the gel.

PLATE 4

A Stained gel on the left of the photograph

A clear gel - second left in the photograph

A selection of gels in various stages of destaining

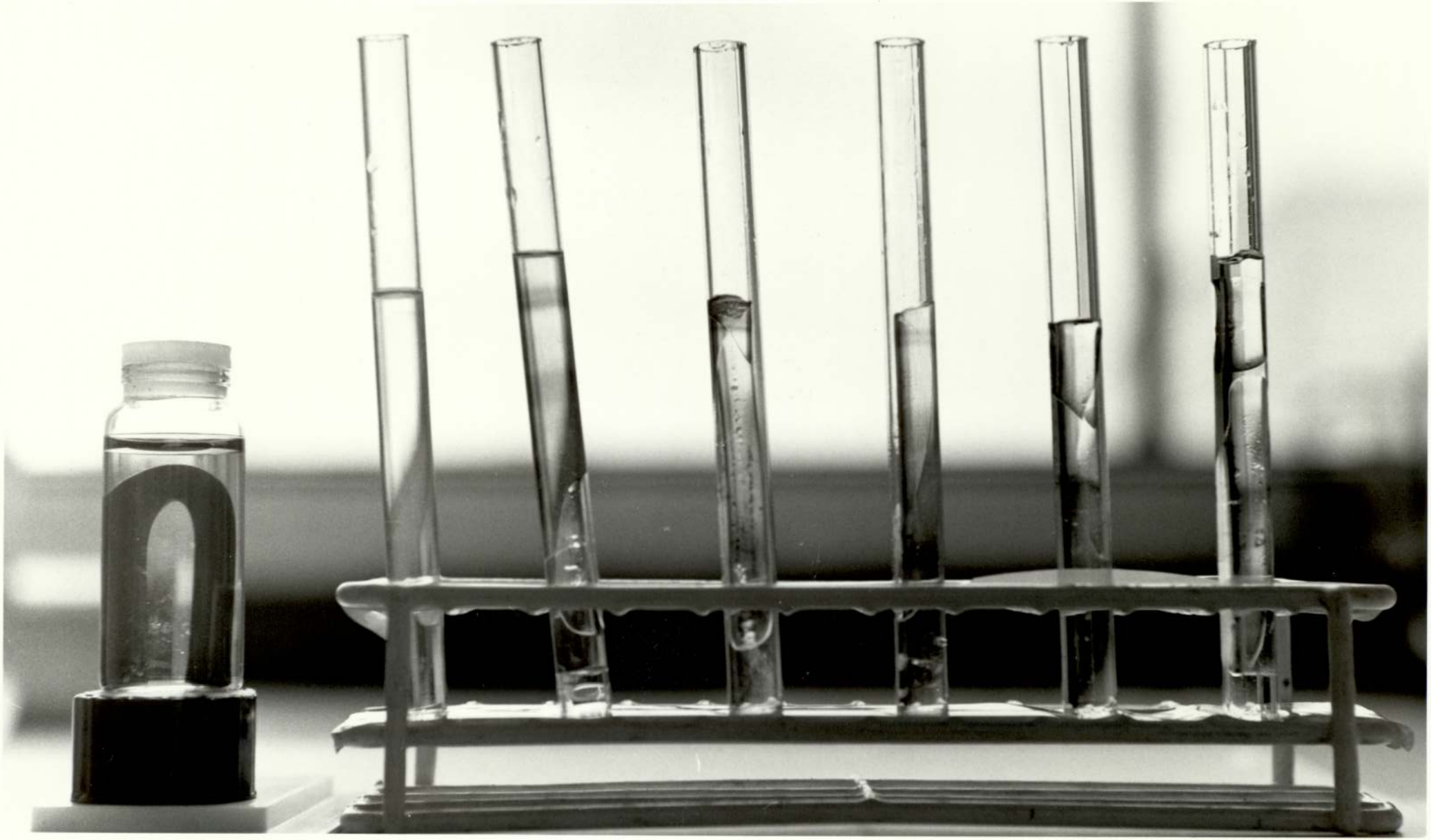


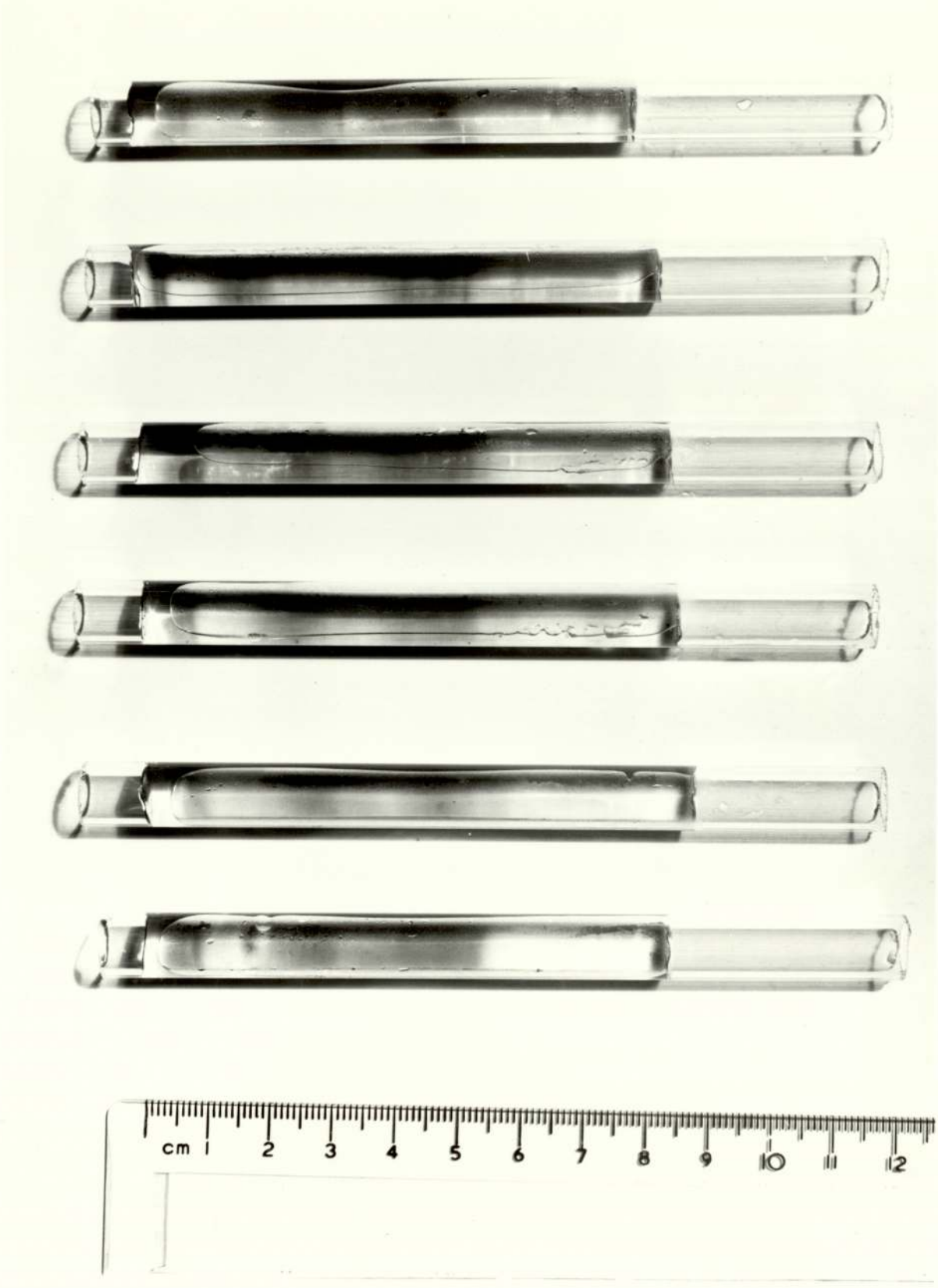
PLATE 5

Joyce Loebl Densitometer


CHROMOSCAN
START
PEN
MAINS
FUSES
OFF
OFF
522/96
JOYCE
LOEBL

PLATE 6

A selection of destained gels showing some separated lacrimal protein fractions. These fractions are specified according to their mobilities, thus the slowest fractions would occur at the top of the gel, around 4 - 6 mms.


cm 1 2 3 4 5 6 7 8 9 10 11 12

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