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Tear Protein Interaction with Hydrogel Contact Lenses

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Doctor of Philosophy

The University of Aston in Birmingham

April 1995

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Tear Protein Interactions with Hydrogel Contact Lenses

A thesis submitted for the degree of Doctor of philosophy
to
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by

Reyhaneh Sariri-Khayatzadeh

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Summary

The design and synthesis of biomaterials covers a growing number of biomedical applications. The use of biomaterials in biological environment is associated with a number of problems, the most important of which is biocompatability. If the implanted biomaterial is not compatible with the environment, it will be rejected by the biological site. This may be manifested in many ways depending on the environment in which it is used.

Adsorption of proteins takes place almost instantaneously when a biomaterial comes into contact with most biological fluids. The eye is a unique body site for the study of protein interactions with biomaterials, because of its ease of access and deceptive complexity of the tears. The use of contact lenses for either vision correction and cosmetic reasons or as a route for the controlled drug delivery, has significantly increased in recent years. It is relatively easy to introduce a contact lens into the tear fluid and remove after a few minutes without surgery or trauma to the patient.

A range of analytical techniques were used and developed to measure the proteins absorbed to some existing commercial contact lens materials and also to novel hydrogels synthesised within the research group.

Analysis of the identity and quantity of proteins absorbed to biomaterials revealed the importance of many factors on the absorption process. The effect of biomaterial structure, protein nature in terms of size, shape and charge and pH of the environment on the absorption process were examined in order to determine the relative up-take of tear proteins.

This study showed that both lysozyme and lactoferrin penetrate the lens matrix of ionic materials. Measurement of the mobility and activity of the protein deposited into the surface and within the matrix of ionic lens materials demonstrated that the mobility is pH dependent and, within the experimental errors, the biological activity of lysozyme remained unchanged after adsorption and desorption.

The study on the effect of different monomers copolymerised with hydroxyethyl methacrylate (HEMA) on the protein up-take showed that monomers producing a positive charge on the copolymer can reduce the spoilation with lysozyme.

The studies were extended to real cases in order to compare the patient dependent factors. The *in-vivo* studies showed that the spoilation is patient dependent as well as other factors.

Studies on the extrinsic factors such as dye used in colour lenses showed that the addition of colourant affects protein absorption and, in one case, its effect is beneficial to the wearer as it reduces the quantity of the protein absorbed.

Keywords: Biomaterials, hydrogels, contact lens spoilation, electrophoresis, lysozyme activity, protein mobility, isotachophoresis, U.V. and fluorescence spectroscopy.

Dedicated to my son

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List of Symbols and Abbreviations

AMO	Acrylo morpholine
ANS	Aniline-naphthalene sulfonate
BBG	Brilliant blue-G
Bis	N, N'-methylene-bis-acrylamide
BSA	Bovine serum albumin
C	Celsius
Ca^{++}	Calcium ion
CHMA	Cyclohexyl methacrylate
Cl^-	Chloride ion
DA	Diacetone acrylamide
DMAEMA	Dimethylaminoethyl methacrylate
DMAPN	3-Dimthylaminopropionitrile
DNS-Cl	Dancyl chloride
EGDM	Ethyleneglycol dimethacrylate
EWC	Equilibrium water content
F	Refractive power
FAD	Flavine-adenine dinuclotide
FDA	Food and Drug Administration (USA)
g	Gram
HCO_3^-	Bicarbonate ion
HEMA	Hydroxyethyl methacrylate
K^+	Potassium ion
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IPN	Interpenetrating polymer network
ITC	Itaconic acid
ITP	Isotachophoresis
KD	Kilo Dalton
mA	Milli ampere
MAA	Methacrylic acid
mg	Milligram
µg	Microgram
Mg^{++}	Magnesium ion
ml	Millilitre
µl	Microlitre
MMA	Methyl methacrylate

mM	Millimole
MPEG	Methoxy polyethylene glycol
μsec	Micro second
Na^+	Sodium ion
NAD	Nicotinamide-adenine dinucleotide
NNDMA	N, N dimethyl acrylamide
NVI	N-vinyl imidazole
NVP	N-vinyl pyrolidone
nsec	Nanosecond
PAGE	Poly acrylamide gel electrophoresis
PEG	Polyethylene glycol
PEGOH	Hydroxy terminated polyethylene glycol
PEO	Polyethylene oxide
pH	1/log the hydrogen ion concentration
PHEMA	poly (2-hydroxyethyl methacrylate)
pI	Isoelectric point
pK	1/log the rate constant of weak acid
PMMA	Poly(methyl methacrylate)
PPO	Para-diphenyl oxazole
PVA	Polyvinyl alcohol
PVP	Poly (N-vinylpyrrolidone)
Rf	Rate of flow
RGP	Rigid-gas permeable
RNase	Ribonuclease
SD	Standard deviation
SDS	Sodium dodecyl sulphate
Sec	Second
SPE	N-(3-sulphopropyl)-N-methacryloxyethyl-N, N-dimethyl ammonium betaine
TEMED	N, N, N', N'-tetramethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
UDP	Uridine diphosphate
USAN	United States Approved Name
UV	Ultra violet
V	Volt
VP	N-vinyl-2-pyrrolidone
WA	Weak acid

Chapter 1

Literature Survey

Chapter 1

Literature Survey

1.1 The Eye

The eye visualises the universe by converting light radiation into nerve impulses. It is a mobile sphere on which the principles of a camera are based. A variable aperture (iris) regulates the amount of light entering; a transparent optical system (lens) focuses this light and a concave film (retina) picks it up. The oldest diagram of visual system which illustrates the mechanism of binocular stereoscopic vision is found in '*The Book of Reflections on the Science of Optics* 'by Kamaloddin Aboul Hassan Farsi a Persian scholar who wrote a commentary on Alhazen's '*Book of Optics* 'at the beginning of the Fourteenth Century (Figure 1.1).

The eye possesses two quite distinct visual faculties, central vision and field vision. Although they are both served by the same retina they are quite separate in position and quite different in function. The eye is frequently compared to a wide-angled camera. There are, however two dissimilarities. First of all the camera film is flat, whereas in the retina is curved and secondly the camera does not discriminate what it records, whereas the eye picks out one object to fix on, whilst recognizing the presence of the others and recognising less the further they lie from the centre of fixation. It would seem that very special tissues are necessary to preform this unique action, but in fact the tissues of the eyes are found in other body sites.

1.1.1 The Structure of the Eye

The anterior aspect of the eyeball, the conjunctiva, is protected from injury by two curtain-like structures, the upper and the lower eye lids. The aperture between the lids, which is known as the palpebral fissure, is approximately almond-shaped when the eye is fully open. The lids close periodically to sweep the eyeball clean and maintain the tear fluid film. The outer coat of the globe (the sclera) is like the ball of a joint which moves with in a socket of muscle, bone and the eyelids. As the eye is formed of collagen fibers, it is open to the same diseases that collagen can suffer elsewhere. A small segment of the anterior half, the cornea, differs from the remainder of the sclera as it is transparent. The cornea is the first structural line of defence for the eye. Damage or destruction of the cornea leads to blindness for many people.

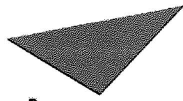

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Figure 1.1 The oldest diagram of visual system comparing the eye with camera [1].

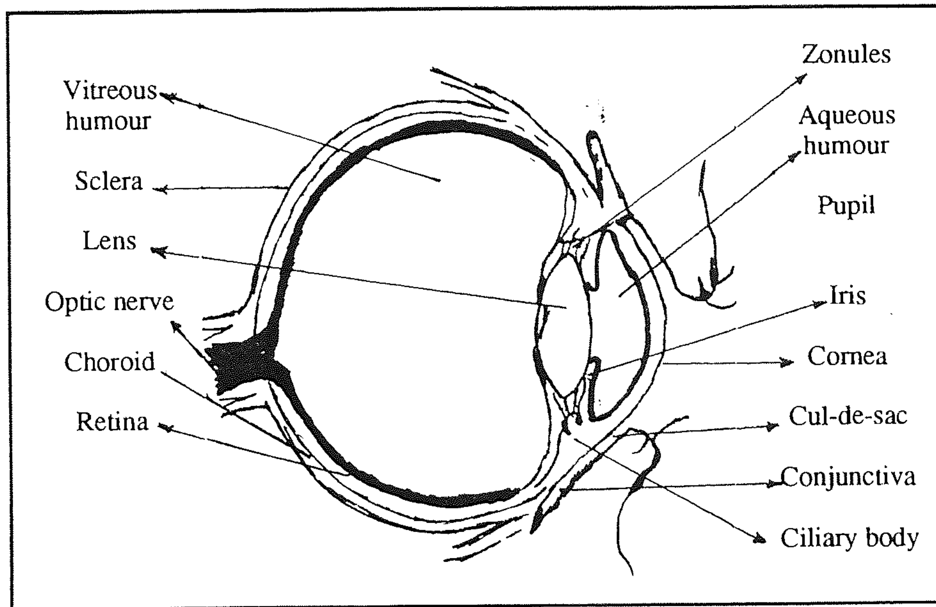

Figure 1.2 Horizontal section of the eye.

Both the conjunctiva and the cornea are kept moist by the lacrimal gland, which is located above the lateral aspect of the eye and ducted into the upper fornix of the conjunctival sac. Figure 1.2 shows a cross section of the component parts of the eye [1].

1.1.2 Nutirition in the Eye

The one unique quality of the eye is that it is partially transparent. Like any other optical system it is fashioned from a series of focusing structures which are transparent, and held together by a series of supporting structures which are not.

Unlike other optical systems, the eye is composed of living tissues and all such tissues require nutirents and waste disposal. The opaque elements supplied with nutirents via a vascular system. The transparent components (cornea, the anterior chamber aqueous, the lens and the vitreous) are supplied by an aqueous fluid. The retinal layers nearest to the vitreous are also light-permeable because the photoacceptors have to contact the pigment retina. Because the cornea is the one continuous fluid structure that forms the external surface of the eye, it can not rely wholly on aqueous. The aqueous supplies the deep layers of the eye, but the superficial layers are dependent the on tear fluid. There is no sharply defined margins in nature and sceleral blood vessels occasionally loop over the corneal margin.

Without the eyelids, the conjunctiva and tear glands, there would be no cornea. Together, these four components form the external eye. The eyelids open to permit

vision. Since the external eye is moist, like the mouth, it is most healthy when shut. However since it is also an optical structure, it clearly has to open some of the time. The eye's construction prevents damage when it is open provided that it opens for short period of time.

When the cornea is damaged, the tear fluid and aqueous fluid are insufficient to meet its requirement and corneal vascularsation may occur. The principle of a synthetic material to replace the damaged cornea has attracted a lot of interest in the last few years. It is, in fact, an in-dwelling contact lens which replaces the cornea and becomes part of the eye. This synthetic cornea material has to be clear and must integrate with the eye by allowing the epithelial cells which cover the surface of the cornea to grow over it.

1.2 Tear Flow

"Tears, idle tears, I know not what they mean." When Tennyson wrote this line he was refering to the fluid that flows from the eye and the nose when one weeps for sorrow, pain, anger, joy, frustration, blackmail, or sympathy. These are *psychogenic tears*, which are only one of four categories of tears shed by man. Hear to be unique to man; in spite of the term : crocodile tears" there is no evidence that any other animal weeps in responce to emotional stress. The tears that flow from the eye when its surface is excessively exposed to light, cold, wind, foreign bodies or irritating gasses and liquids are called *reflex tears*. Reflex tears occur in man, domestic animals and perhaps all terrestrial and amphibian vertebrates with the probable exception of snakes. Every animal that has a backbone and spends all or part of its life out of water sheds a third type of tears; *continuous tears*. A fourth type of tearing is induced when such substance as war gases are administrated not to the eye but elsewhere in the system, ultimately reaching the tear glands by the way of the blood stream. These "secretagogues" are distinct from the irritant substance contained in many tear gases (and perhaps in onions) that induce reflex tearing.

Although well known as manifestations of sorrow, emotions, frustration and blackmail, tears have a more prosaic and important function as a lubricant and as a blood substitute for the cornea. They are not just bitter salt water as the poets would have us believe. They contain in addition proteins,lipids and glycoproteins which increase the wetting effect of the aqueous component and delay evaporation.

The mechanical action of tears in protecting the external eye has been recognized. Tears coat and lubricate the ocular surface, maintain a distortion-free optical surface,

and remove foreign and cellular bodies and detritus from the external surface of the eye. Tears transport oxygen and carbon dioxide, and play a central role in the cellular economy of the ocular surface and conjunctiva.

The tears are produced by the lacrimal and accessory lacrimal glands, entring the eye via the superior cul-de-sac. A portion of the tear fluid flows over the eye and around the cul-de-sac during the blinking action of the lids. Any remaining tear fluid drains away via small holes in the eyelids called puncta and along minute canals to collect in a tear sac. Normally tear fluid flow is about 1μl/minute, with about 7μl of the tear fluid in the eye at one time [2]. The cul-de-sac can hold about 24μl maximum with excess tearing. The turnover period for the tears is about 5 to 6 minutes.

1.2.1 Structure of the Tear Film

The pre-corneal film is probably the most regularly arranged fluid to be found within the body. The thickness of the film has been variously estimated as: 6μl [3], 10 μl [4] and just below 10μl [5]. Even though the film is very thin, it is composed of three distinct layers and attains its thickness from the moment after the blinking, slight thining of the film as a result of the evaporation may be observed [6]. The division of the aqueous layer into two separate layers, however, is less acceptable and the mucoid layer is in fact a part of the aqueous layer.

The superficial lipid layer is principally derived from the tarsal (meibomian) glands. The aqueous phase is wholly drived from the main and accessory lacrimal glands, while the mucus layer is derived from the conjunctival goblet cells.

The function of the lipid layer is the reduction of evaporation from the aqueous phase [7]. It is, however, suggested that the non-polar nature of the surface layer is an important factor in preventing surface contamination of the film with highly polar skin lipids.

The aqueous phase of the tears contains a wide variety of organic and inorganic substances. In addition to the principal inorganic ions, as many as sixty proteins are present in the tears, together with a variety of biopolymers, glycoproteins, glucose and urea. Figure 1.3 shows the three layers of tear film and the relative thickness of each layer.

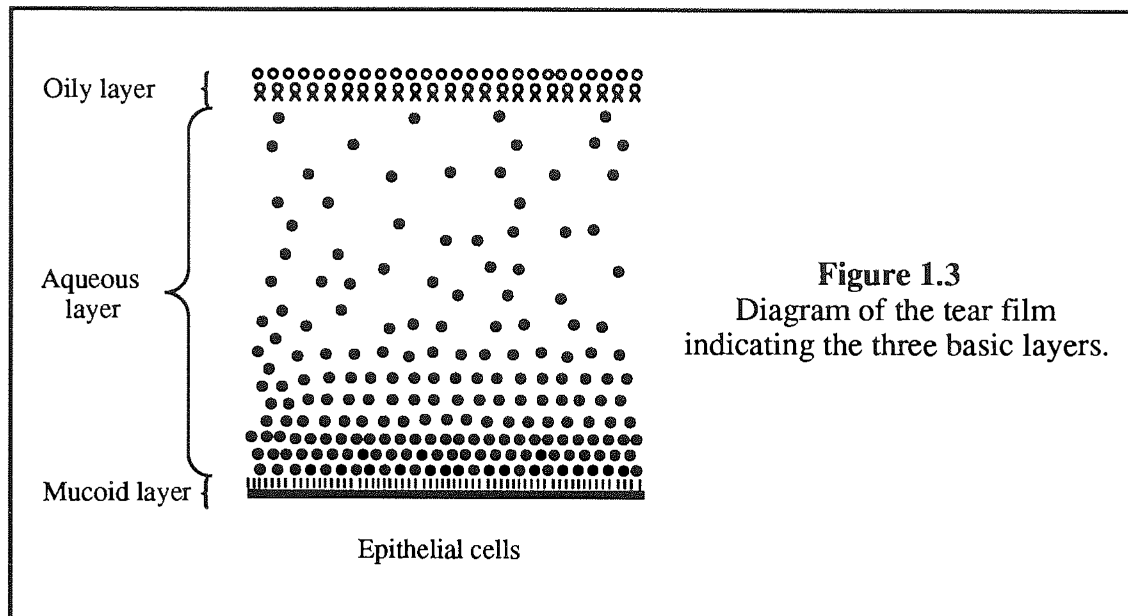

Figure 1.3
Diagram of the tear film indicating the three basic layers.

1.2.2 Tear Volume and its Distribution

The tear volume has been estimated to be 7μl (+ 0.2μl) [9], with a production rate of 1.2 μl/minute. Tear production rates reduce with age, declining from an average of 2μl/min at fifteen years old to less than 1μl/min at sixty-five years of age [10]. The tear film is not evenly distributed over the ocular surfaces, but forms a distinct meniscus at the lid margins; the marginal tear strips, or lakes. The volume of tears in this area is approximately 3μl, while the volume covering the cornea is about 1μl and a further 3 to 4μl are distributed in an even manner over the conjunctiva [9].

Although it is possible to ascribe differing volumes to the tear film located in differing parts of the anterior surface of the eye, the tears are not static [11]. The act of blinking has a substantial effect upon the film and the marginal strips. Holly [4] has described dynamics of the tear film due to the blinking. As the upper lid moves downwards, the superficial lipid layer is compressed. As it thickens, it begins to exhibit interference colours. When the eye opens, at first the lipid spreads in the form of a monolayer against the upper eyelid. In this spreading process, the limiting factor is the motion of the eyelid. The spreading of the excess lipid follows, and in about one second the duplex (multimolecular) lipid layer is formed. The spreading lipid drags some aqueous tears with it, thereby thickening of the tear film. The magnitude of this effect is controlled by the size and the shape of the tear meniscus, a local thinning adjacent to the meniscus takes place, which effectively prevents further fluid flow from the meniscus to the tear film.

1.2.3 Tear Film pH and Buffering Capacity

Due to the active transport of sodium and chloride ions (Na^+ and Cl^-) in the cornea, the maintenance of corneal transparency depends on the pH [12]. Knowing the pH value in the three-layered pre-corneal fluid is the key to understanding the corneal physiology [13]. Using semi-micro- and micro-glass pH electrodes, Fischer [14] reported the mean pH value of pre-corneal lacrimal fluid of the human eye to be pH 7.6 (± 0.4). The pH value shifts toward the alkaline range, the longer the lids remain open. The alkalization is a consequence of the equilibration of the bicarbonate in the lacrimal film with the pressure of the CO_2 in the surrounding air.

Although the complete array of the buffering capacity mechanisms that act in the tears have not been determined, the bicarbonate system seems to be very important. It has been shown that the more shift of tear pH toward alkaline (upward) the weaker the buffering capacity of the tears [15].

1.2.4 Chemistry of the Tear Film

Tear film possess a complicated chemical structure which contains many proteins, lipids and a number of inorganic substances. A full understanding of the structure of the tear film is necessary in order to explain its interaction with hydrogel contact lenses.

1.2.4.1 Lipid phase

The composition of human tear lipids was probably first described in 1897 as cholestrol, fatty acids and fat [16]. However, the large size and number of meibomian glands suggest that the eye has a considerable requirement for fatty materials. Lipids are required to prevent wetting of the skin of the lids adjacent to the eye and to contain the tears. Some lipids are spread over the tear film surface forming the outermost layer of the film and reducing evaporation. This spreading action may be aided by the particular nature of the fatty acids found in lipids which form an unusual group of high molecular weight compounds. It has been suggested that considerable variations in lipid composition exist between different individuals [17]. However, there are considerable technical difficulties in the analysis of the very small samples that can be obtained from subjects and the close similarities between post-mortem samples of human and bovine fluid suggest that the requirement for lipid on the eyelids of man and animals is similar. Table 1.1 shows the lipid composition for bovine and human meibomian samples.

Table 1.1 Comparative lipid composition for bovine and human meibomian samples (all values % by weight) [18].

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1.2.4.2 Aqueous phase

The aqueous phase of the tears forms the major component of the film, comprising about 98% of its total thickness. It is a complex dilute solution of both inorganic electrolytes and low and high molecular weight organic substances.

1.2.4.2.1 Electrolytes

The main cation found in the aqueous phase of the tear film is sodium, and its concentration is similar to that found in serum. Potassium is another principal cation found in tears, but its concentration is about 3-6 times higher than its concentration in serum. Calcium and magnesium cations are found in small quantities in tears. The two principal anions in tears are chloride and bicarbonate ions, and their concentrations are very similar to those found in serum. Table 1.2 compares the concentrations of tear and serum electrolytes.

1.2.4.2.2 Organic substances

1.2.4.2.2.1 Glucose

Glucose is present in tears only in very low concentrations. Raised glucose levels may occur with diabetes, but these values are attributable to the raised tissue fluid levels rather than raised tear levels.

Table 1.2 Average concentrations (mmol/l) of human tear and serum electrolytes [19].

1.2.4.2.2.2 Amino acids

The free amino acids present in tears have not totally been identified, but may be present in a concentration that is three to four times the serum levels.

1.2.4.2.2.3 Urea

The concentration of urea in tears is similar to that of plasma (20-40%). This suggests an unrestricted passage of urea across the blood/tear barrier of the lacrimal gland.

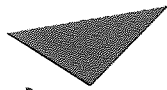
1.2.4.2.2.4 Tear proteins

The aqueous phase of tears contain a remarkably complex mixture of both locally produced and serum derived proteins. Using the cross immuno-electrophoresis technique, Gachon *et al.* [21] have identified at least sixty protein components, some of which are immunologically indistinguishable from serum homologous, while others are clearly distinguishable and of specific tear origin. Table 1.3 lists some identified tear proteins.

Although a complex mixture of proteins have been identified in tears, the most important tear proteins are lysozyme, lactoferrin, albumin, tear specific pre-albumin and globulins.

The main objective of this project is to understand fully the role played by proteins in contact lens spoilation. Therefore, details of the structure and the biological activities of proteins would be discussed later in this chapter.

Table 1.3 Average concentrations of important tear proteins [20].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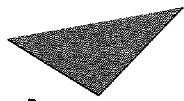
1.2.4.3 Mucous phase

The major source of mucus is conjunctival goblet cells. The essential function of mucoid layer over both corneal and conjunctival surfaces is to render this hydrophobic surface hydrophilic. In addition, mucus plays a role in removing lipid and debris from the surface of the anterior eye [22]. The effect of the surface bound mucous is to increase the surface tension to a level at which surface wetting will occur (about 42 x 10^{-5} N/cm).

Mucus is made up of high molecular weight glycoproteins. Each polypeptide chain has, at about every tenth residue, a carbohydrate chain, each chain is about ten saccharide units long. The glycoprotein component of mucus has been separated by Moore and Tiffany [23]. The principal mucin complex GP1 (molecular weight > 2 x10^6) and its subunit GP3M (Mw~200,000) were detected in an un-reduced saline extract of mucus. Reduction of disulphide bonds in the extract gave rise to GP3 (Mw > 1.3 x10^6), but a considerable patient variation between people was detected.

The ocular mucus is composed of mucin-type glycoproteins, many of the tear components found in the aqueous phase are also found in mucus. The presence of some components such as IgA, lysozyme and lactoferrin in mucin layer can provide bacteriostatic properties of the mucin.

Table 1.4 Some proteins identified in tears [21].



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1.3 Vision Correction

If the whole point of the eye is to see, the whole point of both eyes is to see biocularly. No one would seriously deny that we walk better when we are wearing shoes. If glasses are needed, we will see better when we wear them. We do not become addicted to the glasses; we become addicted to the pleasure of seeing clearly. In spite of these facts, unfortunately, many people do not use their glasses properly. They either forget, loose or, simply, do not like them. The idea of replacing glasses with contact lenses has certainly helped patients with these sorts of excuses. According to a survey that was conducted by the U.S. Food and Drug Administration (FDA) in 1986, approximately 18.7 million people in the United States wear contact lenses [24]. Currently, it is estimated that some 10% of all Americans wear contact lenses; this represents an increase of more than 35% during the past 10 years.

1.3.1 Historical Developments of Soft Contact Lenses

Correcting refractive errors by placing a lens on the eye was described many years before a practical method to accomplish this feat was developed. The earliest reference to contact lenses was by Leonardo da Vinci in 1508. He described using glass contact lenses to neutralize (eliminate the refractive power) the cornea.

For more than a hundred years there was no further reference to the development of contact lenses. Then, the investigations started again in 1636 when Rene' Descartes, described a tube of water placed on the eye neutralizing the human cornea. The research in this area was carried out slowly but progressively over a long period of time until, during 1950s, various lenses were made of poly methacrylic acid (PMMA). The next and more important change in the contact lens field was the introduction of hydrogel contact lenses. Table 1.5 shows a summary of the developments in this area.

In the early 1960s, Otto Wichterele of Czechoslovakia invented hydrophilic contact lenses made of hydroxyethyl methacrylate (HEMA). These plastics differ considerably from PMMA in that the hydrogels absorb relatively high quantities of water (30-85% by weight) and become soft and flexible.

Hydrogels became commercially available in the early 1970s, and brought about a great revolution in the field of contact lens practice. Their ease of fitting, longer wearing times and ease of adaptation soon resulted in rapid market growth.

Table 1.5 Summary of historical developments of contact lenses.

Year	Individual (s)/Company	Development
1508	Leonardo da Vinci	Described glass contact lenses.
1636	Rene' Descartes	Tube of water used to neutralize the cornea.
1801	Thomas Young	Used Descartes principle to study the eye.
1827	John Herschel	Described how a contact lens could be ground; concept of moulding the eye.
1887	F. E. Muller	Fitted a glass-blown lens for a patient to protect the eye.
1888	E. Kalt	Designed and fitted glass corneal lenses. Used ophthalmometer to fit lenses.
1888	A. E. Fick	Described first glass lens to be worn to correct vision.
1936	W. Feinbloom	Made lens with glass central optic and plastic surround (first plastic used in contact lens).
1938	Mullen and Obrig	First all-plastic (PMMA) contact lens.
1947	N. Bier	Fenestrated minimum-clearance haptic lens.
1947	K. Touhy	All-plastic corneal lens.
1950	Butterfield	Designed corneal lens to parallel the cornea. Used peripheral curves.
1960	Wichterle and Lim	Hydrogel polymers for contact lenses.
1968		U.S. FDA became involved in regulating contact lenses.
1971	Bausch and Lomb, Inc.	First hydrogel lens approved in United States.
1970s	J. De Carle	Extended wear with high water contact hydrogel lenses.
1970s		First clinical marketing of soft silicone lenses.
1978	Danker Laboratories	U.S. FDA approval of CAB lenses.
1979	Syntex Ophthalmics	U.S. FDA approval of a PMMA-silicon copolymer lens.
1984		disposable lens introduced in Denmark (Dana lens).
1987	Vistakon, Inc.	First disposable lens approved by U.S. FDA (Acuvue™ lens).

1.3.2 Lens Power

A contact lens has a certain specified refractive power dependent on the refractive index of the material, front and back surface radii, and the lens thickness. The refractive index of a material is a specification indicating its ablility to slow and bend light waves. The higher the refractive index, the greater the slowing and bending the light waves. For example, the refractive index of air is 1.0, water is 1.33, soft contact lenses is 1.43, polymethyl methacrylate (PMMA) contact lenses is 1.49, and rigid gas-permeable lenses is approximately 1.47.

The greater the difference in the refractive index on the two sides of a surface, the greater the bending of the light. Also the steeper (shorter radius) the surface, the greater the bending of the light. The refractive power of a surface is determined by the following formula:

$$F = \frac{n' - n}{r}$$

where F is the refractive power in diopters (D), n' is the refractive index of the second media, n is the refractive index of the first media, and r is the radius of curvature of the surface in metres.

If the lens front radius is longer than the back surface radius, the lens has minus refractive power (Figure 1.4).

The refractive power of a lens can be calculated by knowing the dimensions, but, clinically, the dioptric power is determined by using the lesometer, a lens power measuring device which gives the power of the contact lens in the air.

1.3.3 Contact Lens Materials

Contact lenses are made of polymers. Chemically, these are very large molecules made up of small units (monomers) that are repeated in long chains. These long molecules may have a branching pattern or adjacent molecules may be connected by cross-linking molecules giving the polymer different properties.

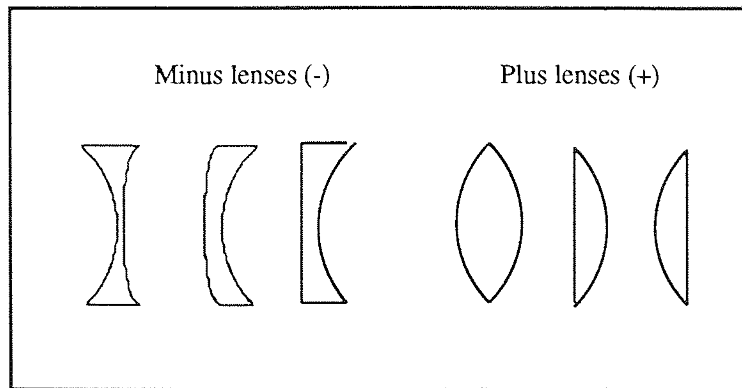


Figure 1.4 Plus and minus-powered contact lens.

Many important properties of these polymers must be considered when used in the manufacturing of contact lenses. An important property of a lens material is its ability to absorb water. Some polymers absorb little or no water, such as the rigid lenses, which will take up about 1.5-2.6% water by weight. Some materials will absorb as much as 80-90% water by weight. There is a whole spectrum of materials with water absorption between these extremes. The polymers that absorb significant amounts of water are called hydrogels. Usually the more water a polymer absorbs, the softer it becomes, likewise it may become more fragile. Another property of the lens materials is their oxygen permeability. In order for the cornea to stay in its normal state, it must receive oxygen from the air. With a lens on the eye, oxygen can reach the cornea via tears flowing under the edge of the lens or by going through the lens. If the lens is permeable to oxygen, all the oxygen must reach the cornea by pumping action of the lens on blinking, causing a flow of tears. If insufficient tear exchange does not occur, corneal swelling (called edema) develops.

A wide range of new materials have been developed to allow some oxygen to go through the lens. The ability of oxygen to move through a material is termed permeability. Non-flexible lenses that allow oxygen to pass through them are called rigid-gas permeable (RGP) lenses. This is a property of the material, and often called the Dk value, where D is the diffusion coefficient and k is the solubility of oxygen in the material. The D and the k of a lens material both increase with increased temperature. Consequently, the temperature during the Dk measurements is important, with the room temperature permeability (Dk_{RT}) being less than eye temperature permeability (Dk_{ET}). *Oxygen transmissibility* is another term, which is a specification of how much oxygen goes through a lens and is the Dk divided by the lens thickness (L). The L in this equation usually stands for lens centre thickness (ct) and not average thickness. Therefore, the oxygen transmissibility of a high-minus lens will overestimate the oxygen transmissibility across the lens if calculated according to the

lens ct. Conversely, the oxygen transmissibility of a high-plus lens will be under estimated. The higher the values of Dk and Dk/L, the better the oxygen transfer to the cornea.

There are many other lens properties that are important, such as elasticity, strength, heat conductivity, scratch resistance, permeability to molecules in the tear film, and density. In addition, the safety of the material is quite important. Some polymers may be toxic; they may damage tissues with which they are in contact. If any lens material dissolves out into the tear film (often termed *leaching*), it may cause a tissue reaction. Likewise, if solutions or medications that are commonly used with the lens react with the polymer, problems may develop. Therefore, the lens materials and care solutions must carefully be tested to ensure their safety for use in the eye.

1.3.4 Advantages and Disadvantages of Contact Lenses

Contact lenses have been used to correct errors of refraction, as protective devices for the eyes against undesirable fluids, gases or solids; as a mechanical aid in the treatment of several pathological eye conditions; for their cosmetic effect in neurotic conditions associated with eye defects; as a valuable aid to vision where the wearing of spectacles is impossible; as a vocational aid to vision, and as the only refractive device which will give useful vision in certain abnormal conditions.

Rain, snow, mud, etc., which may interfere with vision in those wearing spectacles, are no problem with contact lens wear. In extreme cold, spectacle lenses frost over, and steam up when passing from a cold to warm atmosphere. In extreme heat, spectacles become spotted with perspiration, but this does not occur with contact lenses. The advantage of contact lenses in swimming is obvious since spectacles can not be worn. They also reduce mental concern that spectacle wearers naturally exhibit in the face of physical obstacles.

The only disadvantages of contact lenses in 1957 was the cost, the time and the skill required for their fitting as described by Obrig and Salvatori [25]. After the introduction of hydrogel contact lenses into United States in 1972, surface deposits and lens spoilation became major problems that were not previously significant with poly methylmethacrylate lenses. The rapid increase in contact lens wear probably has led to a greater incidence and renewed awareness of lens related problems. When problems arise because of deposit formation and spoilage of the contact lens, the clinician takes an imperic approach that often include multiple changes in the cleaning regimes and in the the types of lens polymer until a successful combination is achieved. Although this

trial-and-error approach may be successful, it often requires a large investment of both time and money on the part of the consumer, clinics, and health care professionals.

Deposit formation and build up on soft contact lenses is associated with discomfort and decreased visual acuity. They may also be responsible for a variety of inflammatory conditions. Giant pappilry conjuctivities, corneal vascularization and infections all may be induced or aggravated by lens deposits. Therefore, the development of lens materials exhibiting minimal protein absorption is desirable.

1.3.5 Polymers Used as Contact Lens Materials

Any polymer from which contact lenses are made has to meet a set of strucure-based requirements. Among other requirements, the polymer must be chemically stable; optically clear, with a stable and appropriate refractive index, a smooth surface that has a low surface energy and low contact angle (the angle between the lens surface and a drop of water on it, a contact angle of zero implies complete wetting of the surface, Figure 1.5) and that does not readily absorb protein; be mechanically strong and stable as it ages; allow for diffusion of gases through the material; and be biologically inert.

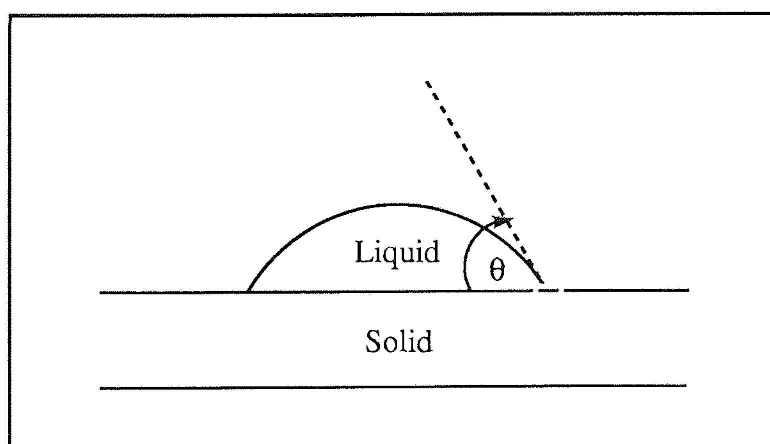


Figure 1.5 The contact angle between a liquid droplet and a solid surface.

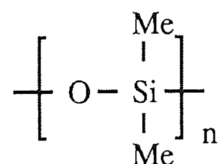
The polymers used or suggested for use as contact lens materials can be devided into four groups [26]: thermoplastics, synthetic elastomers, hybrid rigid "gas permeable" copolymers (RGP), and hydrogels. The most commonly used of these groups for contact lenses are hydrogels. Therefore, the other three groups will be mentioned very briefly.

1.3.5.1 Thermoplastics

Thermoplastics are rigid at room temperature, but can be shaped by the application of heat and pressure. Some thermoplastics such as polyethylene and polyvinyl chloride has been suggested for contact lens use, but none of them achieved any success because of poor surface properties. In the case of poly (4-methyl pent-1-ene) and cellulose esters such as cellulose acetate butyrate, however, the results are more reliable. Although these two polymers can be used to make hard lenses, those sort of lenses are not used widely.

1.3.5.2 Synthetic elastomers

These are rubber like polymers which can be stretched or compressed and return to their original shape after removal of the force. Their polymer chains are highly mobile and are cross-linked along the polymer backbone. The synthetic elastomers have properties intermediate between thermoplastics and hydrogels. They, however, have hydrophobic surfaces and require some form of surface treatment to make them hydrophilic for use as contact lenses. The most important of this group is poly (dimethyl siloxane) the structure of which is shown below, has an oxygen permeability which is 1000 times greater than that of poly methylmethacrylate. Therefore, although it has been used widely as a contact lens material, needs a surface treatment to become hydrophilic. This can be achieved by the addition of some low quantities of methacrylic acid (MAA) or hydroxyethyl methacrylate (HEMA) or both.



Poly (dimethyl siloxane)

1.3.5.3 Hybrid rigid gas permeable materials (RGP)

In this group of contact lens materials, the ease of the preparation of poly methylmethacrylate (PMMA) is combined with high oxygen permeability of silicon rubber. PMMA is the main polymer that has been used in the manufacture of contact lens materials over the years. It is a very transparent polymer with a refractive index of 1.4. The polymer is non-toxic, stable, and resistant to most solutions and chemicals with the exception of organic solvents such as acetone. The main disadvantage of

PMMA as a contact lens material is its lack of permeability to oxygen. For this reason, the RGP lens mateials are now replacing PMMA. PMMA-silicone copolymers are also clear materials that can be used for contact lenses. As a result of combination of PMMA and silicone, the oxygen permeability is increased because of silicone while the properties of PMMA such as solubility, wettability, optical properties, machining, and ability to be modified have been maintained.

Fluorinated polymers have been also used as contact lens materials. They have a good oxygen permeability (Dk) together with a low surface tension. The decreased surface reactivity of the fluorinated lenses improves lens surface wettability and tends to decrease deposition of tear components such as proteins and lipids on the lens. Figure 1.6 shows the structures of some RGP lens materials.

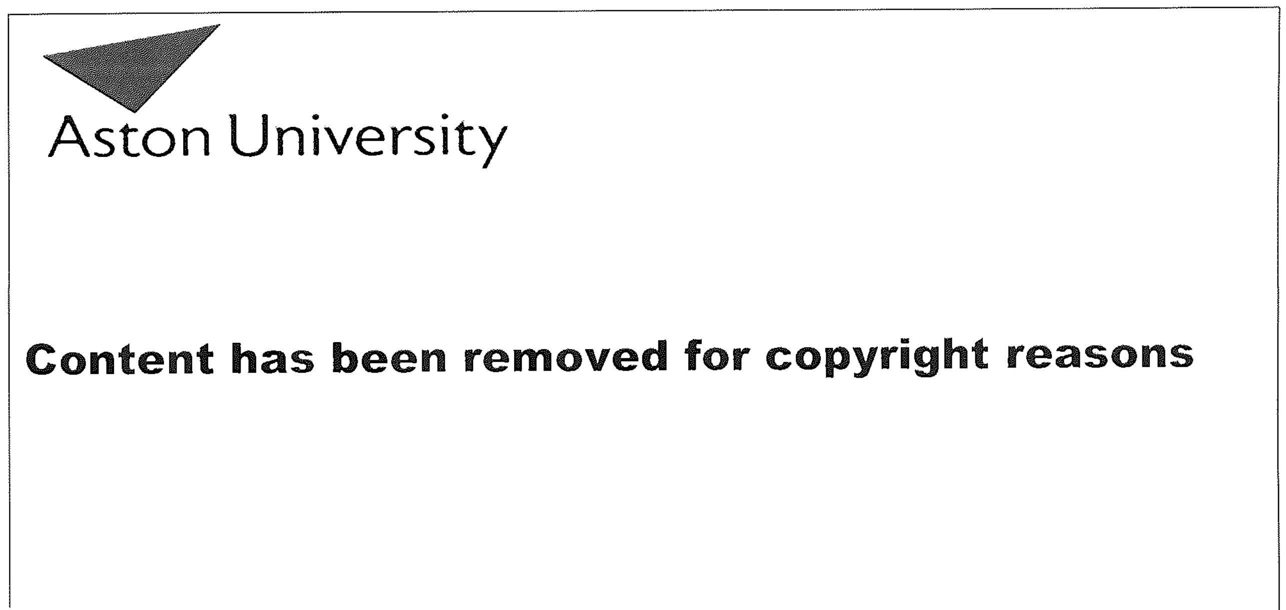


Figure 1.6 The structures of some known RGP materials [26].

1.3.5.4 Hydrogels

Hydrogels are hydrophilic polymer molecules which are cross-linked by water. They do not dissolve, but swell in water. The capacity of hydrogels to absorb water is enormous and can be as much as 1000 times the weight of the polymer. The amount of water adsorbed by a hydrogel is expressed as the equilibrium water content (EWC) and is defined as:

$$\text{EWC} = \frac{\text{Weight of water in the gel}}{\text{Weight of the hydrated gel}} \times 100\%$$

Water in polymers can exist in more than one state [26] and these states, as well as the EWC, affect the properties of the hydrogels. The water in a hydrogel network exists in a continum state between two extremes. The "bound" or non-freezing water which is strongly associated with the hydrogel network through hydrogen bonds, whereas the "free" or freezing water has a much greater mobility and is unaffected by the polymeric environment.

Hydrogels have been used in many fields due to their ease of preparation, their capacity of absorbing and releasing water, and the excellent oxygen permeability.

Hyrogels are used in pharmaceutical preparations to enhance the solubility or as biodegradable polymeric systems for controlled release drug delivery. Because of the unique properties of hydrogels, biodegradable hydrogels are expected to find wide applications in the improvement of existing dosage forms and development of new and better drug delivery systems [27]. They can also be used to provide a continuous release of moisture to plants.

Hydrogels are used as thickening agents (e.g., starch and gelatin in foods), as they absorb a large volume of water from the food causing the concentrating of it.

Technical and electronic instruments can be protected from moisture by enclosure in highly absorbent hydrogel-forming agents.

Hydrogels can be used in electrophoresis and chromatography techniques, bearing in mind that they should possess a limited range of swelling for these purposes [28].

Hydrogels are used in photographic technology because they are light-permeable and can also store light-sensitive substances.

The use of hydrogels as synthetic articular cartilage has met with little clinical or commercial success, because of their relative poor mechanical properties. However, by the use of composite structure of natural cartilage as a model, a new family of hydrogels based on interpenetrating polymer network (IPN) technology have been synthesized [29].

Hydrogels have been widely used in the manufacture of soft contact lenses. Many commercial soft contact lenses are based on poly 2-hydroxyethyl methacrylate (PHEMA) more commonly referred to as HEMA, which has an EWC of 40%. Figure 1.7 shows a range of monomers used in hydrogel synthesis.

Hydrogel lenses are generally composed of four basic hydrophilic monomers: HEMA, glycerol methacrylate, vinyl pyrrolidone and methacrylic acid. In addition, cross-linking agents are typically added to produce mechanical strength and thermal stability. All of these hydrophilic monomers, with the exception of methacrylic acid, yield non-ionic polymers that interact with the polar molecules of water without generating a formal electrostatic change on the molecule. Low water content lenses of this type usually have water contents of 38-45%. High water content lenses, that are mostly vinyl pyrrolidone-based polymers, have water contents of 70-80%. These lens materials constitute the low and high water content non-ionic lens groups. On the other hand, ionic lens materials with low or high water contents are made using methacrylic acid, a formally charged, ionic monomer. Table 1.6 shows some examples of commercially available soft hydrogel lens materials.

The ionic lens materials have been shown to be more reactive with tear components and lens care products than non-ionic materials. Therefore, one of the major problems with hydrophilic contact lenses is their spoilage from tear film. The spoilage of contact lenses is due to different factors such as calcium films, organic plaques and protein films. The most important of these surface coatings appear to consist of proteins.

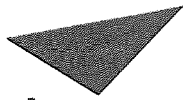

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Figure 1.7 Structures of some monomers used in hydrogel synthesis [30].

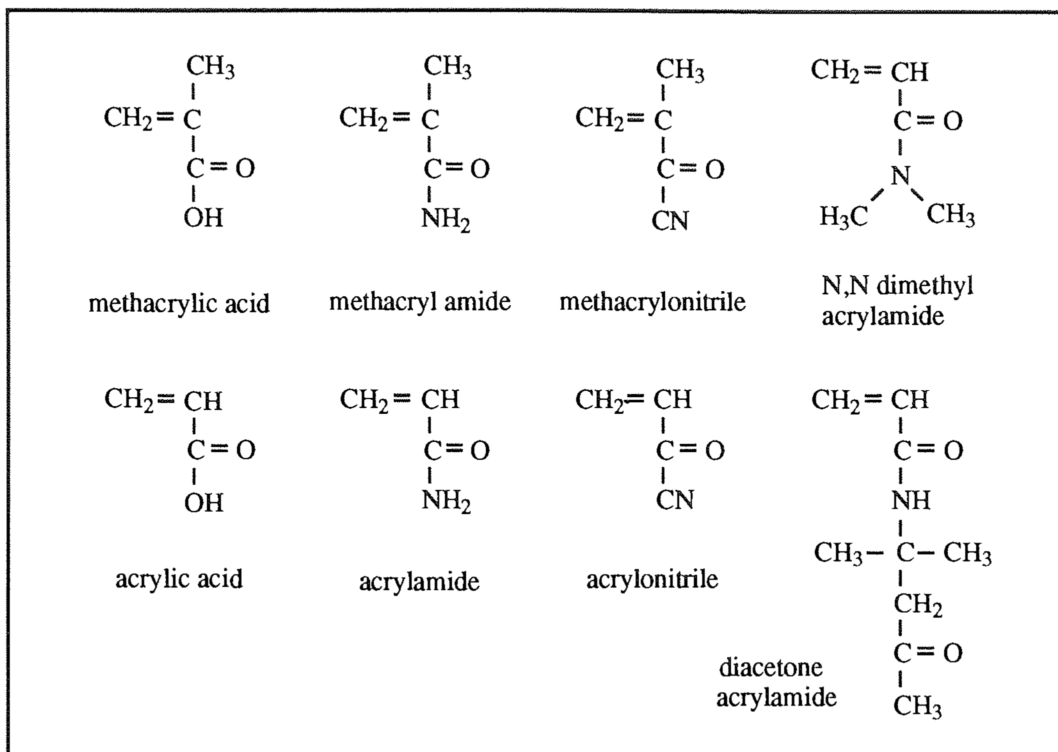


Figure 1.7 Monomers used in hydrogel synthesis (continued).

Table 1.6 Examples of soft contact lenses and lens materials.

Name	Principle component (s)	Water content (%)	Manufacturer Supplier	UASN Nomenclature
Acuvue	HEMA, MA	58	Vistakon	Etafilcon-A
A O Multivue	HEMA	38	American Optical	Telfilcon
Accugel	HEMA, PVP, MA	47	Strieter gel	Droxifilcon-A
Cristelle	VP, MMA	78	Lunelle	-----
Classic	HEMA, VP, MMA	42.5	Pilkington Barnes-Hind	Tetraficon-A
CSI	MMA, GM	41	Pilkington Barnes-Hind	Crofilcon-A
Excelens	PVA, MMA	64	Ciba	Atlafilcon
Frequency 38	HEMA	38	Aspect	Polymacon
Hydro-Curve(II)	HEMA, DA, MAA	55	Soft Lenses Inc.	Bufilcon-A
Hydron	HEMA	38	Hydron Europe	Polymacon
Medalist	HEMA	38	Bausch & Lomb	Polymacon
Permalens	HEMA, VP, MA	71	Pilkington Barnes-Hind	Perfilcon-A
Permflex	MMA, VP	74	Pilkington Barnes-Hind	Surfilcon-A
Softlens	HEMA	38	Bausch & Lomb	Polymacon
Surevue	HEMA, MAA	58	Vistakon	Etafilcon-A
Tresoft	HEMA, MAA	46	Alcon Optics	Ocufilcon-A
Vistagel A	MMA, VP	60	Vista Optics	---

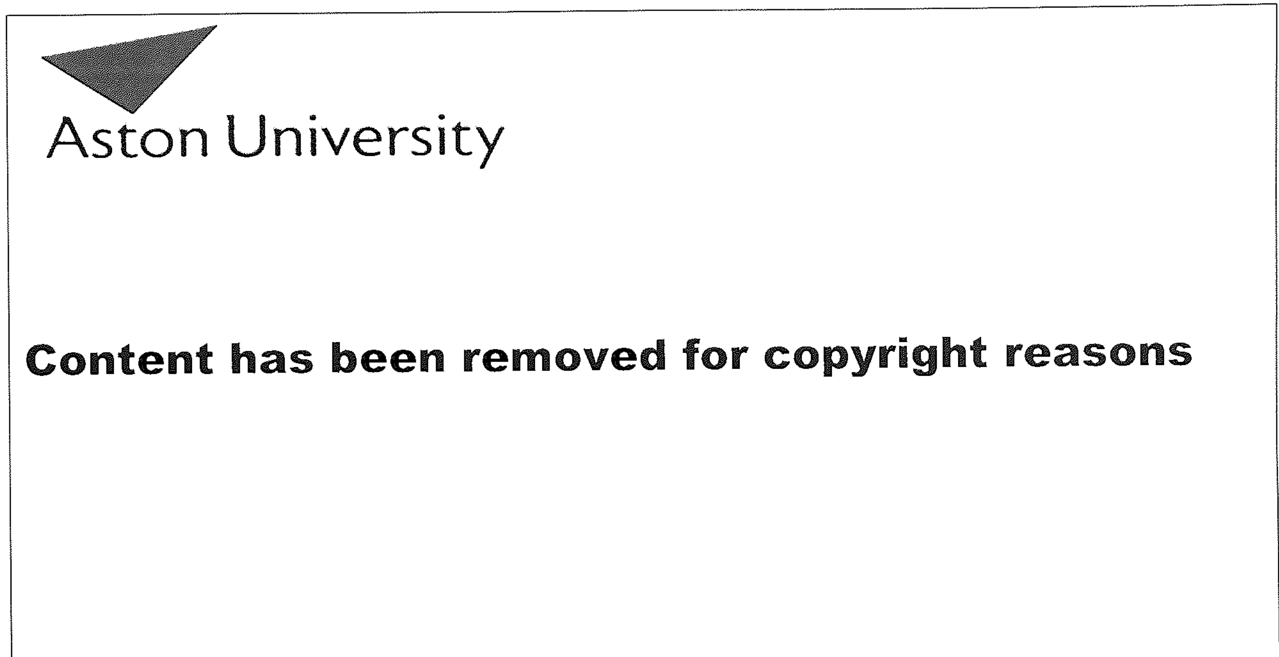
1.4 Biomaterials

Biomaterials are a group of materials designed to be used in physiological interfaces. Progress in biomaterials synthesis and research has been very rapid in the recent years. One extended area is the design and development of new materials, particularly polymers, to fulfil the growing number of biomedical applications. The use of biomaterials in biological environments, however, is associated with a number of particular problems. Table 1.7 shows some biomaterials together with their applications and related problems.

It is apparent from Table 1.7 that the most common problem with any kind of biomaterial is its biocompatability. If the implanted biomaterial is not biocompatible with the environment, it will be rejected by the biological site. This may be manifested in many ways depending on the environment in which the biomaterial is used. For example, a biomaterial rejected by the blood can cause thrombosis, while a non-

biocompatible contact lens biomaterial may deposit more tear components leading to sever infection and conjunctivities.

Table 1.7 Problems associated with some known biomedical polymers [31].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1.4.1 The Problem of Biocompatability

To develop a biomaterial with high biocompatability, one must take into consideration both bulk and surface properties of the material which is to be synthesized. Although

the combination of these two may complicate the design of the biomaterial, both are very important and challenging.

Biocompatability or biotolerence can not be predicted by any exact design of a material, however, it can be suggested that the surface energy of the biomaterial is an important factor to be considered. Baier et al [32] produced materials with high blood biocompatability and comparatively low surface tensions. Andrade [33] claimed that an optimum balance between polar and non-polar sites on the biomaterial is important for its biocompatability.

1.4.2 Factors Affecting Biocompatability

In 1972, Baier defined three factors which are important in biomedical problems and which measure the biocompatability of a material, he called these as magic numbers and Figure 1.8 illustrates an overview of three of these confining factors.

The surface quantities of biomaterials are not the only factors requiring consideration when designing the biomaterial, other factors such as the conditions of biological environment in which the material is being used e.g. volume, flow rate, biochemical composition and pH of the media are important.

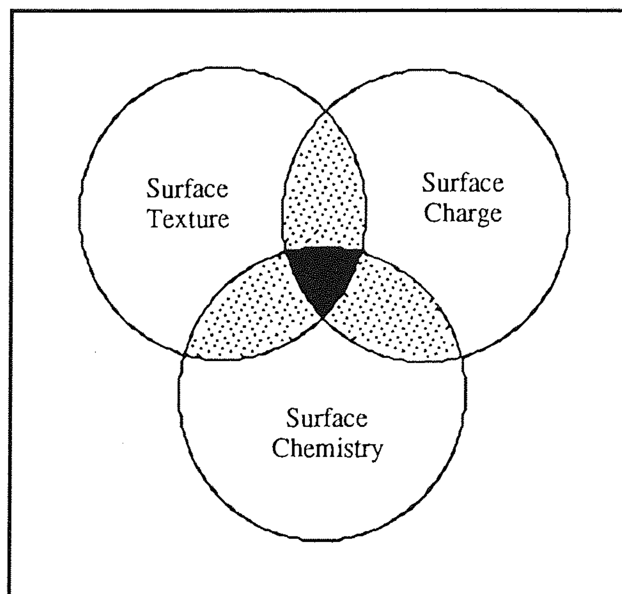


Figure 1.8 Surface factors affecting biocompatability.

1.5 Protein Deposition on Biomaterials

In all areas of biological and medical research today there is an increasing need to know about proteins. These complex macromolecules with molecular weight ranging from the thousands to the millions, comprise in essence the working machinery of life. Hundreds of proteins have been identified in the category of enzymes, catalyzing complex biochemical reactions. Others are responsible for the basic structural framework of living organisms, in higher animals these structural proteins include collagen of bones, cartilage, and tendons; keratin of hair and nails; elastin of blood vessel and ligaments; and myosin of muscle.

1.5.1 Historical Background

The name protein (Greek, *proteios*, of the first rank) was first used by Mudler in 1838 when he carried out a systematic study of the elemental composition of proteins. Most proteins were found to contain 50-55% carbon, 6-7% hydrogen, 20-30% oxygen and 12-19% nitrogen. Trace elements were also identified in certain proteins. The most common of these were sulphur (0.2-0.3%), up to 3% phosphorous in some proteins and 0.34% iron in haemoglobin.

During the late 1800s, amino acids were identified as the basic building units of proteins. Eventually 20 different amino acids were shown to occur as components of most proteins; a number of others were found in special cases.

1.5.2 The Behaviour of Proteins at the Solid-Liquid Interfaces

The natural habitat of most proteins is an aqueous environment. When a protein solution contacts another phase (either a solid, liquid, or gas) with which it is immiscible, protein molecules tend to accumulate at the interface between the two phases. This tendency has a great effect on various natural and technological processes. Adsorption of proteins takes place almost instantanously when a solid surface comes into contact with most biological fluids. The protein film which forms may then act as a substratum for subsequent adhesion of other components such as eukaryotic cells or microorganisms.

Protein adsorption is the overall result of various types of interactions between the different components present in the system, i.e. the sorbent surface, the protein molecules, the solvent (water) and any other solutes such as low-molecular mass ions.

The surface of a protein is often complex in nature, with different characteristics such as hydrophilicity and charge [34]. The surface view of the human lysozyme molecule shown in Figure 1.9 indicates the complexity of the surface groups which can participate in interaction with other surfaces. The fact that many real surfaces are heterogeneous together with the complex nature of the protein surface complicates the prediction of how a protein interacts with a surface.

One major factor influencing protein adsorption is the surface energy, and it has been reported that hydrophobic surfaces adsorb more protein than hydrophilic ones [35]. Prediction of the effect of surface charge on protein adsorption is not staightforward. Proteins with net charge similar to the surface might bind through local patches of the opposite charge. Norde [35] suggested that the driving force in this case was an increase in entropy, due to conformational changes of the protein resulting in the loss of secondary structure. He pointed out that the groups at the surface of a protein are the ones most likely to interact with a solid surface, although interior groups might be exposed through conformational changes. In the case of proteins with strong internal coherence, "hard" proteins, structural rearrangements do not significantly contribute to the adsorption process. The "soft" proteins which have lower structural stability will adsorb even under unfavourable conditions due to the structural rearrangments.

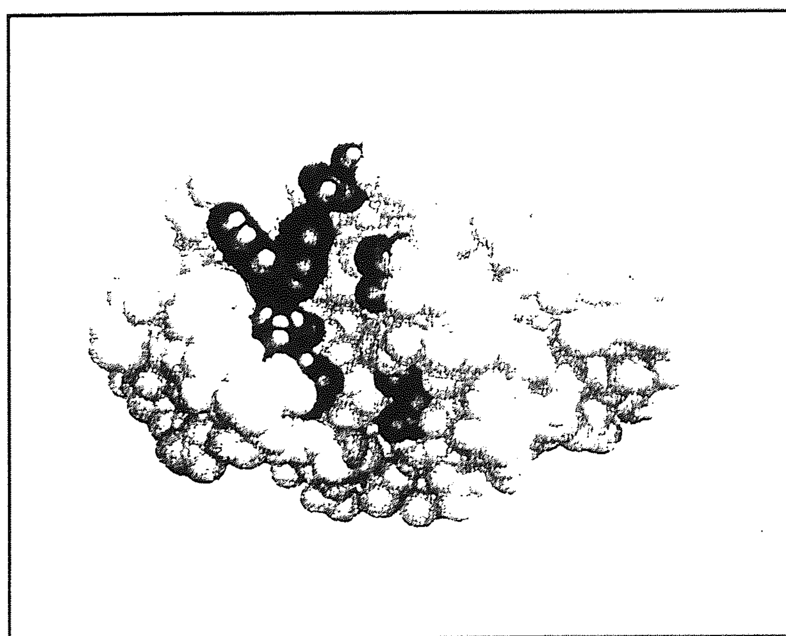


Figure 1.9 The compact structure of human lysozyme.

Certain ions can bind to specific sites in proteins and thereby change the molcule's adsorption behaviour [36]. For example, the binding of insulin to zinc ions increases the amount adsorbed onto chromium surfaces [36]. Binding of small molecules such as fatty acids to proteins may also influence the amount of adsorbed protein [37]. The effect of temperature on adsorption is not always predictable, increases [38] and decreases [39] in the adsorption with increasing temprature have been reported.

Protein adsorption has an impact on the performance of many processes; its effect may be beneficial or detrimental. In fouling of heat exchangers, ultrafiltration membranes and other process equipment, for example, protein adsorption needs to be minimized [40]. However, chromatography [41] and immunoassays [42] require the binding of the protein.

One major area where protein interaction with solid surfaces is of interest is in the field of biocompatible materials. Soft and hard tissue implants [43] and blood compatible materials [44] have been investigated. Adsorption and adhesion from tear fluids [45] and saliva [46] have also been studied.

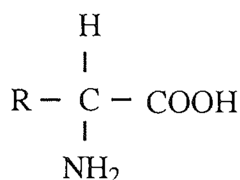
Analysis of protein adsorption onto polymer surface is important in biotechnological and biomedical fields. The eye is a unique body site for the study of protein interactions with biomaterials because of its ease of access and deceptive complexity of the tears. It is, for example, easy to introduce a contact lens into the tear and take it out in a few minutes without surgery and causing any trauma to the patient.

The use of contact lenses for either vision correction and cosmetic reasons or as a route for the controlled drug delivery, has significantly increased recently. Currently, 60 million people requiring vision correction wear some type of hydrophilic soft contact lenses.

The widespread use of hydrophilic contact lenses has demonstrated the problem of lens spoilage. Polymer deterioration and protein deposition will change the optical quality and permeability of the lenses and may reduce wearer tolerance [47]. The protein adsorbed is also a primary layer for the subsequent adsorption of other proteins and tear components. Most of the previous studies have concentrated on the proteinaceous deposits on the surface of the lens. The presence of proteins inside the hydrogel matrix, however, has received little attention. This work is concerned with the study and development of various techniques for the measurement of the protein deposited on and within the contact lenses, as well as considering the stability and activity of the proteins involved.

1.5.3 The Structure of Some Proteins

The general structural formula of the 20 amino acids commonly found in proteins can be shown as:



Amino acids found in proteins can be divided into four major groups according to their side groups. Table 1.8 lists the amino acids which occur most frequently in proteins. The detailed structures of these amino acids can be found in all text books [34].

Normally, chains formed by polymerization of 100-1000 or more amino acid molecules form the *primary structure* of proteins. The monomer units in chain are known as amino acid residues, each amino acid having lost one molecule of H_2O during the polymerization. These are called polypeptide chains, each of which has a free terminal amino acid group (N-terminal) and a terminal carboxyl group at the other end (C-terminal). In such abbreviations the N-terminal amino acid residue is always placed at the left and the C-terminal amino acid residue at the right hand.

The pattern of folding of a peptide chain into α-helix or a β-sheet structure is often refered to as the *secondary structure* of the protein. The further folding which involves interaction between groups that are distant in primary sequence, is called *tertiary* structure. The aggregation of monomeric protein subunits into oligomers (low molecular weight proteins) provides the *quaternary structure*. Three levels of the structure in protein structure are shown schematically in Figure 1.10.

Full understanding of the function of a particular protein in relation to its biological phenomena, a knowledge of its chemical structure is necessary. Proteins possess a great diversity of size and properties. Individual polypeptide chains in most proteins fall within the molecular weigh range of about 10,000 to 100,000 Daltons. Thus, roughly 100 to 1000 amino acid residues are contained in each chain. Most proteins in their functional form contain more than one polypeptide chain, either of the same or different types.

Table 1.8 Amino acids most commonly found in proteins.

Name	Symbol	Molecular weight	Nature of the R group
Alanine	Ala	89	Non-polar
Valine	Val	117	
Leucine	Leu	131	
Isoleucine	Ile	131	
Proline	Pro	115	
Phenylalanine	Phe	165	
Tryptophan	Try	204	
Methionine	Met	149	
Glycine	Gly	75	Uncharged Polar
Serine	Ser	105	
Threonine	Thr	119	
Cysteine	Cys	121	
Tyrosine	Tyr	181	
Aspargine	Asn	132	
Glutamine	Gln	146	
Aspartic acid	Asp	133	Polar Negatively charged
Glutamic acid	Glu	147	
Lysine	Lys	146	Polar Positively charged
Arginine	Arg	174	
Histidine	His	155	

In this section, some of the most important tear proteins and some other proteins which were used for our *in-vitro* spoilation studies are discussed.

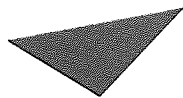

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Figure 1.10 Level of structures in proteins [34].

1.5.3.1 Albumin

Serum albumin is the first member of the albumins family which are a group of proteins characterized by heat coaggulability and solubility in dilute salt solutions.

Albumin accounts for about 60% of total protein in blood serum with a concentration of 42g/L (0.63mM). It is composed of about 580 amino acids with a total molecular weight of 66.5 kDa. Serum albumin has a wide range of chemical and laboratory uses, and physiological functions. For example, albumin is involved in binding, transport and delivery of fatty acids, bilirubin, tryptophan, thyroxin, steroids and a range of pharmaceuticals and dyes [48].

Albumin is a single-chain protein composed of three structurally similar domains. Its amino acid sequence contains a total of 17 disulphide bridges, one free thiol (Cys 34), and a single tryptophan residue (Try 214). The disulphide bridges are positioned in repeating series of nine loop-link-loop structures centred around eight sequential Cys-Cys pairs. In addition to blood plasma, albumins are also found in tissues and secretions throughout the body; the extravascular protein comprises 60% of the total albumin.

Structurally, albumin consists of 28 helices which range in size from 5 to 31 amino acids in length, and can be grouped into 10 principle helices within each domain. Figure 1.10 is a stereo view of human serum albumin. A strikingly high content of

disulfide bridges is found in bovine serum albumin (molecular weight 66,500, 17 disulfide bonds).

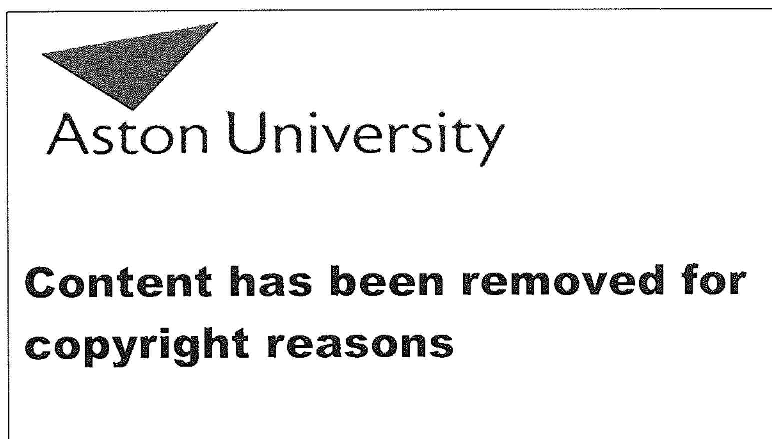


Figure 1.11 A stereo view of human albumin [49].

The isoelectric point of albumin is 4.9 at pH 7.0 leaving the protein with an overall negative charge at this pH (Table 1.9).

Albumin is a relatively unstable protein, despite the many disulfide bridges, and may be denatured at high temperature or due to large changes in the pH as well as other denaturing conditions.

Tear albumin is a unique protein which never occurs in serum. It is a pre-albumin which electrophoretically migrates to a similar position to serum pre-albumin, but the antiserum raised to serum pre-albumin does not react with tear pre-albumin [50]. Trauma during tear collection can affect the levels of serum albumin in tears.

1.5.3.2 Lysozyme

Lysozyme is a mucolytic enzyme with antibiotic properties which was first discovered by Fleming in 1922. Lysozyme was the first protein whose structure was determined by crystallography, but until then very little was known about its catalytic properties. It preferentially hydrolyzes the β-1,4 glucosidic linkage between N-acetylmuramic acid and N-acetyl-glucosamine which occur in the mucopeptide cell wall structure of certain micoorganisms. It is also called muramidase, N-acetylmuramide glycanohydrolase, N-acetylmuramyl hydrolyse and globulin G1. Lysozyme is distributed in animals and plants. Avion egg white lysozyme has been most extensively studied and considerable physiochemical information is now available about it [51, 52]. Lysozyme is also found

in mammalian urine, saliva, tears, milk, cervical mucus, leucocytes and kidneys. Egg lysozyme is consisted of a single polypepetide chain of 129 amino acid subunits of 20 different kinds cross-linked by four disulfide bridges (Figure 1.12).

Lysozyme is very stable protein with a compact structure which is resistant to denaturation, but some chemicals such as dodecyl sulphate, alcohols and fatty acids may inhibit its enzymatic activity. In Figure 1.13 the compact structure of lysozyme is compared to some other proteins with similar molecular weights and sizes. The molecular weight, isoelectric point and dimensions of this protein together with some other proteins, which were used in our *in-vitro* study, are shown in Table 1.10.

There is a close cluster of basic groups, Arg 45 and 68 in one region, Arg 61 and 73 in a second, and Arg 5, 125 and 128 in another, which form the highly positively charged surface regions in lysozyme. The very high isoelectric point of the enzyme is due to the presence of this high positive charge.

Table 1.9 The isoelectric points of some proteins.

Protein	Isoelectric Point (pH units)
Pepsin	~ 1.0
Egg albumin	4.6
Serum albumin	4.9
Urease	5.0
Hemoglobin	6.8
Myoglbin	7.0
Ribonuclease	9.6
Cytochrome c	10.6
Lactoferrin	9.6
Ferredoxin	5.8
Insulin (human)	5.3
Insulin (bovine)	6.8
α-Lactalbumin	4.3
Lysozyme	11.0

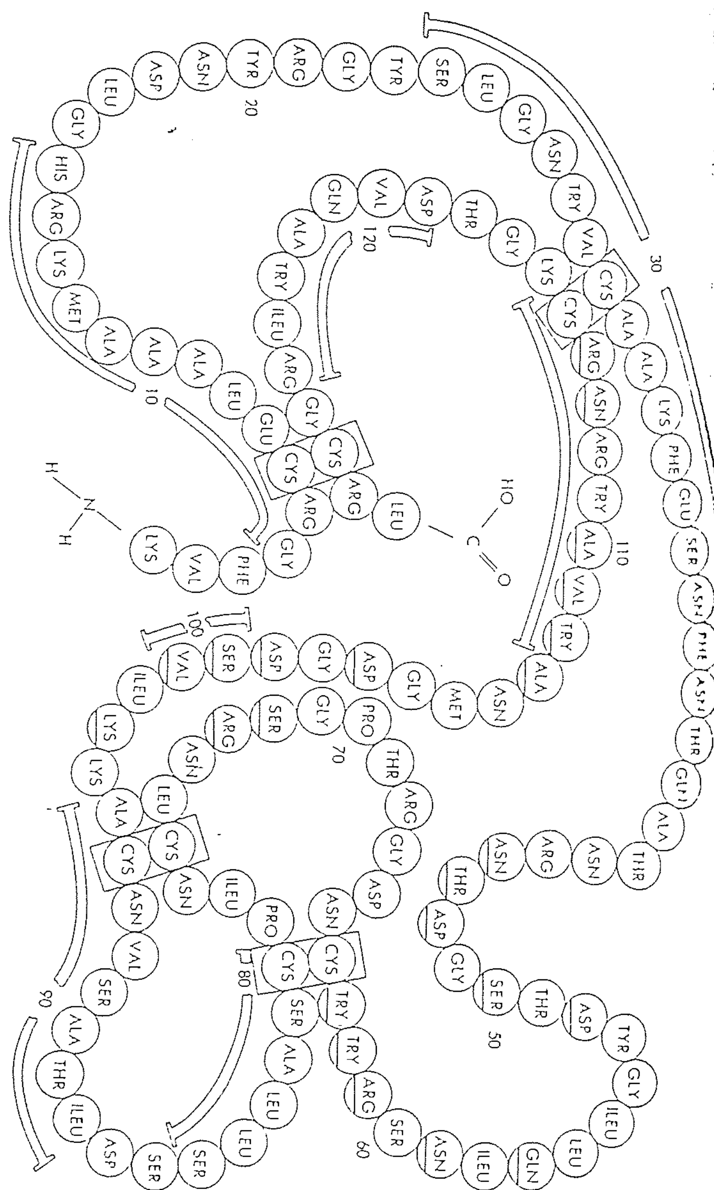
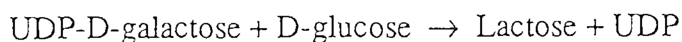


Figure 1.12 The chain structure of chicken egg white lysozyme.

1.5.3.3 Lactalbumin

Lactalbumin is one of the protein components of lactose synthetase enzyme system, which catalizes the final step in lactose biosythesis in the lactating mammary gland.



UDP-D-galactose + D-glucose $\rightarrow$ Lactose + UDP

Structurally, bovine milk α-lactalbumin is very similar to hen's egg-white lysozyme. Of 123 residues, 47 are identical, and many others have similar chemical property. Lysozyme's function is to degrade the polysaccharides of bacterial cell walls, whereas α-lactalbumin regulates lactose synthesis by binding to a membrane-bound galactosyl transferase. α-lactalbumin is produced only in mammary gland and it is probably similar to lysozyme in conformation, but with lower isoelectric point 4.3. Purified α-lactalbumin contains 1 mole of bound Ca^{++}/mole of protein.

Table 1.10 Molecular sizes and molar masses of some known proteins.

Protein (source)	Molar mass (g/mol)	Dimensions (A°)
Lysozyme (hen)	14,320	45 x 30 x 30
Ribonuclease (bovine)	13,690	38 x 28 x 22
α-Lactalbumin (human milk)	14,200	37 x 32 x 25
Myoglobin (sperm whale)	17,800	44 x 44 x 25
Cytochrome c (equine)	12,310	25 x 25 x 37

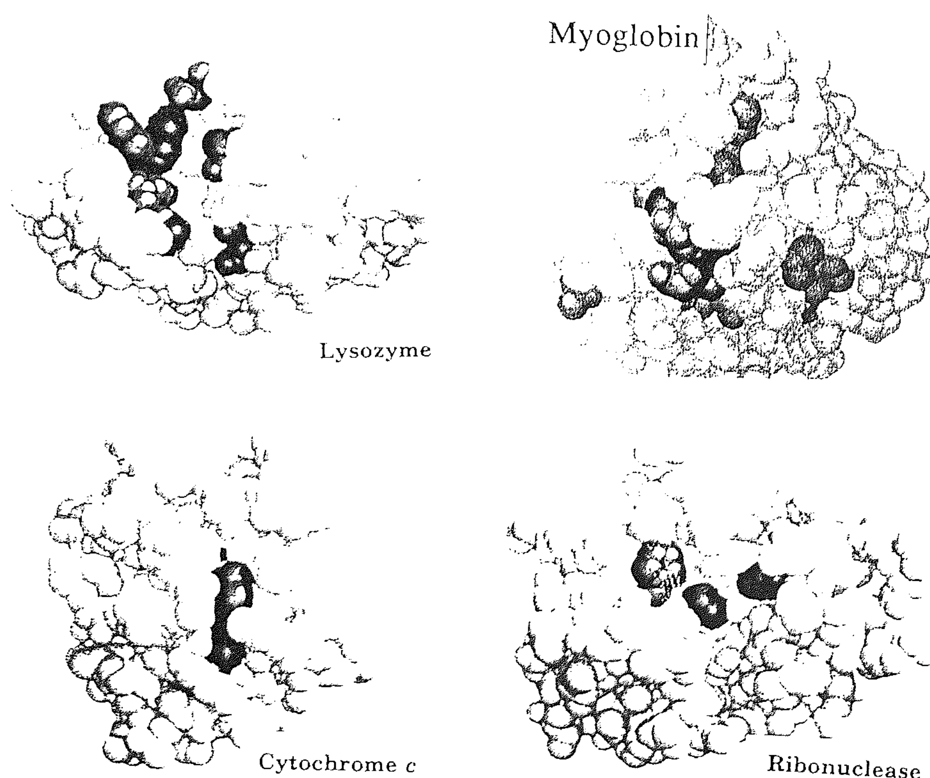


Figure 1.13 The compact structures of some proteins with similar sizes.

Although the charged side chains in α-lactalbumin are located on the surface and appear to be accessible to water as in lysozyme, they are distributed rather differently. In all, there are only two fewer basic residues in α-lactalbumin, but the close cluster of basic groups, which forms the highly positive charged region in lysozyme, are not found in lactalbumin. On the other hand, there are more acidic groups than in lysozyme and, therefore, the isoelectric point in α-lactalbumin is much lower than that of lysozyme (4.3 compared with 11.0 for lysozyme).

1.5.3.4 Lactoferrin

Lactoferrin or lactotransferrin is a member of a family of non-haem, iron-binding proteins called transferrins which includes serum transferrin. Lactoferrin is the red iron-binding protein of milk which has a different sequence from transferrin of blood. It is present in high concentration during the dry period, and confers the bacteriostatic properties of the complexed mammary gland [53]. During lactation, the lactoferrin concentration in milk is low and its bacteriostatic action is reduced by citrate, a normal component of milk [54]. Most studies on the antimicrobial action of lactoferrin have demonstrated a bacteriostatic effect that is reversed by the addition of excess iron [55].

Lactoferrin is also found at high concentrations in exocrine secretions such as salvia, tears, mucosal and genital secretions, as well as in the blood serum. It has been shown that lactoferrin is a growth factor which was first isolated from both human and bovine milk in 1960 [56]. It has a number of biological activities such as regulation of absorption of iron and other metals in the gastrointestinal tract, modulation of the growth of animal cells and antimicrobial activity against bacteria and yeast.

Lactoferrin is a monomeric glycoprotein, having two similar oligosaccharide chains. It possesses two independent metal binding sites, each of which can bind a ferric ion together with bicarbonate anion. A key feature of the structure and function of lactoferrin is the importance of its flexibility, at various levels, ranging from small variations in the positions occupied by different metal and anions, to large-scale rigid body movements of the entire domains.

It has been shown that human lactoferrin has a high tendency to associate with acidic proteins such as albumin or casein [57]. These interactions influence its electrophoretic behaviour and, in fact, it shows different mobilities in different media [58].

Lactoferrin is one of the most important human tear proteins with an apparent molecular weight of 78,000. Its isoelectric point is very high in comparison to other transferrins (~8.4 to 9.0 compared with ~5.4 to 5.9). The high isoelectric point is due to the concentration of charged side chains on the molecule near the N-terminus [59].

1.5.3.5 Ribonuclease

Ribonuclease (RNase) is an enzyme which digests ribonucleic acid (RNA), which was first isolated from beef pancrease in 1920. It is a relatively small compact protein consisting of a single polypeptide chain. A high concentration of disulfide bonds relative to its size (molecular weight of 13,700, four disulfide bonds) probably contributes to its resistance to denaturation and ease with which its structure can be re-formed (renaturation). It is a stable protein which is stable for years as a refrigerated dry powder or in frozen solution. The enzyme activity can be inhibited by magnesium ions at very low concentrations and also competitively inhibited by deoxyribonucleic acid (DNA). Ribonuclease is a positively charged protein with isoelectric point of about 8.0 (for secretory RNase) and is soluble in water. Reduction of all of the disulfide bonds in ribonuclease (as well as in lysozyme and other disulfide proteins) produces a concomitant loss in biological activity. Activity may be recovered by reoxidation in the presence of air at pH 8.0. It is very interesting to consider that even the reduction of two of the four disulfide bonds in ribonuclease and lysozyme does not inactivate the enzymes [60].

Ribonuclease is present in animals, plants, and microorganisms. In human systems, two types of RNase, secretory and non-secretory, have been shown to be widely distributed in various tissues and body fluids [61]. These RNase exist in multiple forms with regards to not only their molecular masses but also their isoelectric points [62].

1.5.3.6 Myoglobin

Myoglobin is one of the most intensively studied proteins, and there is a lot of biochemical, biophysical and spectroscopic data available about this protein [63]. It was the first protein the structure of which was determined to a high resolution by x-ray crystallographic analysis. The total accessible surface area of myoglobin is similar to that of lysozyme and ribonuclease. In general, the total accessible surface area of a protein is approximately proportional to the two thirds the power of the molecular weight, as would be expected for approximately spherical objects. However, the

accessible surface area is nearly twice that expected for a sphere of the same size, which is some measure of the roughness of the surface. Figure 1.14 compares myoglobin with lysozyme and ribonuclease in terms of their polar and non-polar surfaces [64].

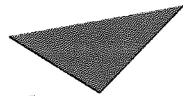


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Figure 1.14 Contributions made to the total accessible surface area by different atoms [64].

Myoglobin is a very compact protein with 153 amino acid residues and a molecular weight of about 17, 000 depending on the source [65].

1.5.3.7 Insulin

Insulin is a hormone, normally produced by the beta cells of the islets of Langerhans situated in the pancreas of all vertebrates. Insulin is synthesized *in-vivo* via a single

chain polypeptide precursor, pro-insulin, and secreted directly into the blood stream where it preforms several important functions such as the control of carbohydrate metabolism.

The insulin monomer (molecular weight 6000 Daltons) is consisted of 51 amino acid residues in two polypeptide chains (A and B). The two chains are connected via two disulfide bridges, and a third disulfide bridge also exists between the 6th and 11th amino acid residues (Figure 1.15).

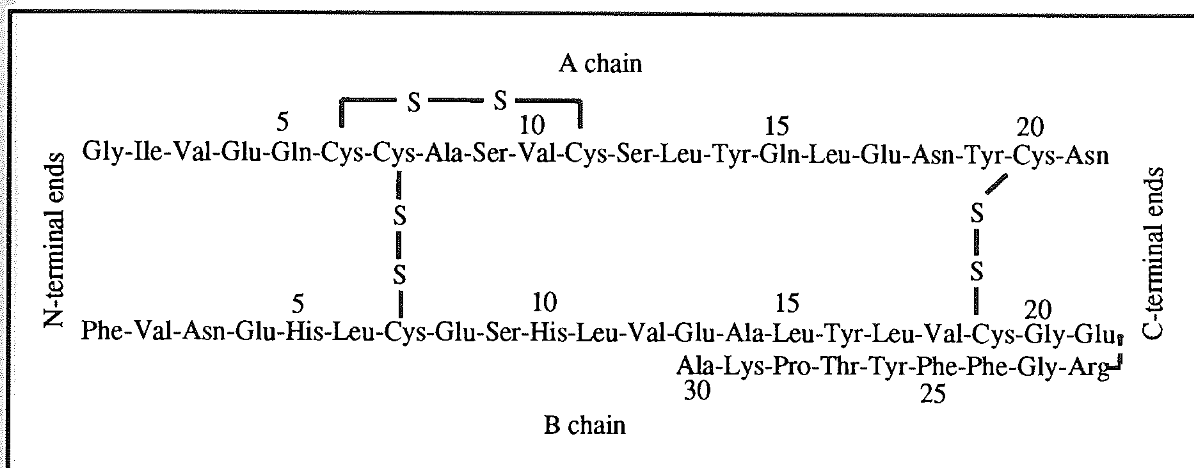

Figure 1.15 The amino acid sequence of bovine insulin.

Insulin is the first protein for which the chemical structure and precise molecular weight were determined [66]. The amino acid composition and physical structure of mammalian insulin varies only slightly from one species to the next. Insulin exist as a dimer in mild acid solutions and it is insoluble from pH 4.5 to 7.0. The isoelectric point of the human insulin is 5.3 and, therefore, its overall surface charge is slightly positive, while that of bovine insulin is about 6.8 and is neutral on the surface.

1.5.3.8 Ferredoxin

Ferredoxins are a group of electron transfer factors found in bacteria and plants. They are non-haem iron-sulfur proteins which play an important part in respiration, nitrogen and carbon dioxide fixation and photosynthesis. The plant ferredoxins primarily have two iron clusters and are sometimes referred to as chloroplast or "plant type" ferredoxins. The molecular weight of the ferredoxins depends on their origin, for example chloroplast ferredoxin is about 12000 Daltons, and chromatium about 10,000. Bacterial ferredoxins are generally four iron cluster proteins with a molecular weight of about 24,000, while the ferredoxin from the clostridium has a molecular weight of 40,000. All types of ferredoxins show an ability to autoxidize and their oxidation-

reduction potentials are negative values. Their acidification and treatment with iron-chelating agents results in evolution of H_2S and loss of visible absorption. Spinach ferredoxin with a molecular weight of 12,600 contains a total of 97 amino acids from which 41 are hydrophobic, 30 neutral, 26 hydrophilic, 21 acidic and 7 are basic. It has a net charge of -14 and shows a maximum absorption at 280 nm. Figure 1.16 shows the primary structure of spinach ferredoxin [67].

1 10 20
Ala-Ala-Tyr-Lys-Val-Thr-Leu-Val-Thr-Pro-Thr-Gly-Asn-Val-Glu-Phe-Gln-Cys-Pro-Asp-
21 30 40
Asp-Val-Tyr-Ile-Leu-Asp-Ala-Ala-Glu-Glu-Glu-Gle-Ile-Asp-Leu-Pro-Tyr-Ser-Cys-Arg-
41 50 60
Ala-Gly-Ser-Cys-Ser-Ser-Cys-Ala-Gly-Lys-Leu-Lys-Thr-Gly-Ser-Leu-As-Gln-Asp-Asp
61 70 80
Gln-Ser-Phe-Leu-Asp-Asp-Asp-Gln-Ile-Asp-Glu-Gly-Trp-Val-Leu-Thr-Cys-Ala-Ala-Tyr
81 90 97
Pro-Val-Ser-Asp-Val-Thr-Ile-Glu-Thr-His-Lys-Glu-Glu-Glu-Leu-Thr-Ala

Figure 1.16 The primary structure of spinach ferredoxin.

1.6.3.9 Cytochrome c

Cytochrome c is a single polypeptide chain protein which consists of 104 or more amino acid residues with a heme group attached through cystein residues at positions 14 and 17. The amino acid composition of equine cytochrome c together with some other representative proteins are shown in Table 1.11.

Precise amino acid sequences are known for a great number of mammalian species with similarities to bacterial cytochrome c. The oxidized form, called ferricytochrome c, has an adsorption at 280 nm, while the reduced form, ferrocytochrome c, does not show absorption at this wavelength, but has three typical absorptions at 550, 521 and 415 nm. The oxidation of succinate to fumarate in the presence of succinate dehydrogenase is one of the most important biochemical reactions in which cytochrome c takes part and can be used *in-vitro* to measure its activity.

$$\text{Succinate} + 2\ \text{Ferricytochrome}\ c \rightarrow \text{Fumarate} + 2\ \text{Ferrocytochrome}\ c + 2\ H^+$$

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Cytochrome c is also called as myohematin, a hematin-protein which plays a vital role in cellular oxidation in both plants and animals. It is generally regarded as universal catalyst of respiration, forming the essential electron-bridge between the respirable substrate and oxygen. The isoelectric point of cytochrome c is 11.0 and its molecular weight is about 13000 [64]. It is stable at room temperature (0 - 25°) and can be denatured by heating to 50° for 5 minutes, which suggests that its structure is not as compact as lysozyme.

Table 1.11 Amino acid composition of some proteins as number of residues per molecule.

Amino acids (3 letter code)	Lysozyme (Chicken)	Cytochrome *c* (Equine)	Ribonuclease (Bovine)	Ferredoxin (Spinach)
Nonpolar				
Ala	12	6	12	9
Val	6	3	9	7
Leu	8	6	2	8
Ile	6	6	3	4
Pro	2	4	4	4
Met	2	2	4	0
Phe	3	4	3	2
Trp	6	1	0	1
Polar (uncharged)				
Gly	12	12	3	6
Ser	10	0	15	7
Thr	7	10	10	8
Cys	8	2	8	5
Tyr	3	4	6	4
Asn	13	5	10	2
Gln	3	3	7	4
Negativelycharged				
Asp	8	3	5	11
Glu	2	9	5	9
Positively charged				
Lys	6	19	10	4
Arg	11	2	4	1
His	1	3	4	1
Percent non-polar	35	31	30	36
Total residues	129	104	124	97

1.5.4 Analytical Techniques for the Study of Protein Deposits

A variety of methods have been employed to detect the effect of exposing contact lenses to protein both *in-vivo* and *in-vitro*. The most widely used techniques include sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), spectrophotoflourimetry, immunochemical assay [68], radio active tracers [69], Ninhydrin method [70], Ultra violet absorption spectroscopy [71], and binding of Coomassie Brilliat Blue-G to proteins [72].

Isotachophoresis (ITP), the latest of the electrophoretic techniques, is a reproducible and rapid analytical method which is used for analysis of serum proteins. In this work, the technique was developed to study the tear proteins and the proteins extracted from contact lenses.

In addition, indirect methods which involve the pre-treatment of the lenses to hydrolyse the protein followed by visualisation with ninhydrin and colorimetric reagents [73] are also available for protein detection. Most of these methods were used in this study, and although some of them are not quantitative, they are useful for providing further detail about protein deposition on and within hydrogels.

1.6 The Scope and Objectives of the Present Work

The complete structure of the protein in terms of its shape, size and surface charge have the most important role in the spoilation of biomaterials. The biological environment is a complex mixture of proteins with different characteristics.

One of the aims of this project is to show the effect of protein relating factors on its interaction with hydrogel contact lenses. A range of analytical techniques have been used to measure the identity and quantity of the absorbed proteins. The chemical structure of the biomaterial and its water content as well as the pH of the spoilation environmen also have significant effect on the absortion phenomena. By the use of different contact lenses and spoilation conditions these factors were studied.

One other important objective of the present reseach is the development of new biomaterials and study of their spoilation behaviour. This part of work is preformed in collaboration with some other members of the research group. The activity and mobility of the absorbed protein have very important effects on the biological action of the protein. Therefore, in other parts of the project it is aimed to measure the activity of the absorbed protein and its mobility once it is absorbed.

The quantity and activity of the proteins absorbed on contact lenses by different wearers will be studied using a series of clinical work in collaboration with an optician colleague. The effect of different factors such as lens material, care system and patient related problems will also be compared.

The effect of different care systems and cleaning solutions on the removal of the proteins will be studied. The quantity of the protein remained after cleaning is important when inserting the lens back into the eye. The activity of the lysozyme extracted from the lenses which have been cleaned with various care systems will be compared.

Chapter 2

Experimental Techniques and Methods

Chapter 2

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2.1 Electrophresis

Proteins are amphoteric compounds which contain both acidic and basic groups, the net charge of a protein being determined by the pH of the medium. The pH at which the proteins have no net charge is called the isoelectric point (pI). At a pH above the isoelectric point proteins are negatively charged, and pH below their isoelectric point they have positive charge. This behaviour of the proteins makes them suitable to be separated by electrophoresis.

$$H_2N-\underset{R}{\overset{H}{\overset{|}{\underset{|}{C}}}}-COO^- \overset{H^+}{\rightleftharpoons} H_2N-\underset{R}{\overset{H}{\overset{|}{\underset{|}{C}}}}-COOH \overset{H^+}{\rightleftharpoons} H_3N^+-\underset{R}{\overset{H}{\overset{|}{\underset{|}{C}}}}-COOH$$

$\longleftarrow$ Higher pH   Lower pH $\longrightarrow$

Electrophoresis is a method whereby charged molecules in a buffer solution migrate in an electric field. Therefore, if the pH of the buffer is higher than the pI of a specific protein, it is negatively charged and migrates towards the anode, similarly, if the pH of the buffer used is lower than the pI of the protein it will migrate towards the cathode. The rate of migration depends on the strength of the field, the net charge, size and shape of the molecules and also on the viscosity, ionic strength and temperature of the medium.

2.1.1 Basic Principles of Electrophoresis

The term electrophoresis is used to describe the migration of a charged particle under the influence of an electric field. Under conditions of constant velocity the driving force on the particle is the product of the effective charge on the particle Q and the potential gradient E, and this is balanced by the frictional resistance f of the medium. In free solutions this obeys Stokes' law so that:

$$f = 6\pi r \upsilon \eta,$$

where r is the radius of the particle moving with the velocity υ through a medium of viscosity η. However, Stokes' law is not obeyed in gels, and f then depends on a number of factors which include gel density and particle size.

The electrophoretic mobility m is defined as the distance d travelled in time t by the particle under the influence of potential gradient E so that

$$m = d/tE$$
$$m = \upsilon/E$$

Most large molecules possess both anionic and cationic groupings as part of their structure and, therefore, are termed zwitterions. Since the dissociation constants (pK values) of these groups will differ widely, the net charge on such a molecule will depend upon the pH of its environment. Thus the pH will also influence the mobility of molecule. The ionic strength determines the electrokinetic potential which reduces the net charge to the effective charge and it is found that the mobility of the charged particle is inversely proprtional to the square root of the ionic strength. The higher the ionic strength of the buffer the greater the conductivity and the greater the amount of heat generated. Increasing temperature causes an increase in the rate of diffusion of the ions and also an increase in the ionic mobility amounting to about 2.4 per cent per degree Celsius rise in the temperature. At the same time, the viscosity of the medium falls with rising the temperature. Thus, the electrical resistance decreases and at constant voltage the current will rise increasing the heat output still further. Therefore, the ionic strength of the buffer is crucial since it effectively determines the amount of the electrical power which can be applied to the system.

Electrophoresis simply refers to the movement of the ions through the medium, so that the factors discussed so far which affect this are applicable to to all forms of electrophoresis, whether in free solution as in moving boundary electrophoresis or when a supporting medium such as starch gel, polyacrylamide gel, cellulose acetate, or paper is employed. However, when a supporting medium is used additional factors may also affect the mobility and sharpness of the separation. These factors include the adsorption effects on to the supporting medium, inhomogeneties within the matrix of the supporting material, and ion exchange with charged groups of support molcules.

The basic principle of electophoresis is a very simple one, but the progress of the charged particles or ions are influenced by a large number of factors. Therefore, the various methods of electrophoresis have a fundamental unity, and their apparent diversity is the

result of their development to exploit to maximum advantage one or more of these influencing factors in order to achieve the desired goal.

2.1.2 Polyacylamide Gel Electrophoresis (PAGE)

This type of electrophoresis uses the supporting medium, polyacrylamide gel, which helps reduce the diffusion so that the separated components remain as sharp zones with maximum resolution between them. Polyacrylamide gel is also inert during the separation process with uniform properties and easily reproduced. The composition of the gel can be modified in a controlled way to achieve the best conditions for the specific separation.

Hjerten (1962) described some of the properties of the gel conditions which form the basis of PAGE. In this nomenclature T represents the total concentration of the monomer (acrylamide+Bis) expressed as grams per 100 ml and the term C is the weight percent of total monomer T which is due to the cross-linking agent, N, N′-methylene-bis-acrylamide (Bis). The best and most easily manageable gels are obtained when T varies between about 3 to 25%, and gelation does not occur much below T = 2.5% even if the C increases up to 20%. The ratio of acrylamide and Bis concentration are important in determining the physical properties of the gel, and if C is much greater than 10 percent the gels become britle and opaque. When C is less than 1% gels become glue-like and very difficult to handle.

Molecular sieving as well as charge play a role in the separation of molecules by PAGE. Therefore, the concentration of acrylamide required to give optimum results depends on the size of the molecules to be separated. Table 2.1 gives the amounts of all constituents to produce gels suitable for the range of molecular weights shown.

2.1.2.1 The chemical structure of polyacrylamide gel

The gel is formed by vinyl polymerization of acrylamide monomers CH_2=CH-CO-NH_2, into long polyacrylamide chains and cross-linking the chains by an appropriate bifunctional monomer, usually N, N′-methylene-bis-acrylamide (Bis) CH_2=CH-CO-NH-CH_2-NH-CO-CH=CH_2. The free radical polymerization of the monomers is catalysed by tertiary aliphatic amines such as N, N, N′, N′-tetramethylenediamine (TEMED) or 3-dimethylamino-propionitrile (DMAPN). The concentration of acrylamide used determines the average polymer chain length while the Bis concentration determines the extent of cross-link formation. Thus both are important in determining physical

properties such as the gel density, elasticity, mechanical strength, and pore size. Figure 2.1 shows the structure of polyacrylamide gel formed by the polymerization process.

Table 2.1 The amounts of reagents required to prepare 100 ml of gel with the separation range for proteins.

Constituent	Amounts required for gels with		
	T = 5 %	T = 7.5 %	T = 10 %
Acrylamide	4.75 g	7.125 g	9.50 g
Bis acrylamide (C = 5 %)	0.25 g	0.375 g	0.50 g
TEMED	0.05 ml	0.05 ml	0.05 ml
Ammonium persulphate	0.05 g	0.05 g	0.05 g
Molecular weight range	Above 100,000	20,000-150,000	10,000-80,000

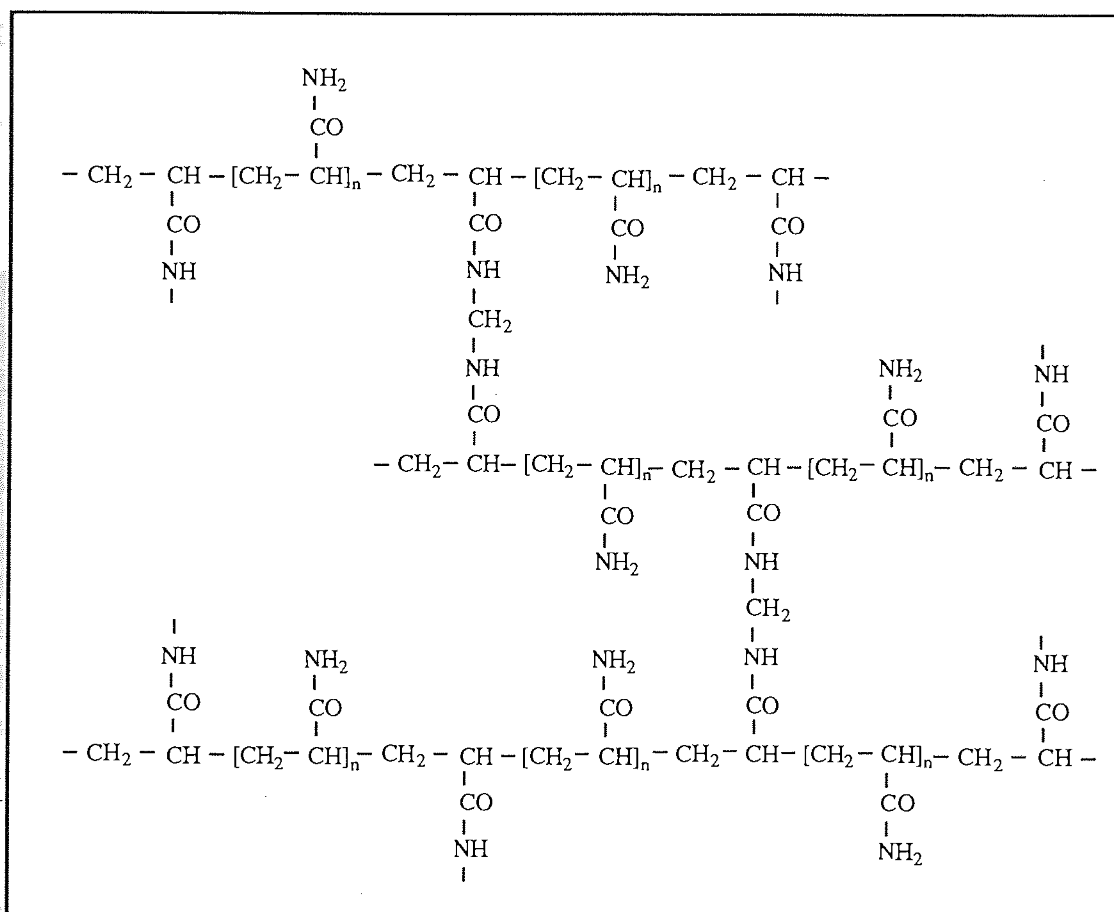

Figure 2.1 Structure of polyacrylamide gel matrix.

2.1.2.2 Pore size effects

The passage of any particle in the gel matrix depends upon the relative size of the particle and the pores in the gel matrix. The pore size of a polyacrylamide gel can be adjusted by

varying the amount of crosslinker as a percentage of the monomer and cross linker. The sum of the weight of the acrylamide monomer and the crosslinker is expressed as % T. For example a 20% T, 5% C_{bis} gel would have 20% w/v of acrylamide plus bis, and bis would account for 5% of the total weight of acrylamide. It has been proven that at any percentage of T, 5% crosslinker produces the smallest pores in the gel. Above and below 5%, the pore size increases [74].

Experimentally it is found that during PAGE both the absolute and relative mobilities of molecules are influenced by many factors which affect the average chain length of the polyacrylamide molecules. These include not only the acrylamide and Bis concentrations and temperature but also the concentration of the catalyst used to initiate the polymeraization reaction and the time between the addition of the catalysts and gel formation (Kingsbury and Masters 1970).

2.1.2.3 Quantitative determination of separated components

The most widely used method for quantifying the results of PAGE separations is densitometry of the stained gel patterns. Quantitative measurements are normally divided into two classes: firstly those where only relative measurements are needed, for example when monitoring the progress of an isolation procedure or of a chemical reaction, and secondly those where the results must be expressed in absolute terms, i.e., concentration or units of activity.

The quality of quantitative densitometry depends on many factors, some of the most important of which are shown schematically in Figure 2.2. In this figure the band paterns are the result of:

a. An ideal pattern, gel stained well into centre.
b. Streaky bands due to particulate material in sample.
c. Curved bands due to uneven gel polymerization, or to too high a current leading to a substantial temperature gradient (and hence mobility differences) between the centre and outer parts of the gel rod.
d. Distorted bands due to an uneven gel surface.
e. Distorted band produced by air bubbles forming during polymerization.
f. Centre not stained due to short staining time.
g. "Halo" effect due to combination of inadequate fixing and high loading.

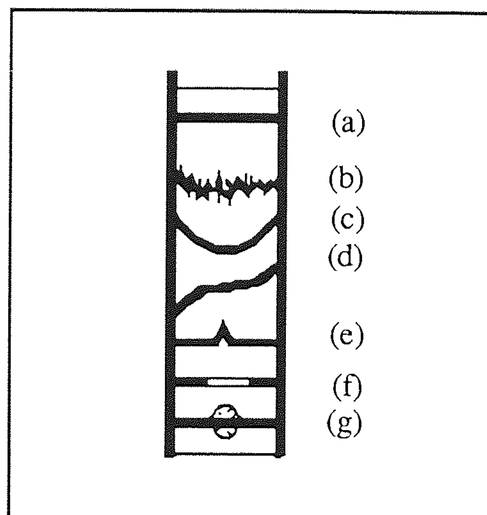

Figure 2.2 Some common band patterns in a polyacrylamide gel electrophoresis.

The densitometer output is often a chart recorder and quantified by measuring the areas under individual peak or by plotting a standard line for the peak height of a series of known standards. In the case of peak height measurements it must be taken into account that the peak heights depend not only to the amount of the material, but also to the distance travelled through the gel. Thus, it is best if a series of standards are run each time with which the peak heights can be compared.

2.1.2.4 Materials and solutions

1. Monomer solution (30% T, 2.7%C)
   58.5 grams of acrylamide (Sigma, A-3553), and 1.6 grams of N, N-methylene-bis-acrylamide (Bis) (Sigma, M-7279) were dissolved in distilled water and the total volume adjusted to 200 ml.
2. Running gel buffer (pH 8.8)
   36.3 grams of tris (hydroxymethyl) aminomethane or "tris" (Sigma, T-1378) was dissolved in distilled water and the final volume was made up to 200 ml.
3. Stacking gel buffer (pH 6.8)
   6.0 grams of tris was dissolved in 100 ml distilled water. The pH of the buffer was adjusted with a pH meter (Mettler delta).
4. 10% sodium dodecyl sulphate (SDS)
   25 grams of sodium dodecy lsulphate (BDH, 10807) was dissolved in distilled water with a final volume of 250 ml.
5. Initiator (10% ammonium persulphate)
   0.5 gram of ammonium persulphate (BDH, 10032) was dissolved in 5.0 ml of distilled water.

6. Running gel overlay

   1.0 ml of solution (4) was added to 25 ml of solution (2) and the total volume was made up to 100 ml with distilled water.

7. Treatment buffer

   2.5 ml of solution (3), 4.0 ml of solution (4), 2.0 ml of glycerol (BDH, 10118), and 1.0 ml of 2-mercaptoethanol (Sigma, M-3148) were mixed with 10 ml of water. The resulting solution was divided into 1.0 ml aliquots and frozen to be used later.

8. Tank buffer

   48.0 grams of Tris and 230.4 grams of glycine (Sigma, G-7126) were dissolved in distilled water and 160 ml of solution (4) was added, the final volume was made up to 16.0 litres.

2.1.2.5 Preparation of the gel

A vertical slab gel unit (SE-600, Hoffer Scientific Instruments) was assembled as shown in Figure 2.3 using a 1.0 mm spacer. The separating gel (10% T, 2.7% C) was prepared by mixing 20 ml of solution 1, 15 ml of solution 2, 0.6 ml of solution (4), and 24.1 ml of distilled water in a 100 ml side arm flask. The flask was then stoppered and evacuated for three minutes (as oxygen inhibits the polymerization). 300 μl of the initiator (solution 5) and 20 μl N, N, N', N' tetramethylene diamine TEMED (Sigma, T-8133) was used as the polymerization catalyst.

The prepared polymerization mixture was then pipetted into the slabs. The formation of the bubbles was prevented to avoid slowing down of the polymerization. The slab was placed in a cold water bath and about 1.0 ml of water saturated n-butanol (5.0 ml water and 50 ml n-butanol) was applied on the surface of the gel. The polymerization was completed when a very sharp interface appeared at the gel surface (about four hours later). The overlay solution was removed and the surface of the gel was washed twice with distilled water. 1.0 ml of solution 6 was added to the gel and allowed to stand for about two hours in a water bath at room temperature. The purpose of water bath was to keep the polymerization mixture at a constant temperature.

The stacking gel (4% T, 2.7% C) was then prepared in exactly the same way as the separating gel. Its composition was 2.66 ml solution 1, 5.0 ml solution 3, 0.2 ml solution 4, 12.2 ml distilled water, 100 μl solution 5 and 10 μl TEMED. After being mixed it was pipetted over the seperating gel and a 1.0 mm comb was inserted carefully to produce the wells in the gel. The slab was left in the water bath at room temperature after about four hours by which time the gel was polymerized.

2.1.2.6 Treatment of the lens extracts and protein solutions

A 0.1 mg/ml solutions of the following pure proteins (listed below), mixture of standards and extracted solutions from the spoiled lenses were mixed with equal volume of treatment buffer and boiled in a water bath prior to placement into the gel wells. The treatment buffer, as previously mentioned contains 10% SDS, which is an anionic detergent that denatures the proteins by wrapping around the polypeptide backbone. It therefore confers a net negative charge onto the polypeptide in proportion to its length. In this way, polypeptides become rods of negative charges with equal charge density or charge per unit. The standard proteins and mixtures of proteins were used as supplied by the manufacture without further purification.

1. Human albumin (Sigma, A-9511),
2. Human γ-globulins: from Cohn fraction (Sigma G-4386),
3. Lactoferrin from bovine colostrum (Sigma, L-4765),
4. Chicken egg lysozyme (Sigma, L-6876),
5. Ferredoxin from spinach (Sigma, F-3013),
6. Ferredoxin from *Red marine algae* or *Prophyra umbilicalis* (Sigma, F-5257),
7. Insulin from bovine pancrease (Sigma, I-5500),
8. α-lactalbumin from bovine milk (Sigma, L-6010),
9. Ribonuclease from bovine pancrease (Sigma, R-5000),
10. Cytochrome c from bovine heart (Sigma, C-3131),
11. Myoglobin from horse heart (Sigma, M-1882),
12. Mucin type I from bovine submaxillary glands (Sigma, M-4503),
13. Mucin type 1-S from bovine submaxillary glands (Sigma, M-3895),
14. Mucin type II: crude from porcine stomach (Sigma, M-2378)
15. SDS-PAGE molecular weight standard solution (Bio-Rad, 161-0317),
16. High molecular weight standard mixture (Sigma, SDS-6H) with molecular weight range of 6,500-200,000.
17. Molecular weight markers for peptides (Sigma, MW-SDS-17S).

2.1.2.7 Loading and running the gels

The comb was carefully removed from the gel and each well was rinsed once with the tank buffer then refilled with buffer. A tiny drop of phenol red was added to each treated sample and 20 μl of the samples were placed very carefully under the tank buffer in each well. The syringe used to apply the samples was washed five times with distilled water between each sample application to avoid cross-contamination with the different proteins. This stage of the preparation was performed as quickly as possible,

because the samples diffuse sideways if they stand for a long time in the wells before the electrophoresis run.

To run the gel, the vertical slab gel unit was filled with the tank buffer up to about 10 cm of the top. The gel was clamped to the upper buffer chamber, which was then filled with the tank buffer to about 1.0 cm above the wires. The buffer surface in the lower chamber was kept below the sample tops and the mixing of the two buffers was prevented. Upper buffer was connected to the cathode and the lower chamber to the anode of the DC power supply (PS-1500, Hoffer Scientific Instruments). A magnetic bar was placed in the unit and the whole was put on a magnetic stirrer (Figure 2.4).

To set the current, the voltage and power knobs were turned to their highest value. The switch on the left hand side of the power supply (Figure 2.4) was on the set limit position. A current of 25 mA/1cm of gel was applied and the switch was turned back to the read output. The ready light was on when the circuit was closed. The timer was set for four hours and the changes in voltage and power were recorded and were used as a guide for the later runs. The voltage started from 86 volts and rose to 140 at the end of the run, while the power had a very small change from 4 to 7 watts. The samples passed from the stacking gel into the separation gel after about 45 minutes and the dye front reached 1 cm above the bottom of the separating gel in about 4 hours, when the run was stopped. The gel was then ready to be stained. Two methods of staining were used, both procedures should be initiated as soon as the electrophoresis run is completed.

2.1.3 Colloidal Brilliant Blue-G Staining of the Gel

Proteins in gels after electrophoresis are generally stained with Coomassie Brilinat Blue R250. This dye forms rather strong, but non-covalent, complexes with proteins. The structure of the dye is shown in Figure 2.5.

Colloidal Brilliant Blue-G (B.B.G) exists in two different colour forms, red and blue [75]. The red form is converted to the blue form upon binding of the dye to the proteins. The protein-dye complex is stable for long periods of time. This behaviour can also be used to determine the quantity of the protein using ultra violet spectrophotometry. It is also possible to extract the protein bound dye from the gels with a solvent and determine its concentration by spectroscopy. In the staining technique applied here, a new form of the B.B.G was used. This form of the dye contained 0.1% w/v B.B.G, 2% w/v phosphoric acid and 15% w/v ammonium sulphate.

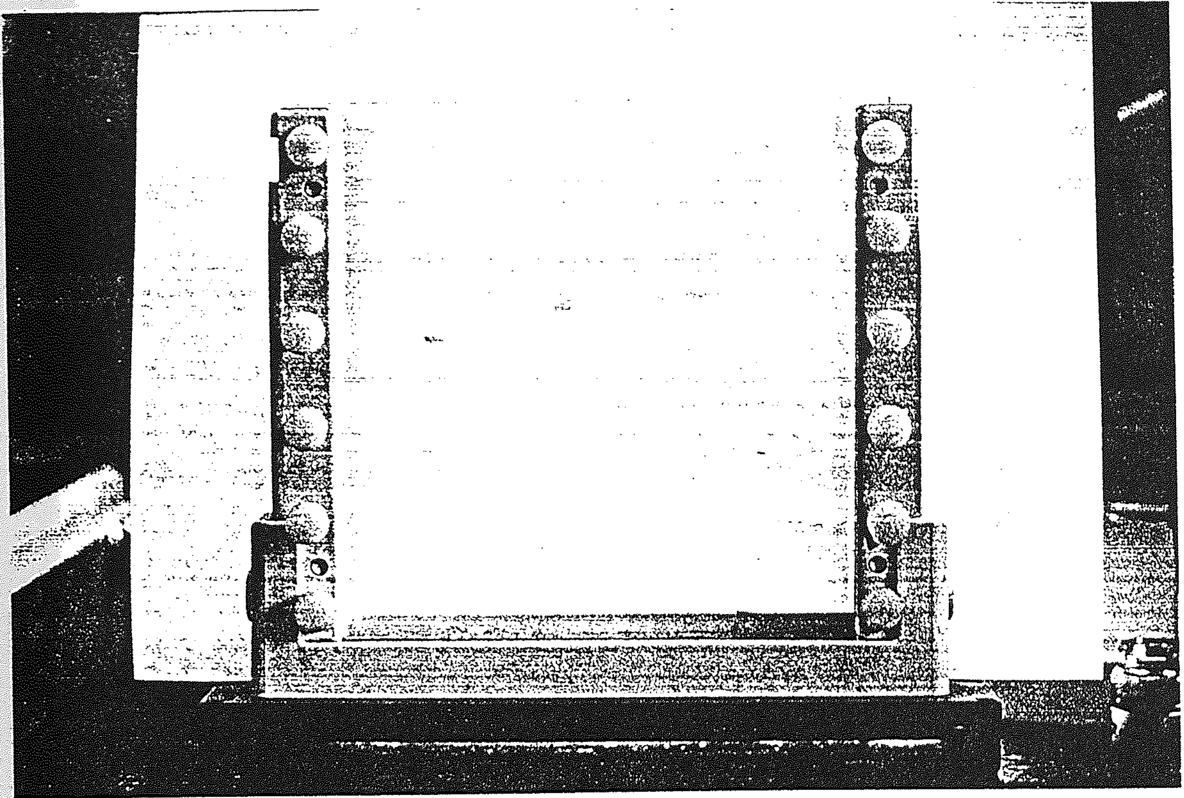

Figure 2.3 The vertical slab gel unit.

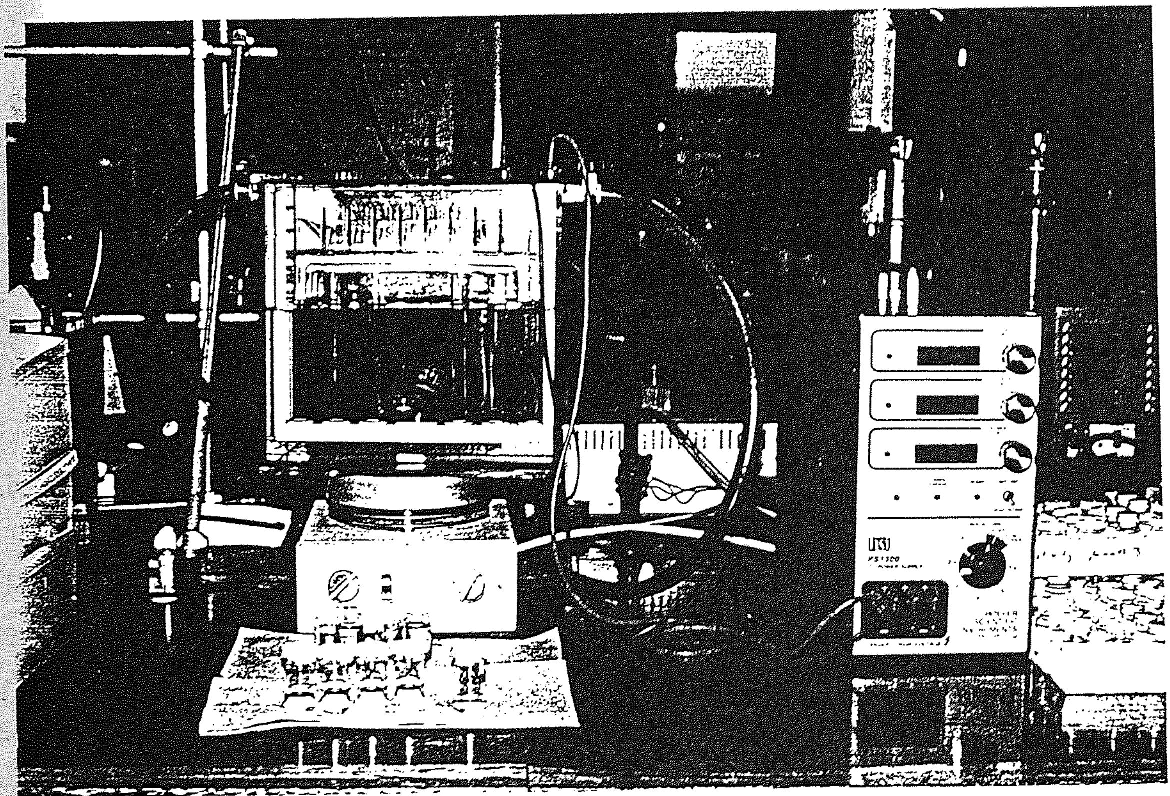

Figure 2.4 Hoffer Electrophoresis Instrument, PS-1500.

The preparation has been shown to be up to ten times more sensitive than methanol-acetic acid preparation of B.B.G. Its sensitivity is comparable to silver staining which will be discussed later in this chapter.

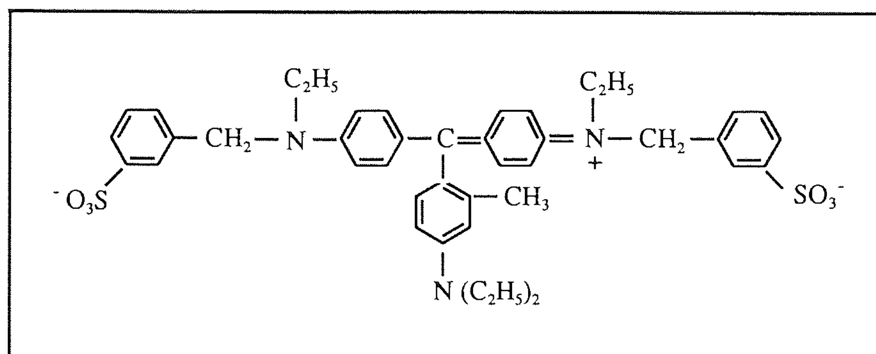

Figure 2.5 The chemical structure of Coomassie Brilliant Blue R250.

2.1.3.1 Materials and solutions

The fixing and staining solutions for this technique were supplied as concentrates and only needed diluting. The destaining solutions were made in the laboratory.

1. Fixing solution
   The fixing solution (Sigma, F-7264) was diluted 1:5 with distilled water.
2. Staining solution
   300 ml of Brilliant Blue-G concentrate (Sigma, B-8522) was diluted to one litre with distilled water.
3. Destaining solution (10% acetic acid, 25% methanol)
   100 ml of acetic acid and 250 ml of methanol were mixed and the volume was adjusted to 1000 ml with distilled water.
4. 25% methanol
   250 ml of methanol was diluted to 1000 ml with distilled water.

2.1.3.2 Procedure

The gel was carefully removed from the sandwiches using gloves to avoid finger prints on its surface. It was fixed in 200 ml of solution 1 for one hour with a gentle agitation. The gel was then placed in solution 2 for about two hours. It was then destained in solution 3 for 10 to 30 seconds, washed with 25% aqueous methanol and left in this solution for one day. The gels can be stored in distilled water for at least four months without the colour fading. A Brilliant Blue-G stained gel is shown in Figure 2.6. The sensitivity of this method can be shown by comparing Figure 2.6 with Figure 2.8 which

is a silver stained gel. It can be seen that most of the bands in Figure 2.6 are as clear as those in Figure 2.8. The lens extracts are more easily detectable on a silver stained gel (lines 7, 8, 9, 10 in Figure 2.6 and 4, 5, 6, 7, 8 in Figure 2.8).

The relative mobility (Rf) for different protein bands were calculated from the following formula:

$$Rf = \frac{\text{The distance migrated by the protein (a)}}{\text{The distance migrated by the dye (b)}}$$

A standard curve was plotted by using the log of the molecular weights for Sigma broad range standards against their relative mobility (Rf). The standard curve is shown in Figure 2.7. The curve was then used to obtain the molecular weight of the extracted protein. In each case the protein was recognized from its obtained molecular weight and comparing to the known proteins (Table 2.3).

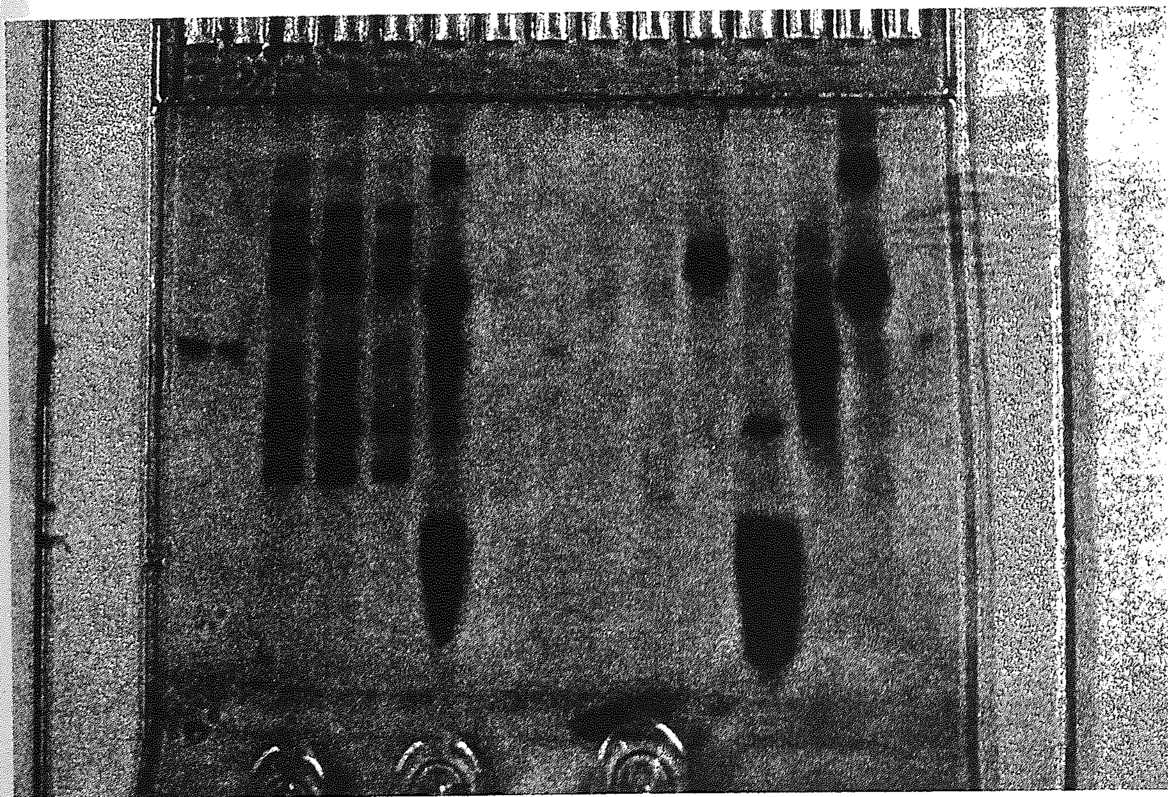

Figure 2.6 A Brilliant Blue-G stained gel. Lines 3, 4, and 5 show high molecular weight standards; line 6 is a mixture of albumin, γ-globulin, lysozyme and lactoferrin; lines 7, 8, 9 and 10 are lens extracts, lines 11, 12, 13 and 14 are lactoferrin, lysozyme, γ-globulin and albumin respectively. (Lines 1 and 2 are due to the contamination from other wells).

2.1.4 Silver Staining of the Gel

Silver staining of the polyacrylamide gels is a highly sensitive visualising technique that allows the detection of most of the protein present even at very low concentrations. Generally, silver staining techniques are much more sensitive than those using Colloidal Brilliant Blue-G and about 100 times more sensitive than Coomassie Brilliant Blue R-250. For this reason the silver staining was used to detect the proteins extracted from contact lenses and the results were compared to the previous results.

Table 2.2 Rf (a/b) of different proteins related to their molecular weight (Mw) for the proteins bands of the gel shown in Figures 2.6 and 2.8. The distance migrated by the dye front (b) was 10 cm.

The distance migrated by protein (a, cm)	The rate of flow (a/b)	Log of MW	Molecular weight (KD)	Suggested protein
2.5	0.25	5.343	220	GP3M
6.7	0.67	4.564	36	Serum pre-albumin
7.0	0.70	4.508	32	---
0.7	0.07	5.680	480	---
0.9	0.09	5.643	440	---
2.0	0.20	5.438	270	IgA
3.4	0.34	5.178	150	IgG
6.5	0.65	4.601	39	Alpha Gl.
9.5	0.95	4.043	11	Lysozyme
2.2	0.22	5.451	280	Secretory IgG
2.0	0.20	5.488	307	Secretory IgG
4.6	0.46	4.907	80	Lactoferrin
5.5	0.55	4.837	68	Albumin
8.8	0.88	4.224	16	Tear pre-Albumin
9.2	0.92	4.149	14	Lysozyme

2.1.4.1 Materials and Solutions

A Sigma silver staining kit (AG-5) was purchased and the following solutions were prepared:

1. Fixing solution (30% ethanol, 10% glacial acetic acid)
   90 ml of glacial acetic acid was added to 270 ml of ethanol, and the final volume was made up to 900 ml with distilled water.

2. Silver equilibrium solution

   1.5 ml of the silver concentrate was diluted to 300 ml with distilled water.

3. Development solution

   30 ml of developer (1) was diluted to 300 ml and 0.17 ml of (2) was added to it.

4. Stop solution (1% acetic acid)

   3.0 ml of glacial acetic acid was diluted to 300 ml with distilled water.

5. Reducer solution

   2.0 ml of reducer (A), 4.0 ml of reducer (B), and 0.7 ml of reducer (C) were mixed and diluted to 300 ml with distilled water. This solution was stable for one day.

2.1.4.2 Procedure

The gel was removed from the electrophoresis chamber very carefully, wearing gloves all the time. It was fixed in 300 ml of solution 1 three times (20 minutes each time). It was then rinsed three times with 300 ml of distilled water (10 minutes each time) and placed in 300 ml of solution 3 with gentle agitation for 30 minutes. Solution 3 was then removed and the gel was rinsed for 20 seconds with 30 ml of distilled water. One half of the solution 3 was placed over the gel for about 10 minutes, it was then replaced with the other half. When the darkness of the bands was sufficiently developed and the background was not too dark, the development was stopped by immersing the gel in 300 ml of solution 4 for five minutes. The stained gel was rinsed three times with 300 ml of distilled water (10 minutes each time), left in solution 5 for 30 seconds, rinsed immediately with distilled water (300 ml three times and 10 minutes each time), and finally stored in distilled water. Figure 2.8 is a silver stained gel which was stored for 45 days in distilled water.

It can be seen from Figure 2.8 that the technique is a slightly more sensitive method compared to the Brilliant Blue-G staining (Figure 2.6). Most of the bands have appeared in both stained gels, but some (especially the ones belonging to lens extracts) are clearer in the silver stained gel. For a faster result, however, the first method is preferred, which is also easier to apply and more economical.

As shown in Figure 2.6 and 2.8, in most cases, the proteins separated on a gel form more than one sharp and distinctive line. Table 2.3 shows the major and most important tear proteins with the number of bands they show on a polyacrylamide electrophoresis gel. Even in the case of proteins with just one band the intensity of the dye is different on the centre with those at the edges.

Table 2.2 Molecular weights and number of the bands for major tear proteins.

Protein	Molecular weight (KD)	Number of bands
GP1	2000	1
GP2	1300	1
IgA	260-360	1
Gp3M	200	1
IgG	150,310,350	3
Transferrin	75	2 dense
Lactoferrin	74,81	2 dense
Albumin	65	1
Secretory component	59,78,260-360	2-3
Alpha globulin	> 40	
Serum pre-albumin	31,70	2 faint
Tear pre-albumin	15-19	
Lysozyme	14.5,23,24	1 dense, 2 faint

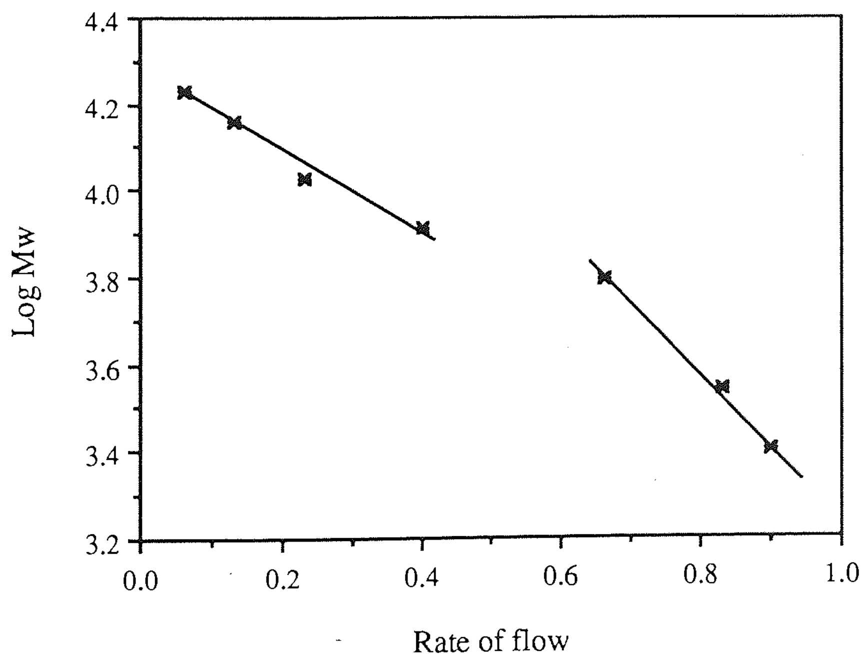

Figure 2.7 The relationship between the molecular weight of Sigma standard proteins and their rate of flow (Rf) in a polyacrylamide gel.

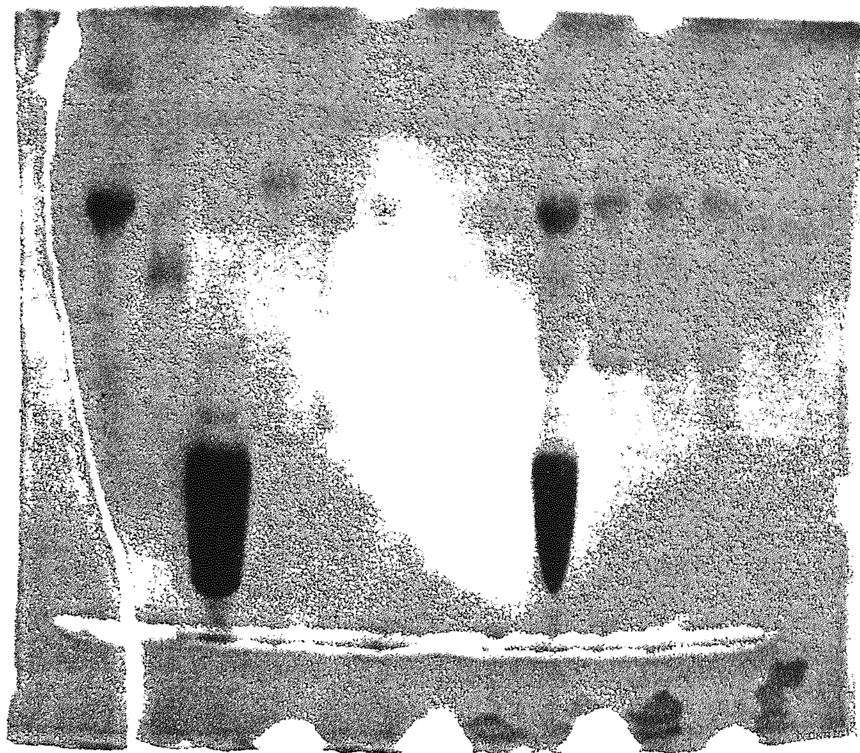

Figure 2.8 A silver stained gel.

2.1.5 Densitometry of the Gels

Densitometry is a quantitative method by means of which the amounts of colour present in each part of a stained gel can readily be obtained. The accuracy depends on whether the proteins bind to the dye-stuff to the same degree or not. However, if this bind is not similar, only an estimation may be used to calculate the amount of the proteins. Figure 2.9 shows the electrophoresis gel with a series of standard lysozyme solutions with different concentrations which was stained with BBG. Figure 2.10 is the densitograms of the standard lysozyme solutions in each well.

2.1.5.1 Instrument procedure

Figure 2.11 shows the instrument which was used to measure the density of the stained proteins on poly acrylamide gel. This was an LKB Ultrascan Laser densitometer to which an Apple II Uroplus computer was attached. The instrument and the computer were turned on and the blue stained gel was placed on the window while the cursor was set for the start and the end positions. The scan speed was set to 100 using the increase/decrease bottom. When all the factors were set, the cursor was pushed in and the lid was closed. The scan started and was followed by the computer program for

each band on the gel. The absorption spectra were obtained with the position and intensity of each peak representing a protein.

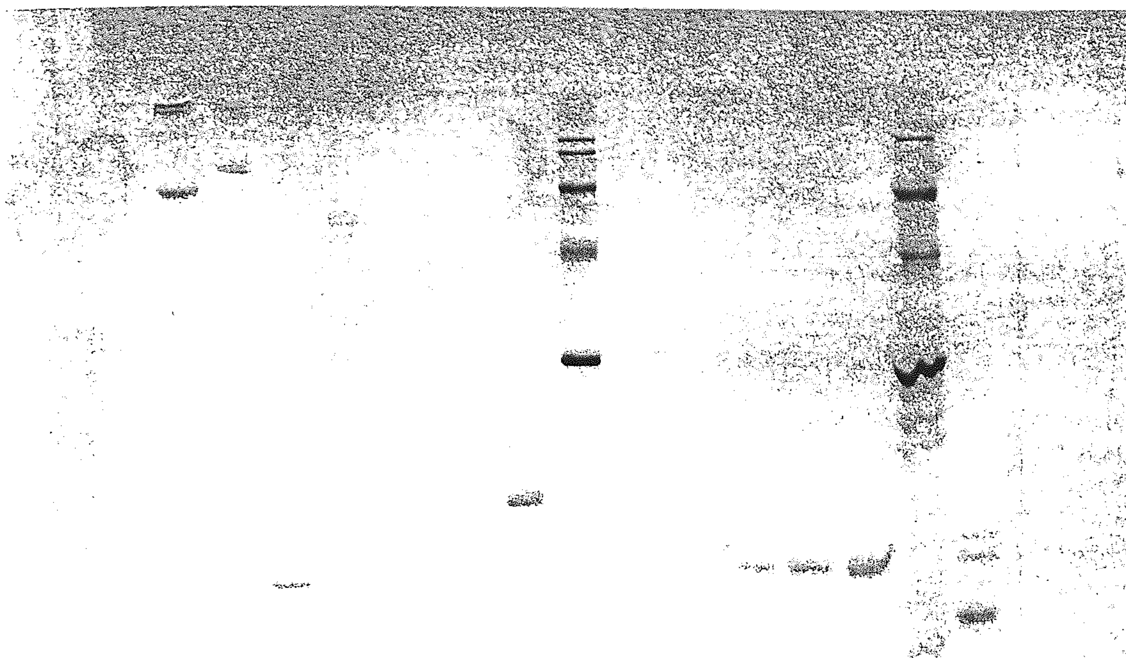

Figure 2.9 A Brilliant Blue stained gel, the wells are:

1-7 0.5 mg/ml Lactoferrin, Albumin, Lactalbumin, γ-globulins, Ribonuclease, Insulin and Ferredoxin,
8. Sigma high molecular weight standards,
9-13. 0.05, 0.1, 0.2, 0.3, 0.4 mg/ml Lysozyme,
14. Sigma high molecular weight standards and
10. Sigma low molecular weight standards.

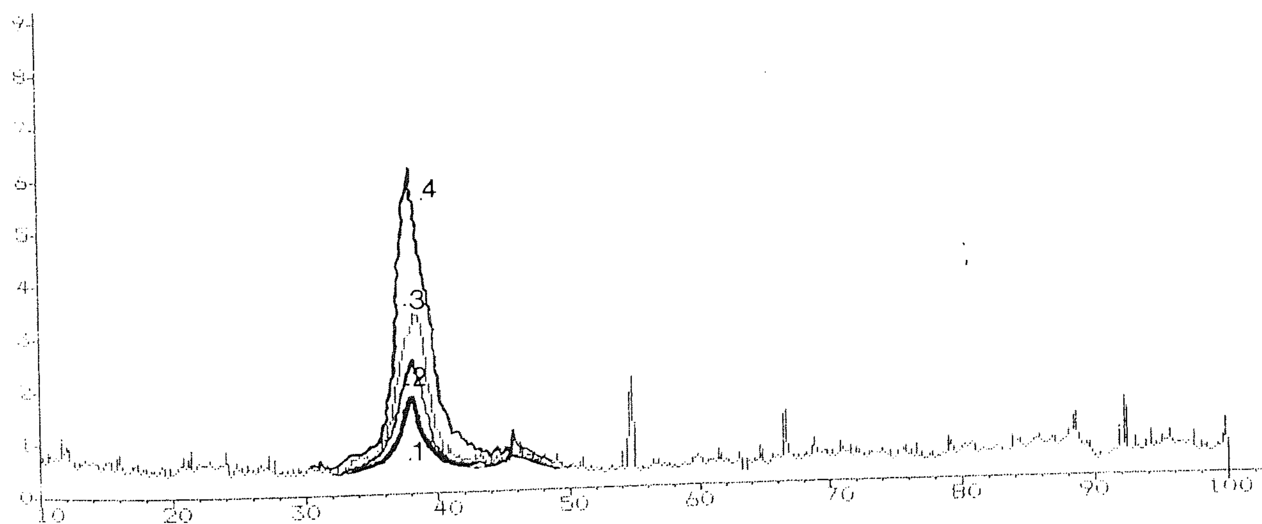

Figure 2.10 The densitogram of lysozyme solutions from the gel in Figure 2.9.

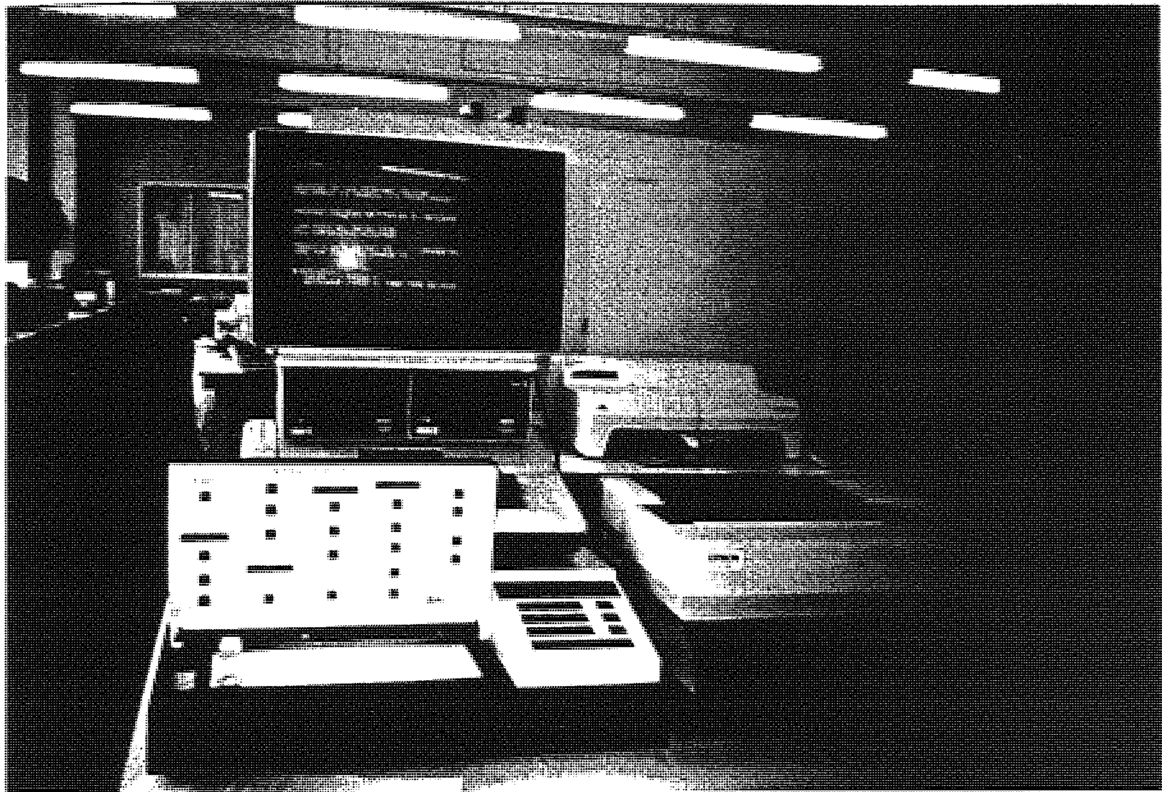

Figure 2.11 LKB Ultrascan Laser Densitometer.

2.1.5.2 Quantitative determinations

A set of standard solutions of lysozyme were prepared and run on a poly acrylamide gel and the resulting gel was stained using Brilliant Blue G technique (Figure 2.9). The densitograms of the standard samples is shown on the same graph in Figure 2.10. The gels were stained with Colloidal Brilliant Blue G as discussed in Section 2.1.3 and the stained gel is presented in Figure 2.9. The intensity of the dye on each of the bands on the gel were measured by the use of the densitometer and a standard curve obtained by plotting the concentrations of the lysozyme samples against the hight of the peak on the densitogram (Figure 2.12). The standard curve was used to calculate the quantity of the lysozyme in the tear samples run on the electrophoresis and in the extraction solutions from the spoiled lenses. The densitograms obtained for some other samples are represented in Appendix 1.

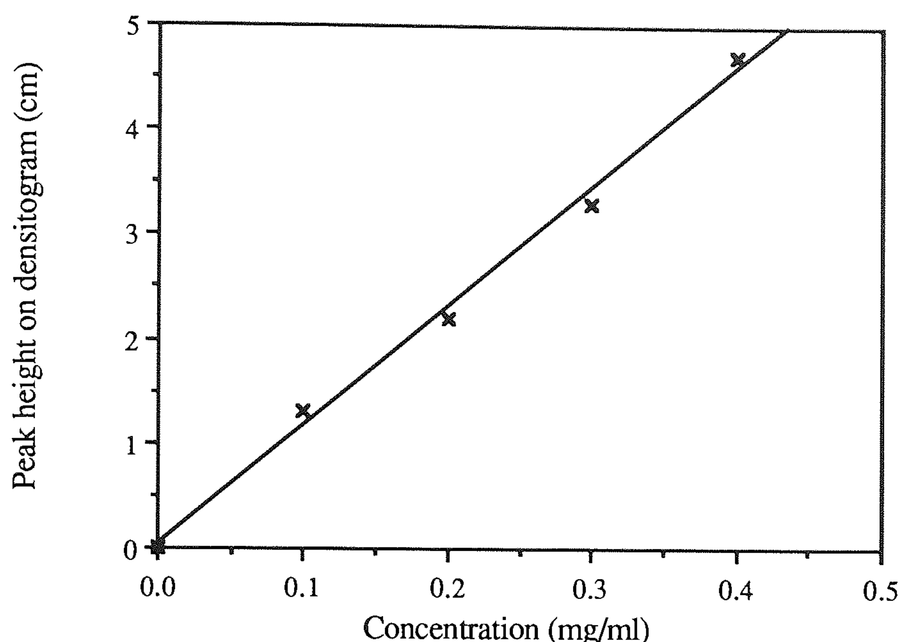

Figure 2.12 The relationship between the peak height on densitogram and the concentration of protein on the electrophoresis gel.

2.1.6 Agarose Gel Electrophoresis (AGE)

Agarose is a highly purified polysaccharide derived from agar, but unlike agar it is not highly contaminated by charged materials. Agarose dissolves in boiling water and remains liquid until the temperature is lowered to 40 °C, at which point it gels. The gel is stable unless the temperature is raised to 100 °C. The pore size of an agarose gel are larger than a polyacrylamide gel. However, the pore size can be pre-determined by adjusting the concentration of agarose in the gel. The higher the concentration of the gel the smaller the pore sizes produced.

2.1.6.1 Procedure

A Paragon electrophoresis system kit from Beckman was used [76]. The agarose gels were supplied ready to use and a 1.0 mg/ml solution of each standard protein (albumin, γ-globulins, lactoferrin and lysozyme) together with a mixture of the four proteins were prepared. 5 μl of each sample was applied across each template according to the procedure given in the kit. When the samples had diffused into the agarose, the gel was squeezed into the Beckman bridge. The bridge was then placed in the cell, covered and the cell inserted into power supply unit. A power of 100 volts was applied for 25 minutes and the gel was silver stained according to the modified method of Morrisey [77]. Another gel with standard proteins was also prepared and stained with Paragon Blue Stain (Beckman, 270-233022-B) which is shown in Figure 2.13.

The sensitivity of this method is much less than polyacrylamide gel electrophoresis. It was shown that, although the gel was silver stained and the concentration of the standard proteins were made 10 times higher than for the other method, the bands were not very clear and the proteins extracted from the lenses were not detectable. It can be seen that, although the concentrations are ten times higher than those used in the SDS-PAGE, the intensity of the blue colour is only similar to the lines in Figure 2.6.

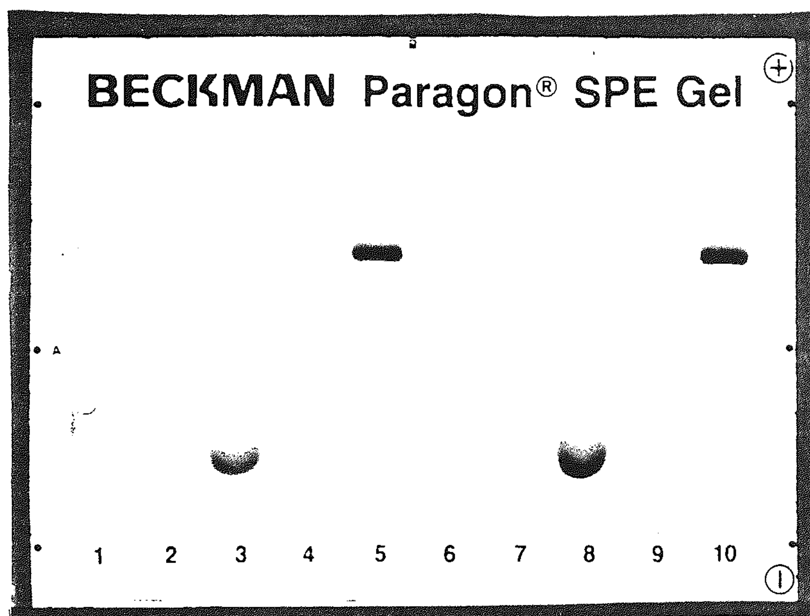

Figure 2.13 An agarose gel stained with Paragon blue stain.

2.2.6.2 The disadvantage of agarose gel electrophoresis

This method is mostly used in diagnostic biochemistry laboratories to identify serum proteins and enzymes which are present in higher concentrations. Although it is a simple, fast and straight forward technique, its sensitivity is very low and it can only detect minimum of 10 mg/ml protein in the solution. Therefore, it was not used for further investigations concerning the tear proteins and the proteins extracted from the contact lenses.

2.1.7 Western Blotting

Protein blotting is a process by which protein bands from a polyacrylamide gel are transferred onto a more stable and immobilising medium, such as nitro-cellulose membrane. Lower concentrations of samples are more easily detected by this technique, since they are not spread throughout the thickness of the gel, but are concentrated at the surface of the membrane.

There are three basic methods for transferring the proteins to the membranes. These are capillary blotting, diffusion blotting and electroblotting [78]. Capillary and diffusion blotting are relatively slow procedures. Electroblotting is now the favoured method for protein transfer. In this method the transfer is facilitated by the use of an electric potential between the gel and the membrane. The principal of electroblotting on polyacrylamide gels is illustrated in Figure 2.14.

The electroblotting buffer was made by adding 12.11 grams of Tris and 56.3 grams of glycine to 1000 ml of methanol and adjusting the final volume to 3500 ml with distilled water. The polyacrylamide gel was placed on three pieces of Whatman 3.0 mm filter paper (Sigma, P-4681) already saturated with buffer. The nitro-cellulose membrane (Sigma, N-8267) was placed on the top of the gel and three more pieces of wet Whatman paper were placed on its top, avoiding any bubbles during the procedure. The sandwich was then held together between two rigid plastic sheets supplied with Hoffer transfer tank (TE-52).

The cassette was placed between parallel platinum electrodes in the transfer tank so that the membrane medium remained between the gel and the anode and the tank was filled with buffer.

A current of 150 mA was applied overnight with the cooling coil and the magnetic stirrer were in operation to overcome the heat developed during the process.

The cassette was opened carefully wearing gloves to prevent contamination and the membrane removed prior to staining by an appropriate method.

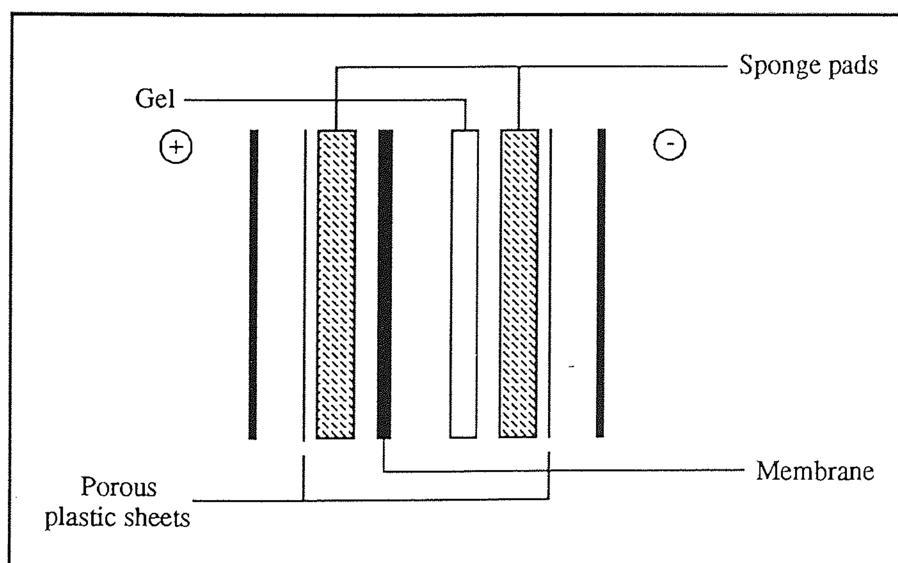

Figure 2.14 Diagramatic representation of electroblotting

2.1.8 Biotin-Avidin Staining on the Blots

In this technique, the blotting principal is dependent on the affinity of biotin for avidin. The membrane is initially reacted with biotin (in the form of N-hydroxysuccinimide biotinate), which binds to all the proteins. Avidin (to which a horseradish peroxidase HRP is conjugated), then reacts with biotin. Finally, a colour reagent is added and the substrate reacts with the catalytic action of the enzyme to give a coloured precipitate at the site of the antigen-antibody complex (Figure 2.15).

2.1.8.1 Materials and solutions

1. Borate-Tween solution (0.05 mM, $Na_2B_4O_7$, 10 H_2O, 0.2% Tween, pH 9.3). 14.07 grams of sodium tetraborate (Sigma, B-9876) was dissolved in distilled water, 2.0 ml of Tween 20 "polyoxyethylene sorbitan monolaurate", (Bio-Rad, 170-6531) was added and then the final volume was made up to one litre with distilled water.
2. Tris-buffered saline (0.02 M tris, 0.05 M NaCl, pH 7.5)
   2.42 grams of Tris and 29.24 grams of sodium chloride (BDH, 1024) were dissolved in one litre of distilled water and the pH was adjusted to 7.5 using HCl.
3. Tris-Tween solution (0.02 M tris, 0.05 M NaCl, 0.2% tween)
   2 ml of Tween 20 was added to one litre of solution (2).
4. Avidine-horseradish peroxidase conjugate solution (A-HRP)
   100 µl of Avidin-horseradish peroxidase (Bio-Rad, 170-6528) was added to one litre of solution (3).
5. Phosphate-buffered saline (0.01 M phosphate buffer, pH 7.2)
   0.105 grams of sodium monobasic phosphate (BDH, 10383), 0.60 grams of sodium dibasic phosphate (BDH, 30132), and 2.55 grams of sodium chloride were dissolved in 300 ml of distilled water and the pH was adjusted to 7.2 using HCl.
6. Biotin solution (7.5 mM N-hydroxy succinimide biotinate in dimethyl formamide)
   This solution was purchased (Bio-Rad, 170-6529) and used as supplied.
7. Horseradish peroxidase colour development solution
   60 mg of 4-chloro-1-naphthol (Bio-Rad, 170-6534) was mixed with 20 ml of ice cold methanol. To this solution, 60 µl 30% H_2O_2 (Sigma, H-1009) and 100 ml of solution (2) was added and used immediately.

2.1.8.2 Procedure

The membrane was washed three times in 100 ml of solution 1 for 10 minutes. It was then agitated in 100 ml of fresh solution 1 to which 200 μl of solution 6 was added and incubated for 15 minutes, washed twice in 100 ml of solution 1 for 5 minutes each time, followed by two washes with solution 3 for 5 minutes. It was incubated in solution 4 for about one and a half hour, washed twice in 100 ml of solution 3 for 5 minutes, followed by two rinses in 100 ml of solution 3. The membrane was incubated in solution 7 for 30 minutes by which time the bands became visible. The colour development was stopped by washing repeatedly in distilled water. It was dried between two sheets of blotting paper and stored in the dark.

2.1.8.3 Results

The bands transferred from the gel to nitro-cellulose membrane. They were visualised by the biotin-avidin technique. The identification of the proteins is easier by this technique. Unfortunately, the Biotin-Avidin staining did not produce good results for the lens extracts. However, the membrane was stained with Brilliant Blue G and a stained membrane with this method was stored for later assessments. The stained membrane was not clear and its quantification was not possible by the use of laser densitometer.

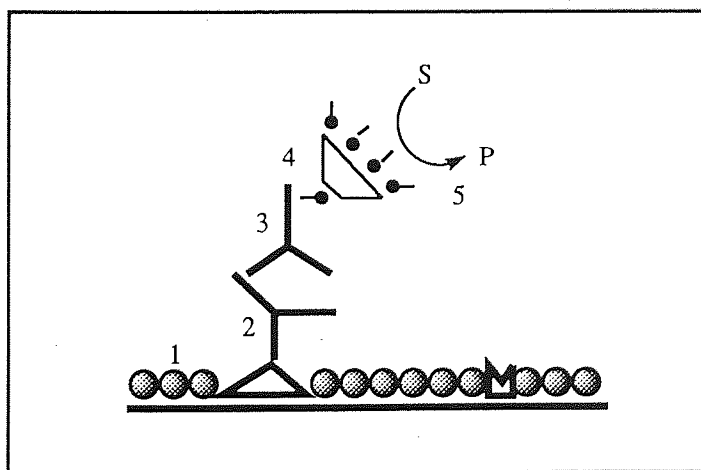

Figure 2.15 The principle of biotin-avidin staining
1) blocked unoccupied sites on the membrane,
2) biotin, 3) avidin,
4) horseradish peroxidase and
5) the colour reaction. [79].

2.2 Spectrophotofluorimetry

Spectrophotofluorimetry is a simple and direct method for the identification and quantification of proteins. It is especially a sensitive and useful technique for the analysis of the protein absorbed to hydrogel contact lenses.

2.2.1 Theoretical Aspects

When a molecule has absorbed a photon and is raised to a higher energy level, it is not in its most stable state and will attempt to return to stable state. This can be achieved by discharging its excitation energy. The molecule that has been excited to an upper vibrational level in the first excited state will very rapidly reach the lowest vibrational level of that state. At that point, several things can happen to the remaining electronic excitation energy. If upper vibrational levels of the electronic ground state are near the $\upsilon = 0$ level of the excited state, weak collisions can remove energy stepwise and the strong collisions reduce the molecule to the ground state in a single step. In the absence of such strong collisions, the molecule may remain excited long enough to emit a proton. This emitted light is called fluorescence. The emitted photons have a range of energies, because a photon may represent a transition from the $\upsilon = 0$ level of the excited state to a $\upsilon > 0$ level of the ground state. Figure 2.16 shows this process schematically. The lengths of the arrows in the figure are proportional to the energy change; it is clear that the range of energy of the fluorescent photons is less than that of the absorbed photons, that is the wavelengths of the flourescence band are longer than those of the absorption band.

Luminescence is the emission of photons from electronically exited states. Luminescence is divided into two types, fluorescence and phosphorescence. In phosphorescence, the emission is from an exited triplet state to a ground singlet state. Since this transition is forbidden the rate of return to the ground state is slow, which means the decay times are long (msec to sec). Fluorescence is the emission from excited singlet states, also yielding a ground singlet state. These are allowed transitions and occur rapidly with rates near 10^{-8} sec or 10 nsec. Stokes (1852) realised [80] that when fluorescence occurs, absorption of light at one wavelength is followed by the emission at a longer wavelength. The decay times for fluorescence are typically near 10^{-8} sec or in the range of a nsec.

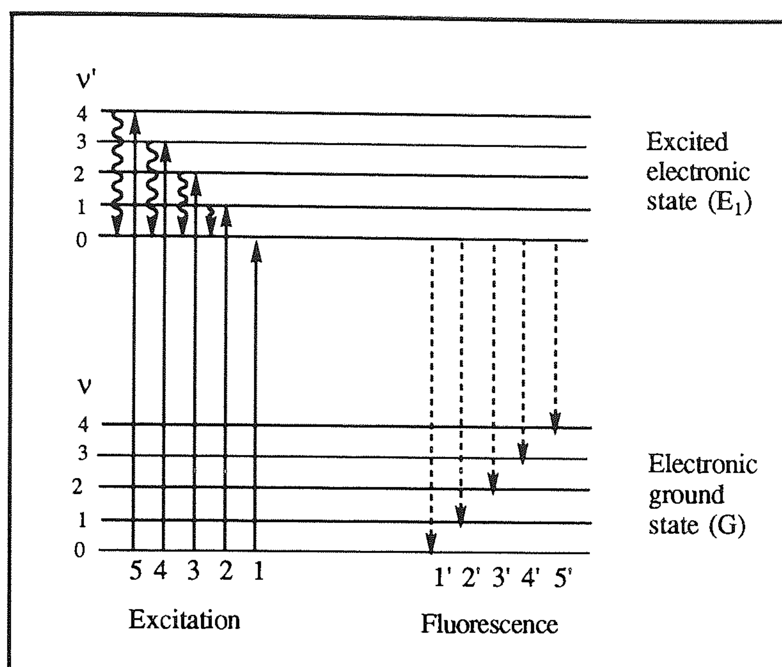

Figure 2.15 Energy level diagram showing the absorption of light (solid upward arrows) from the ground state G to the first excited state E_1. The vertical wavy lines represent vibrational non-radiative losses. The dashed downward arrows represent fluorescence transition.

Substances which display fluorescence are generally delocalized aromatic systems with or without polar substitutes. Some typical molecules which exhibit fluorescence are shown in Figure 2.17.

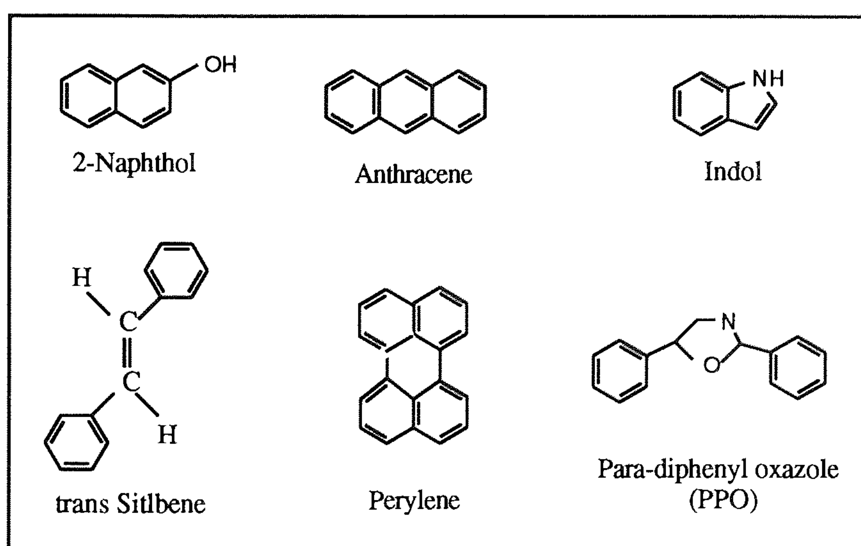

Figure 2.17 Typical fluorescent molecules [81].

Biological systems contain a variety of intrinsic (natural) fluorophores. The intrinsic fluorescence of most of the proteins is due to the presence of the fluorescent amino acids, tryptophan (Ex. 280 nm, Em. 345 nm), tyrosine (Ex. 280 nm, Em. 308 nm) and

phenylalanine. However, tryptophan is the most highly fluorescent amino acid accounting for 90% of the emission from most proteins. Emission from tyrosine is particularly observed in proteins without tryptophan, in denatured proteins, or in those with a high ratio of tyrosine to tryptophan. Tyrosine is highly fluorescent in solution, but its emission is often quenched in native proteins due to the quenching effects of hydrogen bonding to the hydroxyl group or because of energy transfer from tyrosine to tryptophan. The emission of phenylalanine from proteins has been studied less. Some proteins may also contain a fluorescent coenzyme such as reduced nicotinamide-adenine dinucleotide (NADH), flavine-adenine dinucleotide (FAD), or prydoxal phosphate (Figure 2.18). In both FAD and NADH the amount of fluorescence depends upon their local environment. For instance, the emission of NADH is usually increased about three-fold upon binding to proteins, whereas the emission of FAD is usually quenched.

It is possible to add extrinsic fluorophore labels to the substances which do not contain an appropriate natural fluorophore. For example dansyl chloride (DNS-Cl) and aniline naphthalene sulfonate (ANS) are among the earliest chemicals used as probes (Figure 2.19). Dansyl chloride can be covalantly attached to macromolecules by reaction with amino groups. ANS often binds spontaneously but non-covalently to proteins and membranes, probably by hydrophobic and electrostatic interactions. The emission of both molecules is sensitive to the polarity of the surrounding environment. ANS is nearly non-fluorescent in water, but fluorescenes strongly upon association with serum albumin, immunoglobulins and other proteins.

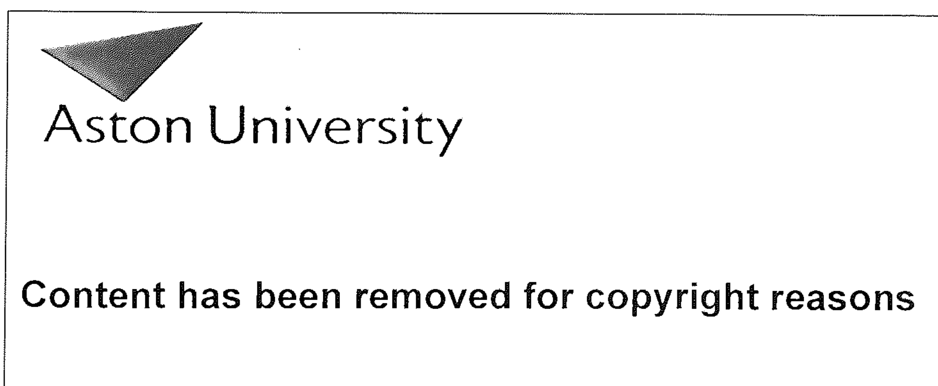
Aston University

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Figure 2.18 Intrinsic biological fluorophores (in the case of NADH and FAD, only the fluorescent part of the molecule is shown [81].

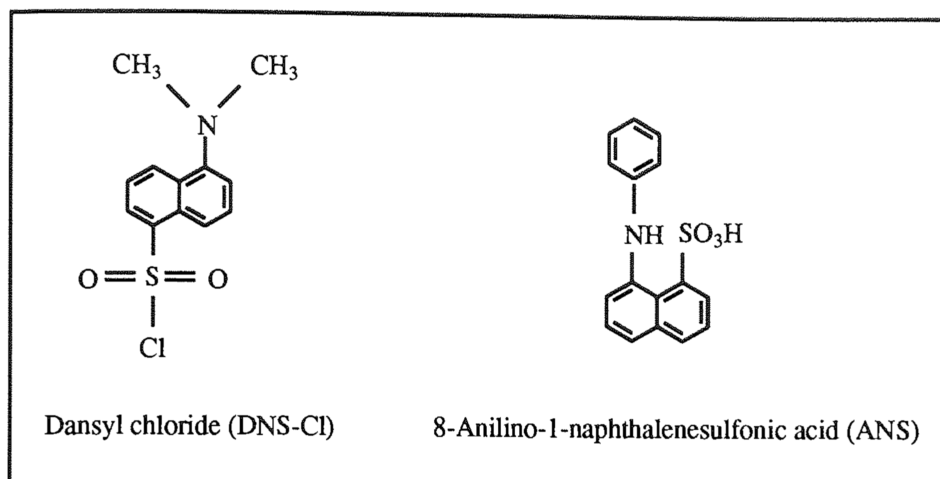


Figure 2.19 Typical extrinsic fluorophores used to label macromolecules.

2.2.2 Aminco-Bowman Spectrophotofluorimeter

The Aminco-Bowman (SPF-125) spectrofluorimeter was used to obtain the excitation spectra of some standards solutions and worn lenses. The instrument is shown in Figuer 2.20. The spectra were then used to study the effect of protein extraction, the effect of care system, penetration of the protein in lens matrix and many other factors related to protein deposition on the lenses. The instrument was checked every week with standard cells. The excitation spectra were taken at 280 nm emission. The spectra were recorded at 0.1 on % full scale. The shutter was open during the run, but was always closed each time the sample was changed. The power was set on HV position during the run and it was turned back to the off position when the run was completed. The lens was placed at the base of the cuvette so that it was faced to the direction of the light. The fluorescence spectra were recorded from 200 to 800 nm in all cases. The application of the fluorescent spectroscopy in this research will be discussed in a later chapter. Figure 2.21 shows the fluorescence spectra of a high water content ionic contact lens (Acuvue™) spoiled for short periods in 1.0 ml of 0.5 mg/ml lysozyme solution. Some typical spectra obtained using this fluorimeter for detection of different proteins are represented in Appendix 2.

2.2.3 Hitachi Fluorescence Spectrophotometer

A newer and more advanced type of spectrophotofluorometer (Hitachi, model F-4500) was used to compare the results obtained from the Aminco-Bawman (SPF-125) spectrofluorimeter. The lay out of the main unit and the data processing unit are shown in Figure 2.22. This was a newly purchased instrument which had more sensitivity and a wider range of application.

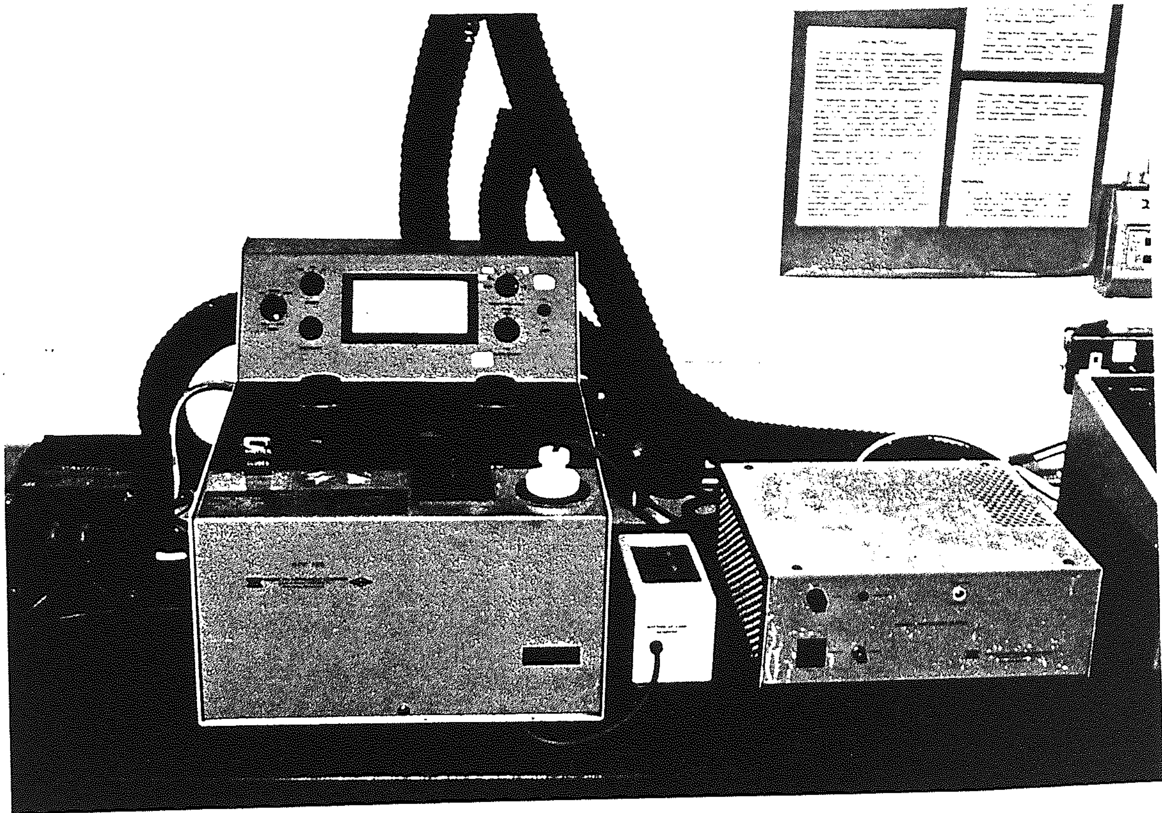


Figure 2.20 Aminco-Bowman fluorimter.

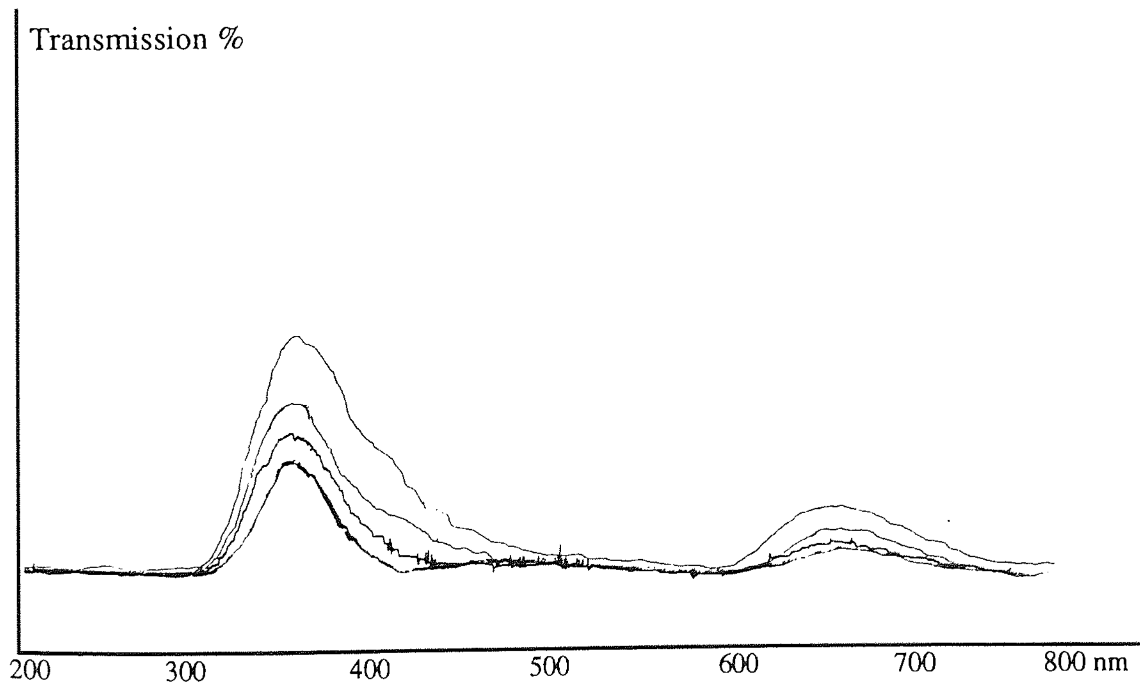


Figure 2.21 The fluorescence spectra of Acuvue™ soaked for 5, 10 ,15 and 20 minutes in the standard lysozyme solutions.

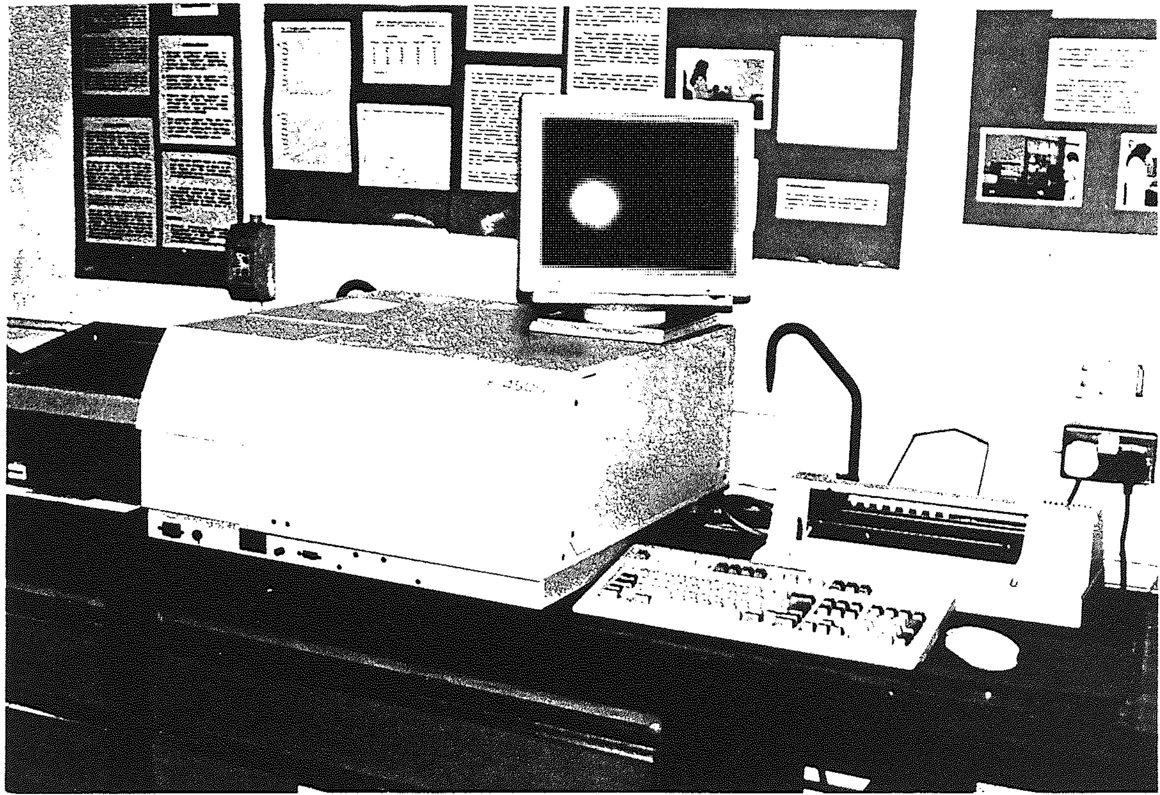


Figure 2.22 Hitachi F-4500 fluorescence spectrophotometer.

The instrument was set up to study the proteins absorbed on the surface of the lens without the need of their extraction. A series of standard lysozyme solutions were measured by Hitachi F-4500 spectrophotofluorometer and their flurorescence was measured at 360 nm exitation. The fluorescence spectra obtained for lysozyme standard solutions taken by Hitachi spectrophotoflurimeter are shown in Figure 2.23. A calibration curve was obtained by plotting the concentrations of lysozyme standard solutions against the peak higth in spectrphotoflurogram (Figure 2.24). Some other spectra of different proteins extracted from the hydrogel contact lenses are included in Appendix II.

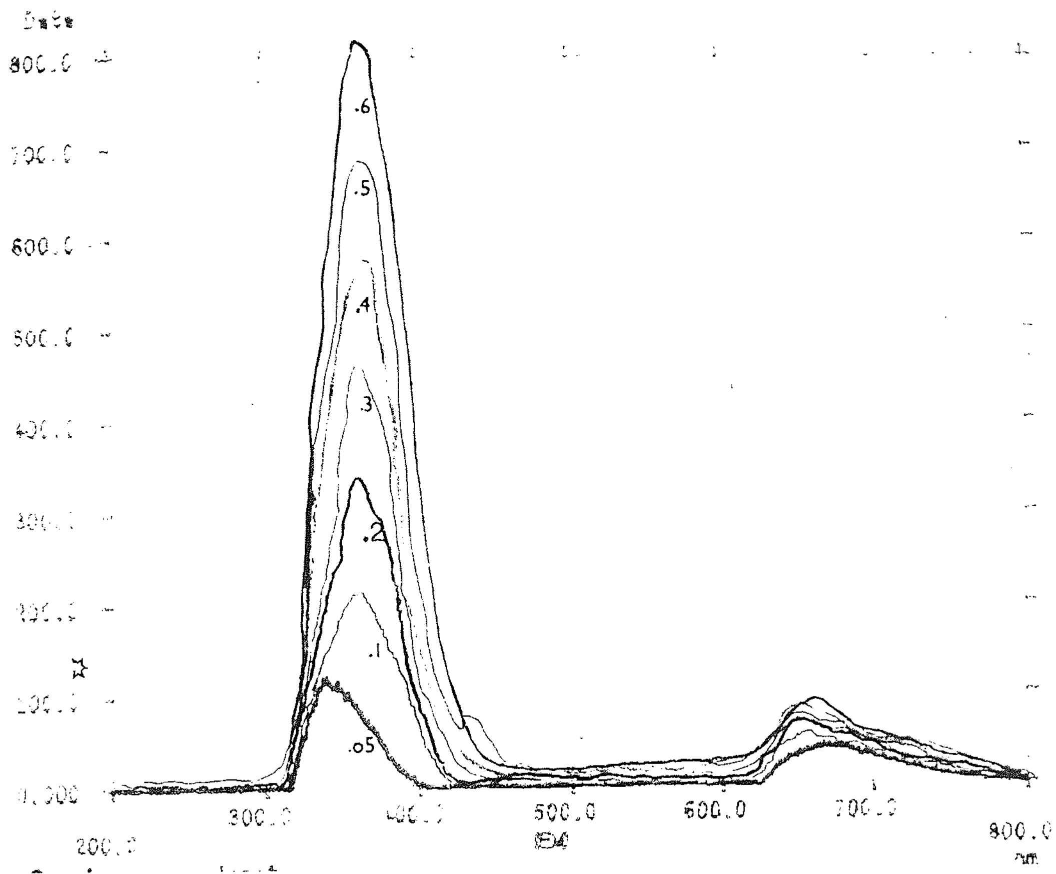


Figure 2.23 The fluorescence spectra of lysozyme standard solutions using Hitachi fluorimeter, the numbers represent the concentrations in mg/ml.

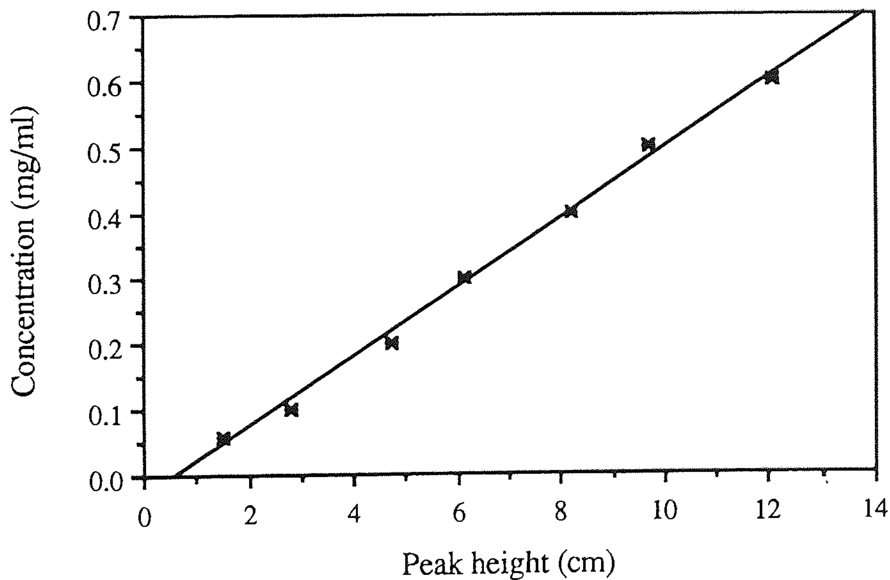


Figure 2.24 The calibration curve for the lysozyme standard solutions using Hitachi fluorescence spectrophotometer.

2.3 Ultra Violet Absorption Spectroscopy (U.V.) as a Quantitave Method

The quantity of proteins deposited on hydrogel lenses can be calculated by U.V. spectrophotometery. Nearly all proteins exhibit an absorption peak near 280 nm due to the aromatic amino acid moieties. Measurement of U.V absorbance is an interesting and direct method which allows the quantitative studies, but background absorbance of the material sometimes interfere with the reading. This can be minimized by using a blank of exactly the same lens.

A U-2000 spectrophotometer (Hitachi) was used (Figure 2.25). A pair of matched quartz 1 cm cells were selected for the sample and the reference. The contact lenses were mounted on the bottom of the cell so that they faced to the direction of the light (Figure 2.26). The deuterium lamp was turned on and the wavelength set to 280 nm. The instrument was zero adjusted using the blank. A calibration curve was produced by measuring the absorbance (A) of a series of lysozyme solutions with known concentrations C (ranging from 0.01 to 0.5 mg/ml), and plotting A against C (Figure 2.27). Similar calibration curves were also obtained for other individual and mixtures of proteins used for the spoilation studies. The calibration curves obtained for each protein and for the mixture of proteins were stored in the memory of the instrument to be used for the calculation of proteins in the unknown samples. The U.V. absorption spectrophotometry was then used in the following experiments to calculate the quantity of the protein absorbed and also to establish the activity of the lysozyme absorbed and adsorbed on and in the soft contact lenses.

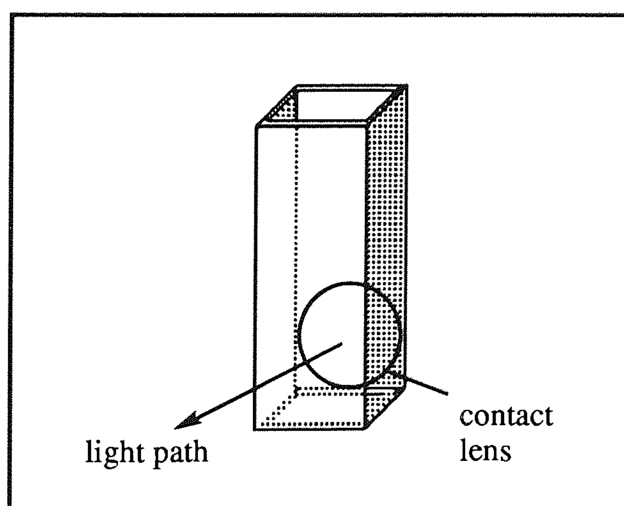


Figure 2.26 The position of the contact lens in a U. V. quartz cell.

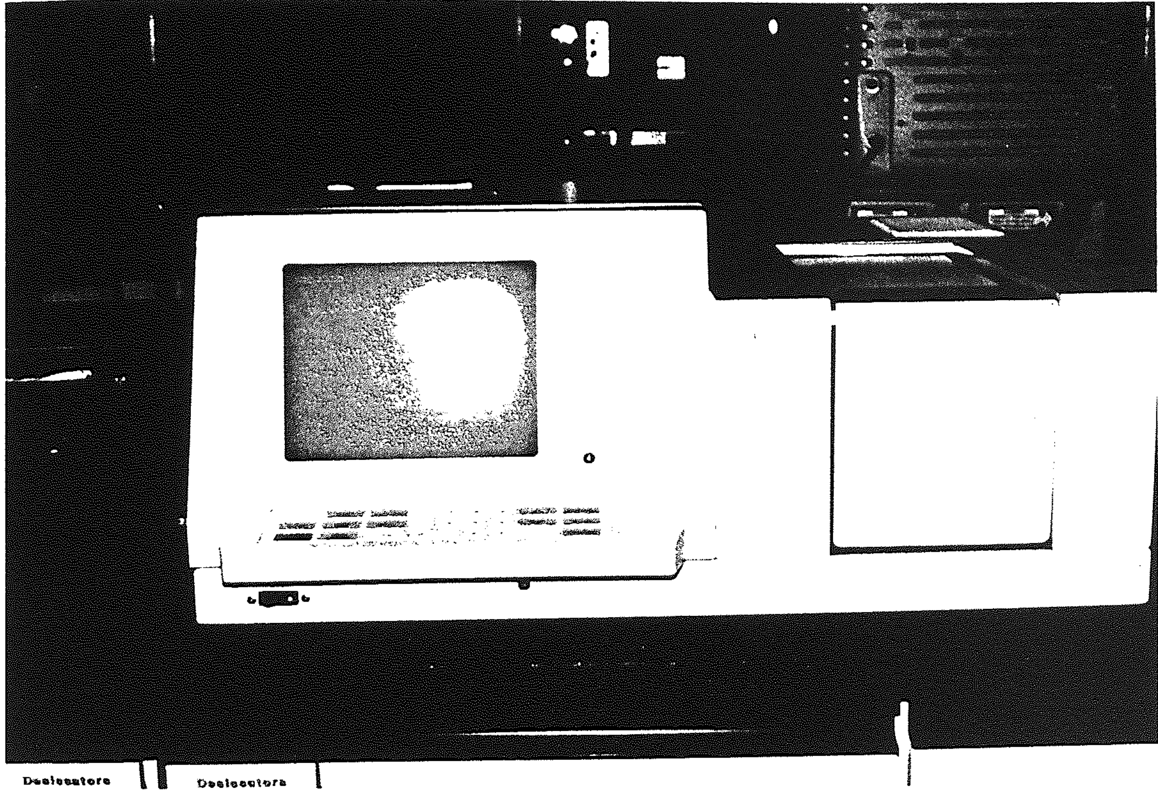


Figure 2.25 The Hitachi U.V. spectrophotometer.

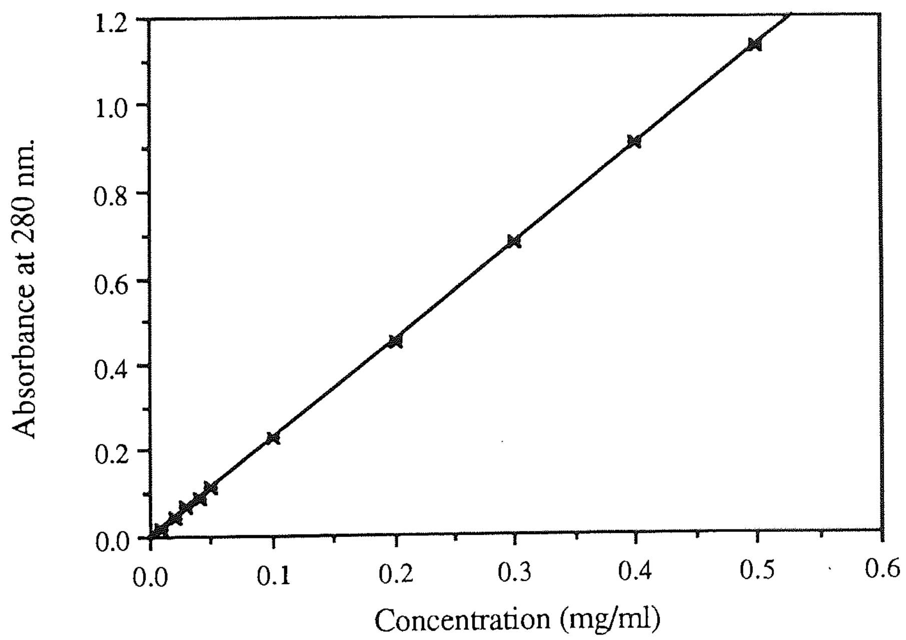


Figure 2.27 Calibration curve for the U.V. absorption of standard lysozyme solutions.

2.4 Chemical Methods

A number of chemical methods were used to measure the quantity and activity of the proteins after extraction from lenses. They were used in conjunction with the spectrophotometry.

2.4.1 Protein Extraction from Contact Lenses

To obtain the most possible extractable protein from the surface and matrices of the contact lenses, a number of different extraction solutions and extraction techniques were used. The following extraction solutions were used:

1. ReNu™ multi purpose solution
2. Saline solution
3. 3M urea solution
4. 10% SDS solution

The extraction techniques using the above solutions are summarised below:

1. Cut the contact lenses in quarters and shake them vigorously in 1ml of the extraction solution.
2. Leave the whole lens in 1ml of the extraction solution while shaking on a low speed (set on 150) vibrator (IKA-Vibrax-VXR) for 24 hours.
3. Cut the lenses into 8-10 small pieces, add 0.5ml of the extraction solvent and centrifuge for 15 minutes at a high speed.

The most effective way was the long term extraction (at least 24 hours) in ReNu™. This was the technique used in most of the experiments which neeeded the lens extracts. However, it was known that most of the solutions and techniques were only able to extract 50-65% of the total absorbed protein. A comparison between the various techniques and different extraction solutions are made in Figure 2.28.

2.4.2 Measurement the Activity of Lysozyme

The following technique, which is based on the decrease in turbidity (A_{580}) following lysis of a suspension of *Micrococcus lysodeikticus* (this is the substrate for enzymatic action of lysozyme) [82].

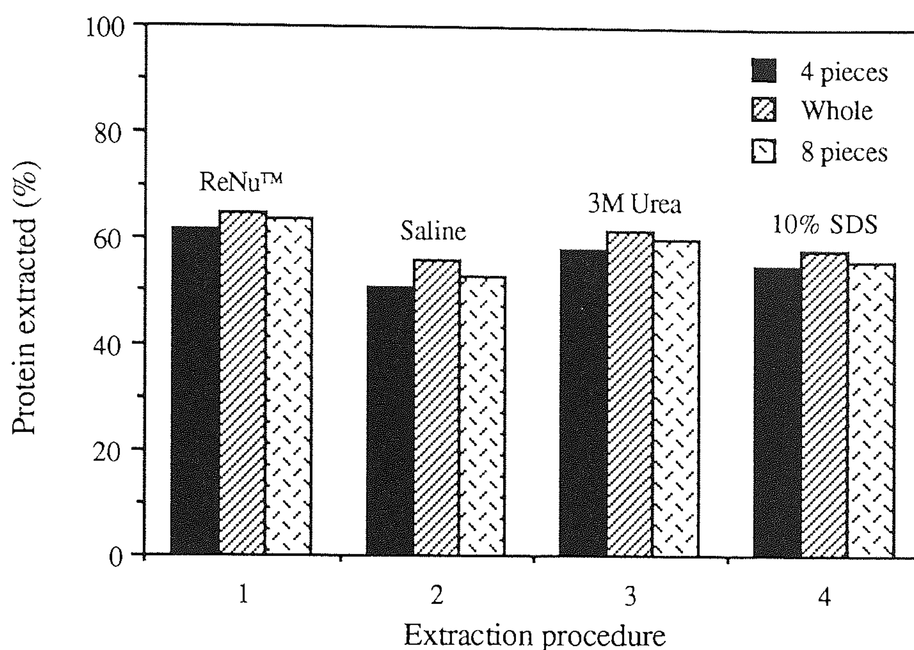


Figure 2.28 The efficiency of the extraction techniques.

2.4.2.1 Materials and solutions

1. Phosphate buffer (pH 6.2, M/15)
   9.08 grams of anhydrous potassium dihydrogen phosphate (BDH, 29608) was dissolved in 1000 ml of distilled water (solution "a"). 9.47 grams of anhydrous disodium hydrogen phosphate (BDH, 10248) was dissolved in 1000 ml of distilled water (solution "b"). 815 ml of solution "a" was mixed with 185 ml of solution "b", and the pH was checked with a pH meter.
2. Buffered substrate
   15 mg of dry *Micrococcus lysodeikticus* (Sigma, M-3770) was dissolved in 10 ml of saline solution (0.9 % NaCl) and diluted to 100 ml with phosphate buffer. The solution was aged overnight at room temperature.
3. Lysozyme standard solution (0.4 mg/ml)
   20 mg of lysozyme was dissolved in 50 ml of 0.9% NaCl solution.

2.4.2.2 Procedure

5.0 ml of the substrate was equilibrated at 37 °C for about five minutes in two test tubes. 0.5 ml of each standard solution and extracts were added to each appropriate tube. The U.V. absorption was recorded at 540 nm after exactly thirty seconds (first reading) and after three minutes reaction at 37 °C in the water bath (second reading).

reading) and after three minutes reaction at 37 °C in the water bath (second reading). The blank used was 0.9% NaCl and it was also equilibrated in 37 °C bath for at least five minutes. To avoid any errors, each sample was determined individually.

2.4.2.3 Calculation of lysozyme activity

The change in absorbance (ΔA) was calculated from the following formula:

ΔA = A (540 nm) at 30 seconds - A (540 nm) at 3.0 minutes

The concentration of active lysozyme in the unknown samples was measured from the U.V absorbance:

$$C = \frac{\Delta A_T}{\Delta A_S} = \times 0.40$$

Where C is the concentation of lysozyme in the unknown sample, A_T and A_S are the absorbances of the unknown and the standard lysozyme solutions respectively, and 0.4 is the concentration of the standard solution of lysozyme.

2.4.3 The Assay of Lysozyme by Radial Immunodiffusion Kit

A radial immunodiffusion kit for human lysozyme, 'NL' NANORID™ was purchased from the Binding Site, Birmingham (GT 073.3). The tear samples and extracted solutions from the contact lenses were diluted as recommended by the kit to values of about 0.01-0.017 mg/ml.

The plates were left while open at room temperature for 10 minutes to warm up before the application of the samples. Each well was checked for the presence of any moisture or dust and 10ml of samples were applied to each well very carefully to avoid contamination. The lid was closed tightly and the plate was stored flat at room temperature (not more than 22°C) for 96 hours which was the minimum recommended diffusion time for completion. After the diffusion was completed the ring diameters were measured to the nearest 0.1mm using a jewellers' eye piece (Binding Site, code: D040). The sample concentrations were then calculated directly from the calibration curve obtained by plotting the ring diameter squared against the concentrations of the standards, the accurate concentration of each sample was then calculated by taking into

account the dilutions made. A calibrator was used each time to check the accuracy of the test.

In some cases when the concentrations of lysozyme were very low, the wells were "double filled". The well was initially filled with 10ml of the sample and it was allowed about 30 minutes to diffuse into the gel with the lid kept on, then a second 10ml of the sample solution was applied into the gel. The results obtained were, of course corrected for the sample volume. The results of the "double fill" are less accurate than a "single fill". It was very difficult to get a precise photograph of the gel with the sample diffused into the gel. The ring diameter was measured for a series of standard lysozyme solution and the data (Table 2.4) obtained were used for plotting a calibration curve. The calibration curve was plotted using the ring diameter squared and the concentrations of lysozyme standard provided by the kit (Figure 2.29).

Table 2.4 The relationship between the ring diameter and lysozyme concentration.

Standard concn. (mg/ml)	Ring diameter (mm)	Diameter square (mm^2)
10	4.4	19.36
60	7.5	56.25
100	9.2	84.65

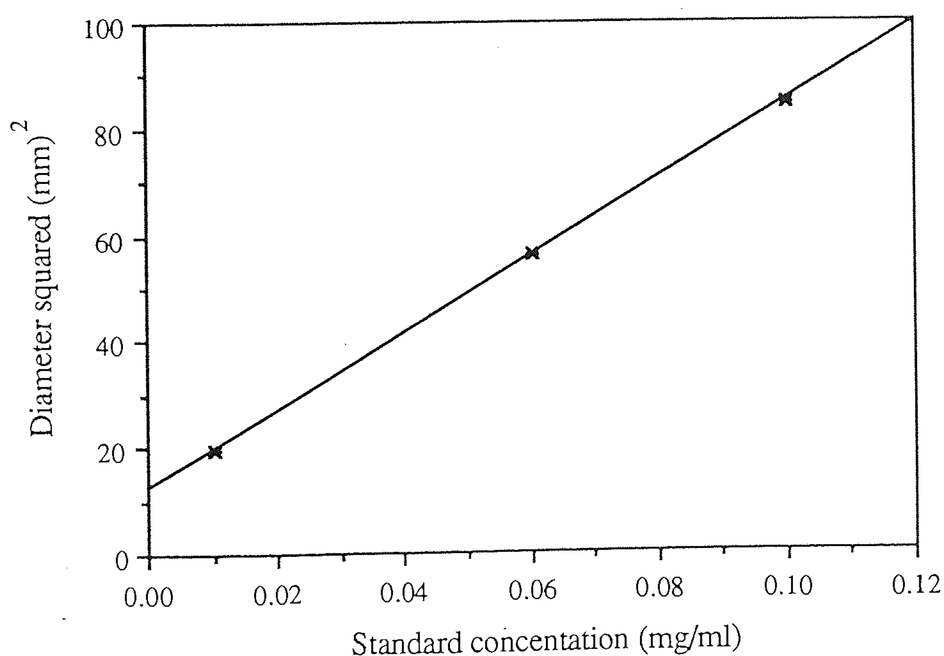


Figure 2.29 The calibration curve for the NANORID™ kit.

2.4.4 Measurement of the Activity of Ribonuclease [83]

This method was based on the enzymatic effect of ribonuclease on ribonucleic acid and it is a modification of Kunitz spectrophotometric assay.

2.4.4.1 Materials and solutions

1. Sodium acetate buffer (0.1 M, pH 4.8)
   8.2 grams of sodium acetate (Sigma, S-8750) was dissolved in distilled water and 3.37 grams of acetic acid (Fisons plc., 2789) was added, the final volume was made up to one litre and the pH was checked with pH meter.
2. Substrate solution (0.05 mg/ml)
   5.0 grams of yeast ribonucleic acid (Sigma, R-7125) was dissolved in 100 ml of sodium acetate buffer (solution 1).
3. The enzyme standard solution (10 mg/ml)
   200 mg of ribonuclease from bovine pacrease (Sigma, R-5000) was dissolved in 20 ml of 0.1 M sodium acetate buffer (pH 5.0). Diluted solutions of 5, 2.5, 1.2 and 0.6 mg/ml were then prepared by mixing the appropriate volumes of the above solution with buffer.

2.4.4.2 Procedure

Equal volumes of each standard solution were mixed with substrate solution and the absorbances at 275 nm was measured immediately (A_0) and after 5 minutes (A_T) at room temperature (20 ± 2° C). The differences in the absorption during the time (ΔA, which is $A_0 - A_T$) were plotted against the concentrations (activities) of ribonuclease standards. The standard line (Figure 2.30) was then used to calculate the activity of unknown solutions of ribonuclease which were treated in the same way as the standards.

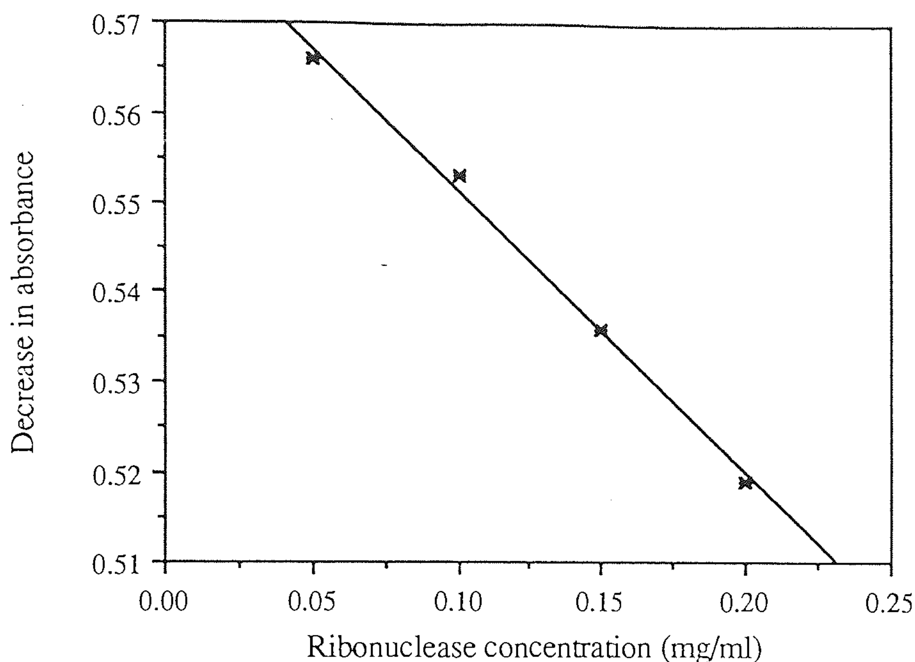


Figure 2.30 The calibration curve for the activity of standard ribonuclease solutions.

2.4.5 Primidine Cyclic Phosphate Hydrolase Assay

This method was a modification of the procedures carried out previously in the references [84] and [85]. A solution of sodium salt of cytidine 2': 3'-cyclic phosphate is freshly prepared and is reacted with the enzyme solution.

2.4.5.1 Materials and solutions:

1. Substrate solution (1mg/ml)
   30 mg of sodium salt of cytidine 2':3'-cyclic phosphate (Sigma, C-9639) was dissolved in 30ml of 3.0 M NaCl solution with pH 7.0. This substrate solution was always made freshly as the cyclic phosphate was unstable.
2. Enzyme standard solutions
   0.2 mg of the pure bovine ribonuclease (Sigma, R-5000) was dissolved in 100 ml of distilled water (2 μg/ml), other dilutions of the standard solution were then made from this solution (1.0 and 0.5 μg/ml).

2.4.5.2 Procedure

5.0 ml of the substrate solution was placed in three small beakers and 0.5 ml of each solution containing enzyme standards were mixed with it while stirring with a magnetic stirrer. The mixtures were stirred with magnetic stirrer at room temperature for 10

minutes to allow the enzyme to act on the substrate. The pH of the solutions were then measured and the volume of the 0.02 N sodium hydroxide solution needed to adjust the pH to 7.0 was recorded for all standard solutions. The volume of sodium hydroxide was plotted against the concentration of the standard solutions and the straight line (Figure 2.31) obtained was used to measure the concentraion of any ribonuclease present in the unknown samples.

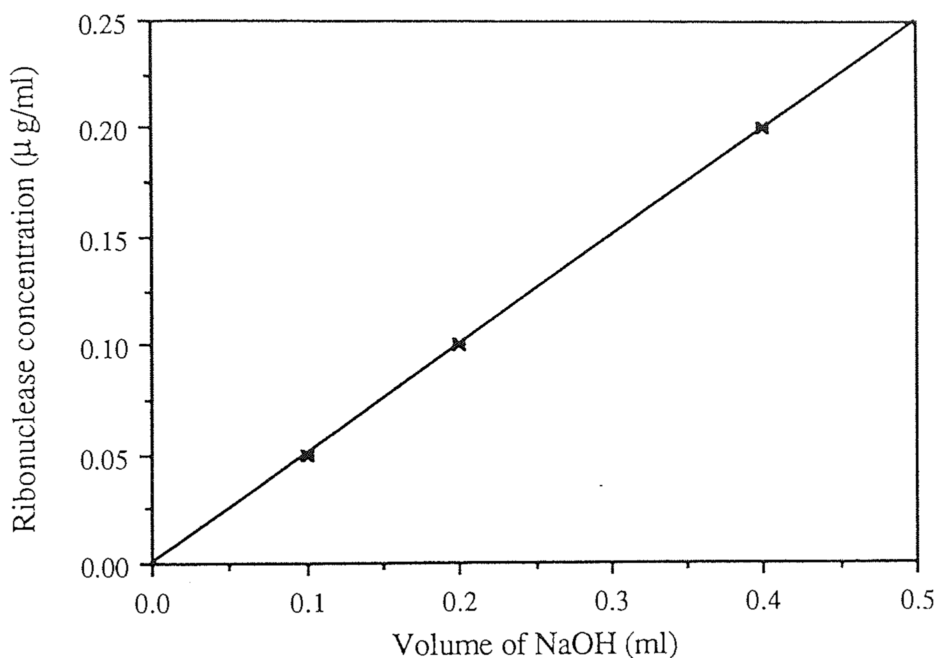


Figure 2.31 The standard curve obtained by hydrolyse assay.

2.4.6 Quantification of Lactoferrin using Lactoplate®

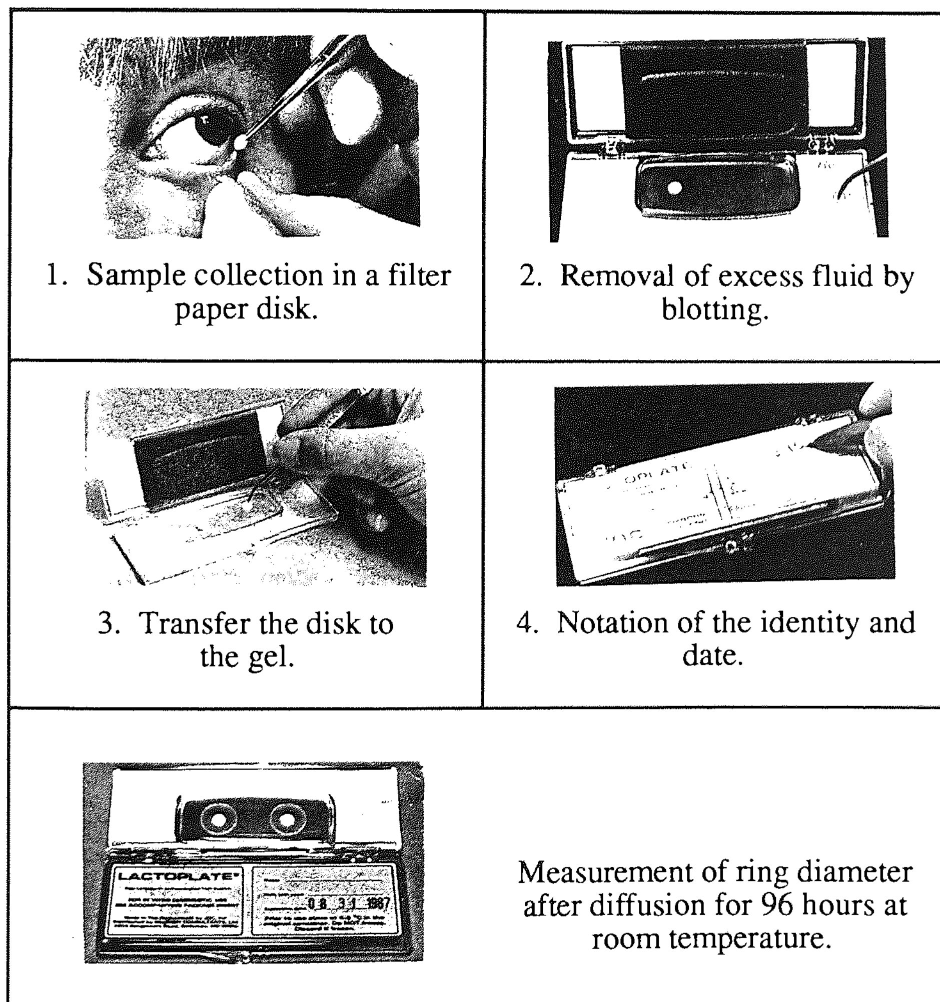
2.4.6.1 Introduction

The concentration of lactoferrin in the extraction solutions from the contact lenses are very low and can not be detected by electrophoresis or other quantitative techniques. The Lactoplate® kit is essentially devised for the semi-quantitative immunoassay of lactoferrin as an *in-vitro* diagnostic of the lacrimal gland function [86]. It is based on the biological action of lactoferrin on the substrate which is contained in the gel matrix provided by the kit.

2.4.6.2 Procedure

The Lactoplate® Immunoassay Test kit (JDC Inc. Culenborg, Netherlands, Lot No./Serial No: 89/17) was purchased directly from the company and used as recommended by the manufacturer.

The filter paper provided by the kit was well soaked into the solution under investigation, the excess fluid was removed by blotting and was carefully placed to the reagent gel. The gel was transferred to its closed container and left at room temperature for three days. The sample diffused into the gel during this period reacting with the reagent and leaving an opaque ring whose size was related to the concentration of the lactoferrin in the sample. The ring diameter was measured after three days and, using Table 2.5, the concentration of lactoferrin was calculated. A freshly prepared standard lactoferrin solution was used with each sample to check the accuracy of the test. Figure 2.32 demonstrates the summary of this procedure.



1. Sample collection in a filter paper disk.

2. Removal of excess fluid by blotting.

3. Transfer the disk to the gel.

4. Notation of the identity and date.

Measurement of ring diameter after diffusion for 96 hours at room temperature.

Figure 2.32 Summary of Lactoplate® procedure.

Using the following standard table, the concentrations of the lactoferrin in the unknown solutions were calculated by the following formula:

$$C = 0.0105 \times D^2$$

where C was the concentration in mg/ml and D the diameter in mm.

Table 2.5 The relationship between the ring diameter and lactoferrin concentration.

Ring diameter (mm)	Concentration (mg/ml)
5.0	0.26
5.5	0.32
6.0	0.38
6.5	0.44
7.0	0.51
7.5	0.59
8.0	0.67
8.5	0.76
9.0	0.85
9.5	0.95
10.0	1.05
10.5	1.16
11.0	1.27
11.5	1.39
12.0	1.50
12.5	1.60
13.0	1.80
13.5	1.90
14.0	2.10
14.5	2.20
15.0	2.40
15.5	2.50
16.0	2.70
16.5	2.90

2.4.7 Determination of Tear Immunoglobulin by RID™

An RID kit for immunoglobulins was purchased from Sigma, and the concentration of immunoglobulins in tears and in the ReNu™ solutions extracted from the *in-vivo* spoiled lenses were measured by the use of this kit. The procedure is very straight forward and was followed according to the route given by the kit.

The plate was placed at room temperature uncovered before the sample application and the presence of any moisture in the wells was also detected. A 10 microlitre sample was applied to each well, the lid was closed and was incubated at room temperature for exactly 18 hours. The diameter of the ring formed was again proportional to the concentration of the immunoglobulin in the sample. A series of standards provided by the kit were used to obtain a calibration straight line and to frequently check the accuracy of the results.

The relationship between the ring diameter and the immunoglobulin concentration is shown in Figure 2.33. The standard curve was used to calculate concentration of the protein in unknown samples.

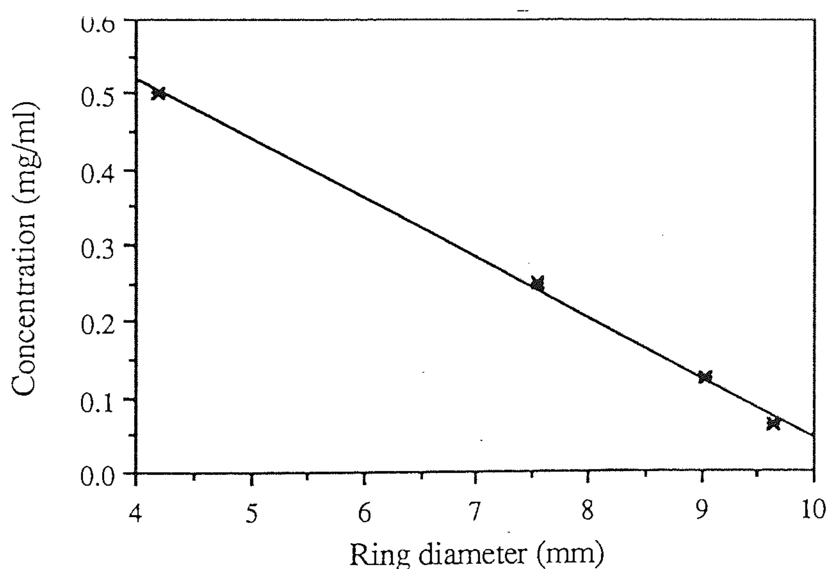


Figure 2.33 The relationship between the ring diameter and immunoglobulin concentration.

2.4.8 Detection of Proteins by Chemical Reagents

One of the most widely used reagents that will detect most amino acids and peptides is ninhydrin. It reacts with the amino groups that are almost invariably present. The reaction is complex, but a major component appears to be first the oxidation

deamination of the amino acid to CO_2, NH_3, an aldehyde containing one less carbon atom than the original amino acid and hydrindantin which the reduced form of ninhydrin (Figure2.34). The hydrindantin then reacts with liberated NH_3 to give the purple product which is intensely coloured with a maximum absorbance at 570 nm. The ninhydrin method is not very specific and is most useful with purified components. The method was used for the detection of some hydrolysed standard proteins, but due to the low sensitivity it could not be used for the determination of tear proteins and those extracted from the contact lenses.

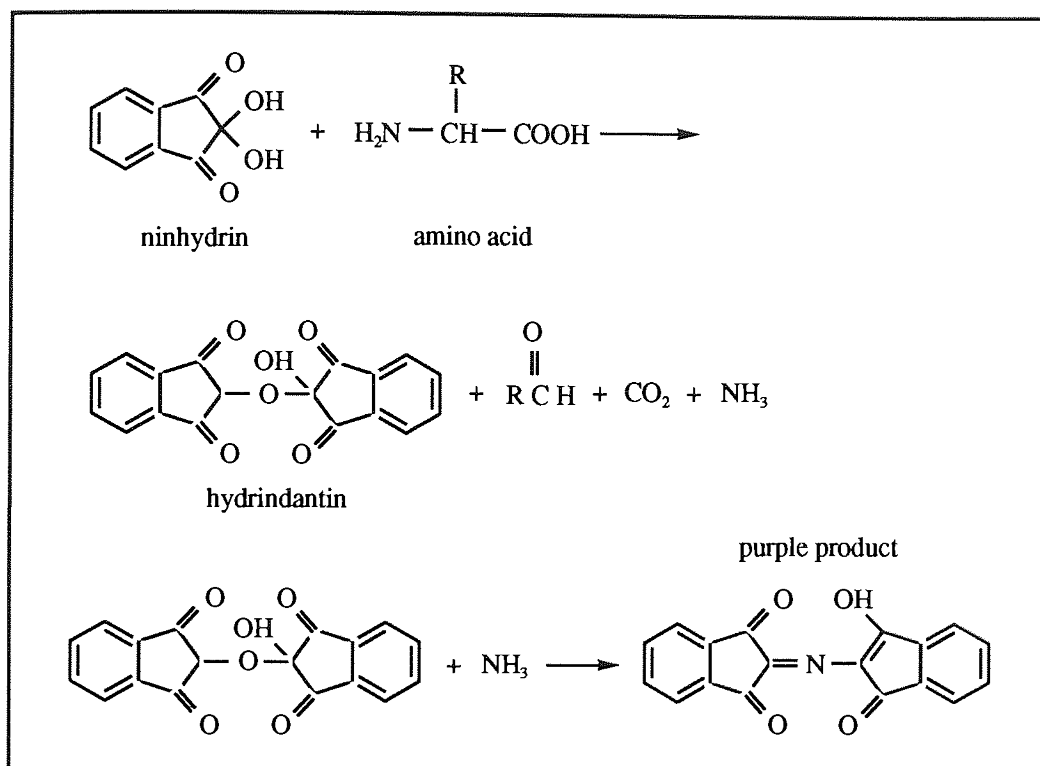


Figure 2.34 The chemical reaction of ninhydrin with amino acids.

The most widely used method of specifically detecting and measuring quantitatively proteins in solution is the Folin phenol method of Lowry. The active contituents of the Folin phenol reagent are the phosphomolibdic-tungstic mixed acids (Figure 2.35).

Proteins reduce the mixed acid causing the loss of one, two or three oxygen atoms from the tungstate and molybdates, to produce a number of reduced species with characteristic blue colour (λ_{max} = 750 nm). Copper ions are generally included, as they facilate the reduction process. The greatest disadvantage of this assay procedure has been the variety of non-protein substances that interfere, either producing the blue colour by themselves or interfering with colour development by proteins.

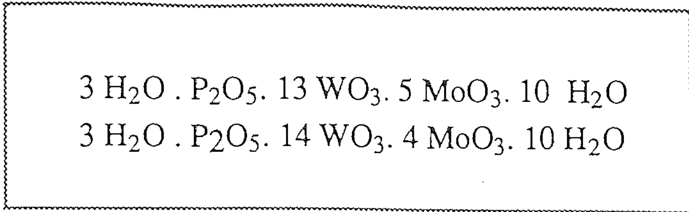

$3\ H_2O\ .\ P_2O_5 .\ 13\ WO_3 .\ 5\ MoO_3 .\ 10\ H_2O$
$3\ H_2O\ .\ P_2O_5 .\ 14\ WO_3 .\ 4\ MoO_3 .\ 10\ H_2O$

Figure 2.35 The active constituents of Folin phenol reagents.

A more specific but less sensitive method of quantitatively assaying proteins is Biuret reaction. A dilute solution of cupric sulphate in strongly alkaline tartrate is added to the protein solution. A purplish-violet colour is formed, with a maximum absorbance at 540 nm. The nature of the coloured compound is uncertain, but the colour is probably due to the formation of tetra-coordiated cupric ion complex with adjacent peptide groups (Figure 2.36).

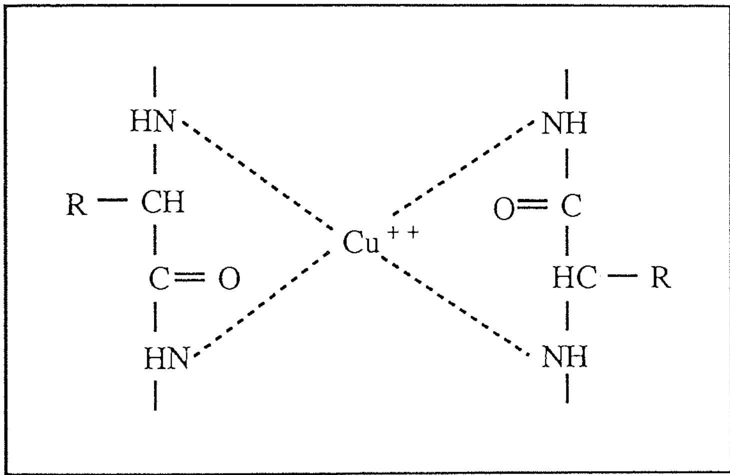


Figure 2.36 The coloured complex formed in Biuret reaction.

2.4.9 Microprotein Determination

The Buiret [87] and Lowry [88] procedures have been used for the determination of proteins for clinical assays from a long time ago. However, the more sensitive Lowry method, has the disadvantage of poor stability of combined reagents, non-reproducibility of the colour especially at low protein concentrations and non-linear chromogenic response with protein concentration. The modification of these procedures include the use of Biuret reagents for Lowry method [89], which is a simpler and more reliable method.

The method used in this study, a modification of reference [89] was followed. In this procedure, the Biuret reagent reacts first with proteins followed by phenol reagent. The colour is developed after about half an hour and the absorbance is read at 700 nm.

2.4.9.1 Materials and solutions

A micro protein determination kit was purchased from Sigma (catalogue No. 690-A) which contained Biuret reagent (active reagent cupric sulphate), phenol reagent (2.0 N)and protein standard solution (albumin 100 mg/ml). A solution of sodium chloride (0.85%) was also prepared.

2.4.9.2 Procedure

The test samples were diluted with sodium chloride solution so that the final protein concentration was between 0.015 and 0.1 mg/ml. 0.2 ml of the diluted samples were added to 1.2 ml Biuret reagent (Cat. No. 690-1), mixed and allowed to stand at room temperature for 10 minutes. Then 0.1 ml of Folin and Ciocalteu's phenol reagent (Cat. No. 690-2) was added to the tube, mixed well and left at room temperature for 30 minutes. The blank was also prepared in the same way using sodium chloride instead of sample. Standard protein solutions were made by diluting the standards provided by the kit (Cat. No. 690-10) to give concentrations between 0.025 to 0.1 mg/ml. The calibration curve (Figure 2.37) was obtained by plotting the total protein (mg/ml) against the absorption at 700 nm.

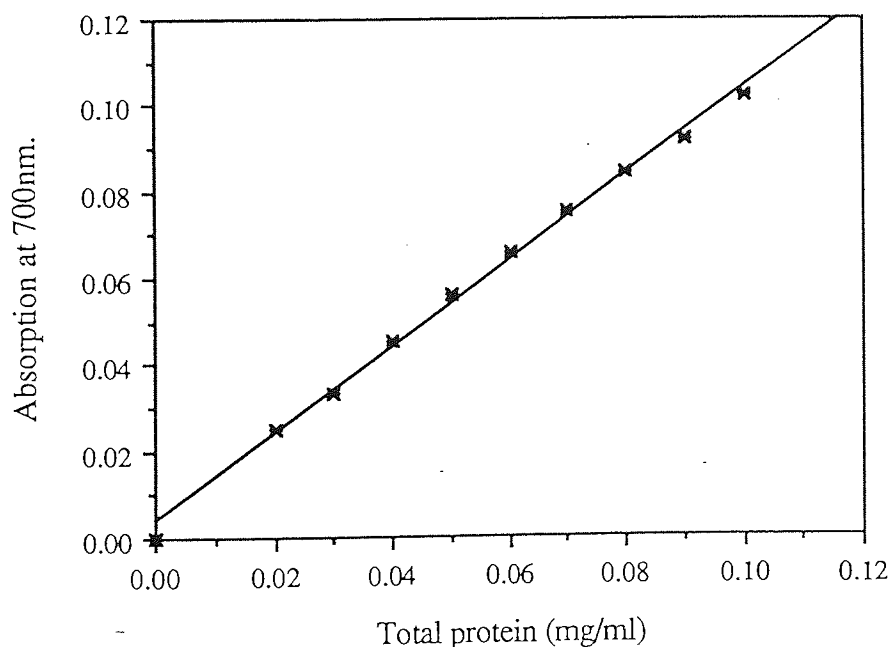


Figure 2.37 The calibration curve obtained by micro-protein determination.

Chapter 3

Development of a Novel Analytical Technique for Tear Protein Analysis

Chapter 3

Development of a Novel Analytical Technique for Tear Protein Analysis

3.1 Introduction

The use of isotachophoresis (ITP), the latest of the electrophoretic separation techniques, has grown very rapidly during the last decades in various application areas. It is characterised by small sample requirement, in the picomole range, short analysis time of about 10 to 30 minutes, and the ease of quantification. One difficulty with the method is the visualisation of protein resolution from U.V. signals. Although a wide range of ampholytes can be used for spacing the cation and anion mixture, use of these spacer results in sample/spacer mixing, reduced sensitivity and broadened zone profiles. Isotachophoresis has been established in many fields as a separation method for small molecules and also high molecular weight biopolymers, such as proteins and nucleic acids. The potential of isotachophoresis separation of proteins in an electrolyte solution has been demonstrated [90].

3.2 Historical Background [91]

The first laws which govern isotachophoresis separation were laid down in 1897 by a German chemist Kohlrausch. However, it was not until 1923 that anyone made use of the basic principle by Kohlrausch for the separation of ions. In 1923, Kendel described the separation of some ions by isotachophoresis. In 1953, Longworth published a paper emphasising the importance of isotachophoresis for the separation of a mixture of metal ions. During 1960s, Kendall's so-called ionic migration technique received full attention. In 1962, Konstantinov *et al* developed a "moving boundary method" for the microanalysis of metal ions. In 1963, Martin started to work systematically with the isotachophoresis techniques from both the theoretical and practical points of view. In 1964, Ornstein and Davis applied the Kohlrausch function to the separation of proteins. Up to 1970 several names had been used for what Kendall initially called the ionic migration technique: these included moving boundary method, displacement electrophoresis, steady state stacking cone electrophoresis and ionophoresis.

In 1970 Hayland, together with a group of researchers in this field, introduced a name based upon an important phenomenon of electrophoretic technique, namely the identical velocities of the sample zones at equilibrium, isotachophoresis Greek: *iso*, equal; *tacho*,

speed) or isotachophoresis for short. The name isotachophoresis was readily accepted and has since been the acknowledged domination.

3.3 Basic Theory of Isotachophoresis

Isotachophoresis is an electrophoretic separation technique, in which ion species of the same sign migrate when an electric field is applied across an electrolyte system comprising of a sample solution, a leading electrolyte, and a terminating electrolyte. The leading electrolyte must contain only one ion species, *the leading ion*, having the same sign as the sample ions to be separated, and a mobility higher than that of any of the sample ions. The terminating electrolyte, on the other hand, must contain only *the terminating ion* which has the same sign as the sample ions and a mobility lower than any other ion species present in the system. The sample solution which contains the sample ions to be separated, must be introduced between the leading and the terminating electrolytes.

The polarity of the electric field must be such that the leading ion migrates in front of the sample ions, as is shown in Figure 3.1.

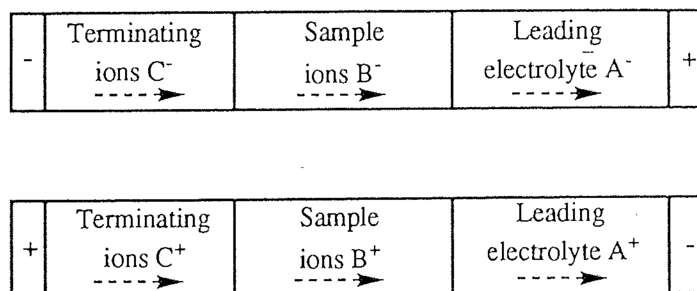


Figure 3.1 The direction of ions in isotachophoresis.

As shown in Figure 3.1, isotachophoresis can be used to separate positively and negatively charged ions. When the system has reached equilibrium, all the ions move with the same speed, individually separated into a number of consecutive zones in immediate contact with each other, and arranged in order of effective mobility.

The basic theory of isotachophoresis was initially described by Kohlrasch [92]. Consider a system of two electrolytes in contact with each other with a common counter ion R^+. Suppose the migration takes place in a narrow tube as shown in Figure 3.2.

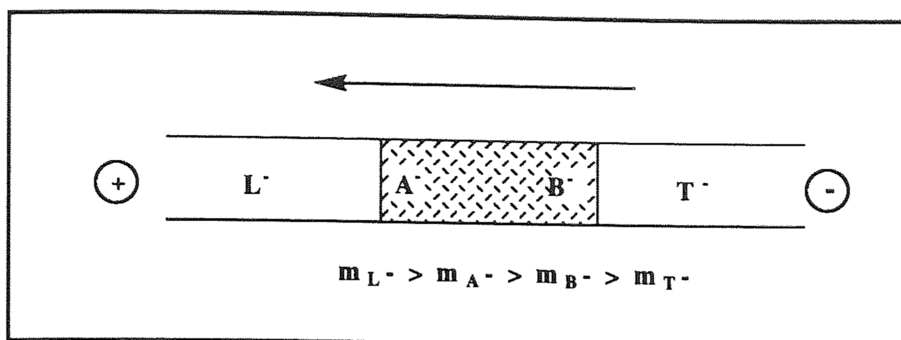


Figure 3.2 The starting conditions for the separation of A^- and B^- which are introduced between the leading and terminating electrolytes (containing L^- and T^-).

The effective mobilities (m) of the participating ions are selected so that $m_L - > m_A - > m_B - > m_T -$, (the counter ion R^+ is not included in Figure 3.2). The mobility is defined as a migration velocity (cm/s) in a unit electric field (V/cm) and has dimension of (cm^2/Volt.sec).

When a constant current is fed through the system, the migration velocity of the ions will be determined by the mobility of the A^- and B^- ions and the strength of the electric field. The ions with the highest mobility (A^-) will move ahead of the B^- ions, which have lower mobility and therefore, in the same electric field, a lower velocity.

However, since the current is constant, the B^- ions are forced to follow immediately behind the A^- ions. Therefore, the electric field strength must be increased in the B^- zone to compensate for the lower mobility of B^- ions (Figure 3.3).

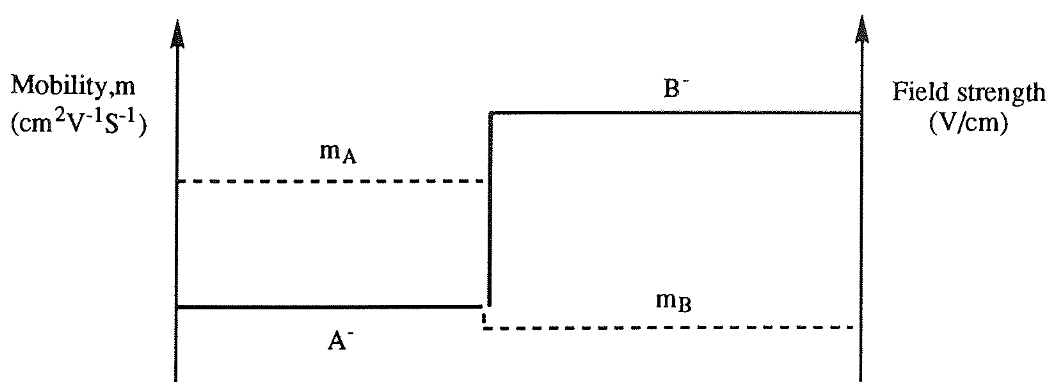


Figure 3.3 The field strength varies inversely with the mobility.

The concentration in the B^- zone will adjust relative to the concentration of A^- ions to give the steady state condition with identical velocity for the ions. This is the principle from which the name isotachophoresis (equal velocity) has been derived. Therefore, the ion

concentration in each zone is constant, and the amount of ions in each zone can only be changed by proportionally changing the length of the zone. Thus, the zone length is directly proportional to amount of ions in it. This is illustrated in Figure 3.4.

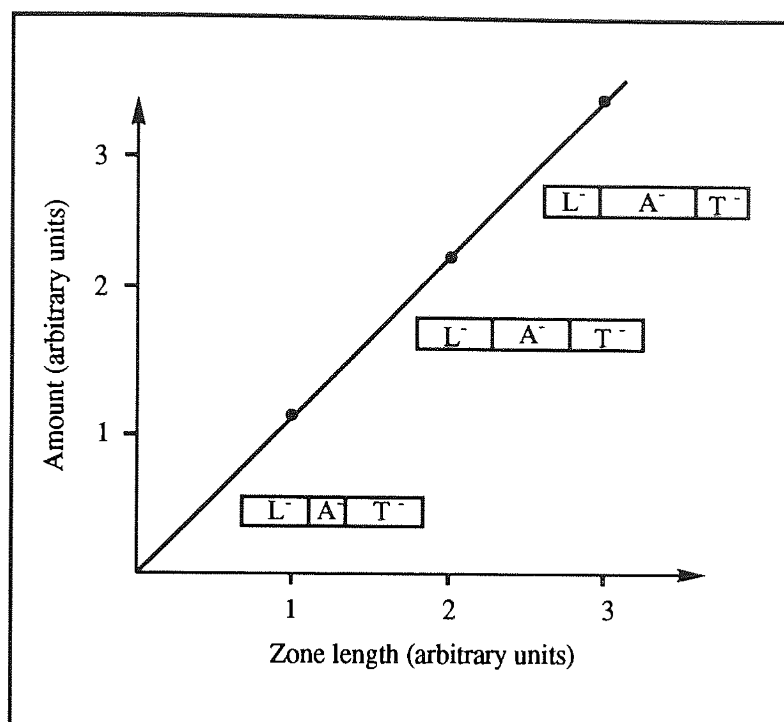


Figure 3.4 A unique feature of isotachophoresis, the amount of ion A^- is directly proportional to the zone length.

The ratio between the concentrations C_A and C_B of ions A^- and B^- is given by Kohlrausch equation:

$$\frac{C_A}{C_B} = \frac{m_{A^-}}{m_{A^-}+m_{R^+}} \times \frac{m_{B^-}+m_{R^+}}{m_{B^-}}$$

If a third electrolyte RC is added to the system, so that the mobility of C^- is between mobilities of A^- and B^-, the above equation can be applied to C^-, i.e., the concentration of C^- will adapt to the concentration of A^-. The three zones will move with the same speed, arranged in the order of their mobilities and the isotachophoresis equilibrium will be reached.

3.4 Self-Sharpening Effect

If a C^- ion diffuses into A^- region, it will experience a lower field strength than it had in the C^- region, it will, therefore, slow down until it is overtaken by the boundary which is moving all the time towards the anode. Conversely, the ion A^- in the C^- region will speed up by the higher field strength there, and soon reaches its own region again.

This phenomenon is called self-sharpening effect and is responsible for the high resolving power of isotachophoresis (Figure 3.5).

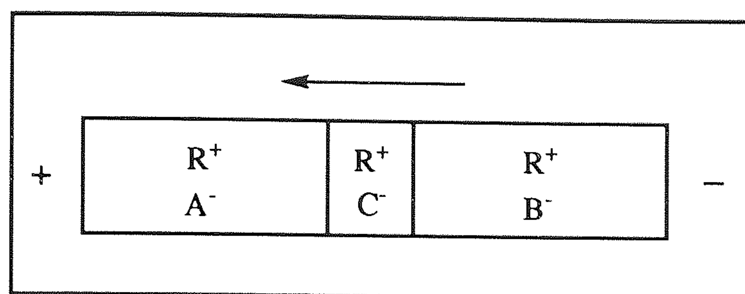
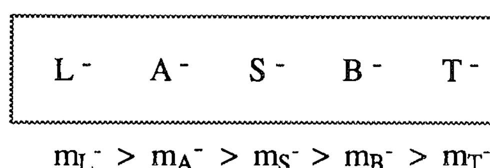


Figure 3.5 The self-sharpening effect in isotachphoresis.

3.5 The Choice of Spacers

When the zones containing A^- and B^- ions are very narrow it will be very difficult to distinguish between them using UV detection, especially if both zones have similar UV absorbances. If the ion S^- with a mobility intermediate between A^- and B^- is added to the sample, A^- and B^- will be spaced apart.



$m_{L^-} > m_{A^-} > m_{S^-} > m_{B^-} > m_{T^-}$

The S^- ion should preferably be non-UV absorbing. The UV detection resolution will be enhanced as A^- and B^- are now spaced apart and show up as two separate peaks on the UV tracer. Usually, a mixture consisting of many components with different mobilties is added to the sample. The advantages of discrete spacers for protein ITP in anionic systems have been discussed, both for serum [91] and model protein [93] separation. A mixture of 22 cations have been used as discrete spacer for improved resolution in cationic isotachophoresis of proteins [93].

3.6 Selection of Electrolytes

According to the principle of isotachophoresis, electrolyte conditions in the leading-ion zone determine all the parameters in the succeeding zone. Therefore, by choosing a certain pH and concentration in the leading electrolyte, all the pH values and concentrations of the sample zones are determined. The separation capacity will reach a maximum for a sample when the differences in the net mobilities for different sample ions are as large as possible.

Therefore, by changing the pH in the leading electrolyte an optimal separation can be obtained. The best way to find the optimal separation is to vary the pH in the leading electrolyte in the pH region where the net mobilities of the sample ions are most affected. This happens when the pH is equal to the pK of a certain ion. Tables 3.1 and 3.2 show some suggested electrolyte systems for the separation of anions and cations respectively.

A series of different electrolyte systems with a wide range of pH have been used for analysis of brain cell proteins [94] and membrane proteins [95]. In this part of work the isotachophoresis technique and electrolyte solutions were developed and modified to meet the requirements for the measurement of tear proteins and some standard protein solutions which are mostly found in human tears. Having developed the isotachophoresis instrument for this purpose, then it will be possible to use the technique for the identification of proteins extracted from the hydrogel contact lenses.

3.7 Quantification

In capillary isotachophoresis the separated components form homogeneous zones. The zone lengths are directly proportional to the amount of ions in them (Figure 3.4), and by measuring the length of the individual zones as they appear on the record chart, the quantity of each ion can be calculated. Figure 3.5 shows the relationship between the quantity of a lysozyme solution and its zone length (the UV-absorbance signal).

Table 3.1 Some electrolyte systems used for the separation of anions.

System No.	Leading electrolyte (pH)	Terminating electrolyte
1	0.01 M HCl 0.013-0.055 M β-alanine (3.0-4.2)	Caproic acid
2	0.01 M HCl 0.012-0.034 M creatinine (4.2-5.2)	Pivalic acid
3	0.01 M HCl 0.018-0.09 M histidine (6.0-7.0)	Glycine, add $Ba(OH)_2$ until pH = 9-10
4	0.01 M HCl 0.001-0.09 M histidine (5.2-7.0)	Phenylacetic acid Cacodylic acid

Table 3.2 Some common electrolyte systems suitable for the separation of cations.

System No.	Leading electrolyte (pH)	Terminating electrolyte
1	0.01M HCl (2.0)	0.01M LiCl 0.01M Tris
2	0.01M sulfanilic acid, adjust the pH with HCl (2.4)	0.01M LiCl
3	0.01M KOH, adjust pH with ascorbic acid (4.1)	0.01M LiCl
4	0.01M KOH, adjust pH with fumaric acid (4.3)	0.01M Li_2SO_4
5	0.01M CH_3-COOK, adjust pH with CH_3-COOH (4.0-5.5)	0.01M b-alanine 0.01M a-alanine
6	5mM $Ba(OH)_2$ 15mM valine (9.9)	20mM Tris 5mMHCl, H - 8.3

3.8 Detection Methods

Normally, four different detection methods are used for measurement in isotachophoretic analysis: ultraviolet (UV)-absorbance detection, conductometric detector, potential gradient detection, and thermometric detection. However, the UV detection and thermometric detection methods are most frequently used.

3.8.1 UV Detection

In isotachophoresis experiments, all ions move with the same speed in individual zones at the dynamic equilibrium stage, and only the counter-ion is homogeneously mixed with them. Therefore, the U. V. detection can give valuable information about some of the ions. In practice, U. V. absorption is measured directly through the capillary. As the total internal volume is in order of 40 to 100 ml only an extremely small cell volume is available for the actual detection.

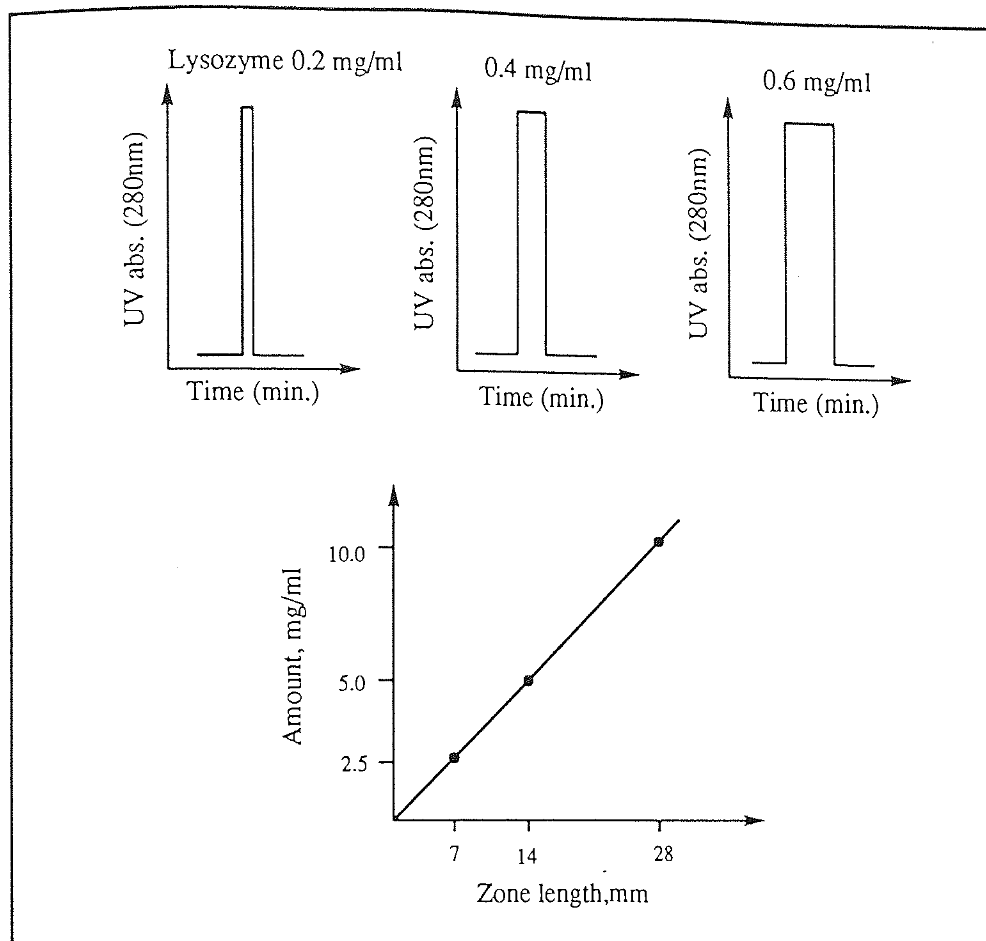


Figure 3.6 Quantification by measuring the zone lengths.

3.8.2 *Thermometric Detection*

In this mode of detection, the relative differences between the separated zones is monitored. As mentioned before, isotachophoresis is performed at a constant current. Since power (P) is the product of field strength (E) and current (I), P=E.I, the Joul heat produced will change stepwise at each zone boundary. The position of each zone boundary is thus indicated by a rise in temperature which can be measured by a sensitive thermocouple. Since the temperature of each zone is related to the net mobility of the ion in it, the height of the thermal record (the "thermal step height") can be used to identify each sample ion qualitatively.

3.9 Isotachophoresis of Tear Proteins

3.9.1 *Introduction*

Capillary isotachophoresis is a very reproducible and rapid analytical technique for quantification of proteins. Only minute amounts (about 30 μg) of protein are required and,

therefore it can be most suitable for the detection of tear proteins and lens extracts. The most interesting feature of capillary isotachophoresis resides in the fact that it provides instant quantitative information during the separation procedure itself without denaturation or coloration of the proteins.

3.9.2 Materials and Solutions

1. Leading electrolyte, 0.01 M potassium acetate (pH 4.6-5.3)
   0.098 grams of potassium acetate (Sigma, P-3542) was dissolved in 100ml of distilled water and the final volume was made up to one litre. The pH of the solution was adjusted to 5.0 by 0.01 M acetic acid (FSA Laboratory Supplies, Code: A/0360). 0.5% of hydroxypropyl methylcellulose (Sigma, H-8384) was added to this solution just before use.
2. Terminating electrolyte, 0.01 M β-alanine
   0.89 grams of β-alanine (Hopkin&Williams Ltd, 1135.5) was dissolved in distilled water and the final volume was made up to one litre.
3. Terminating electrolyte (System 2), 10 mM acetic acid
   This was another possible terminating electrolyte for the separation of proteins and was made by diluting the appropriate volume of glacial acetic acid with water.
4. Ampholyte solution, 1% pH 3.5-10
   5 µl of Ampholine® carrier Ampholyte (Sigma, A-5174) was diluted with 200 µl of distilled water.
5. Model protein mixture
   5 mg of model proteins, lysozyme, lactoferrin, lactalbumin and ribonuclease were dissolved in 10 ml of distilled water.

3.9.3 Running the LKB Isotachophoresis Instrument

The LKB 2127 Tacophor isotachophoresis instrument is shown in Figure 3.7. The temperature was set on 20° C and the high voltage power was connected. All parts of the Tachophor which were in contact with the electrolyes were cleaned before it was used. The leading and terminating electrolyte reserviors were filled with the prepared buffers. The capillary and the injection port were rinsed and filled with the leading and terminating electrolyes respectively. The presence of bubbles was prevented as they may adhere to the membrane and cause a trip voltage.

The sample was injected in the boundary between the leading and terminating electrolytes by the use of a microsyringe. The perspex cover on the analyser unit was

then lowered and about one hour was allowed before the instrument was warmed up. The U. V. recorder was set and the zero line was adjusted. The trip voltage was set on 300 mA so that when the voltage rose higher than this value an automatic trip occurred.

The Tachophor was kept absolutely clean especially, those parts which come in direct contact with the electrolyes as it is a very sensitive analysis instrument. The electrolytes were changed every fifth run and the unused electrolyte was kept in the refrigerator. The whole system was washed once with distilled water every time the electrolyte system was changed.

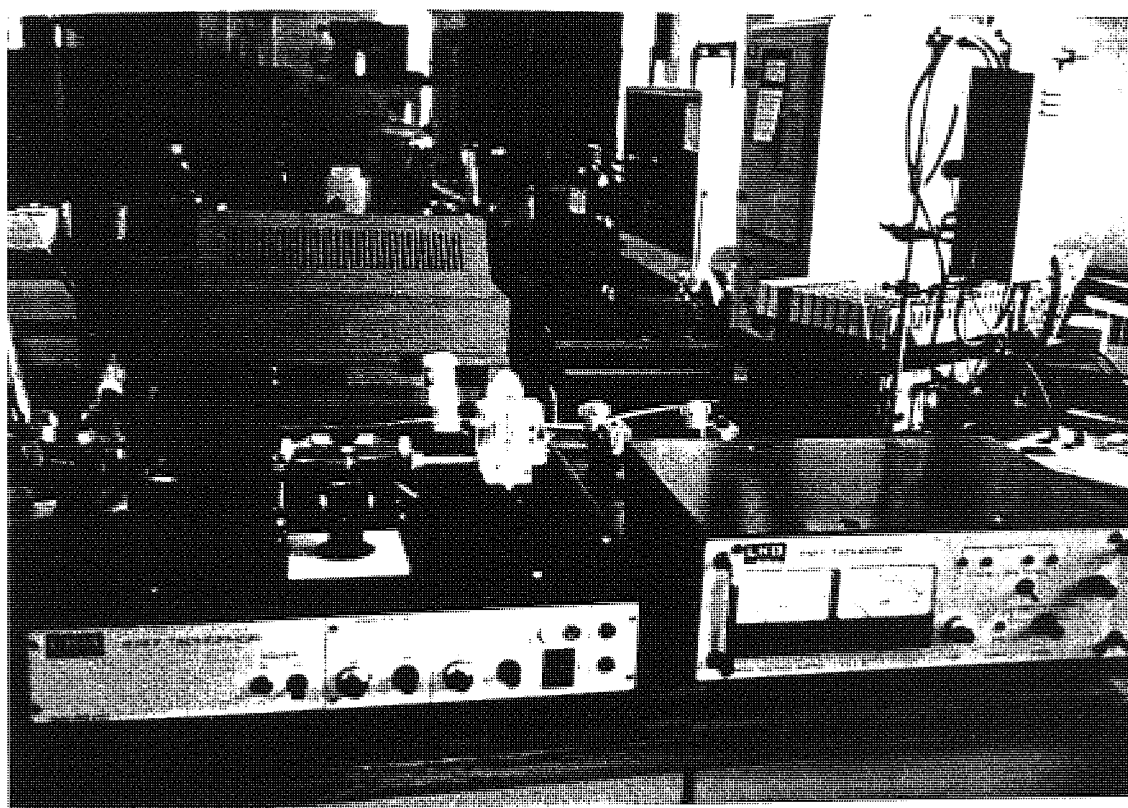


Figure 3.7 The LKB isotachophoresis instrument.

3.9.4 Experimental Procedure

Cationic isotachophoresis of the model proteins (0.5-1.0 mg/ml of lysozyme, ribonuclease, lactalbumin, myoglobin, lactoferrin and albumin) and tear samples was run on an LKB 2127 isotachophoresis instrument (Figure 3.7). A capillary of 0.8 x 340 mm was used and an LKB U. V. detector was preformed at 280 nm. Separation currents were 250 mA for 10 minutes which were then reduced to 200 mA and detection currents were 50 mA. The U.V. signals were recorded on Servoscribe 1S and Howe FT 1000 chart recorders for the first few experiments the typical isotachograms of which are presented in Appendix III. A Trio personal computer (Trivector) equipped

with an Epson LX-80 printer was then attached to the isotachophoresis instrument for faster and more accurate detections. This was set to give comprehensive peaks for the separated proteins. The procedure set on the Trio computer is shown in Appendix III. The spectra of protein were recorded by both chart recorders and the results are presented in Appendix III.

3.9.5. Results and Discussions

One of the advantages of isotachophoresis for the analysis of tear proteins is that the sample volume is quite low in comparison to any other analytical method. The other important advantage of this technique is the sensitivity for the separation of the proteins. It is a fast and reliable way to identify the tear proteins in very small volume of tears. The isotachograms of a tear sample is presented in Figure 3.8, where the effect of the injection of 2 μl of 1% spacer ampholytes pH 3.5-10 is also shown.

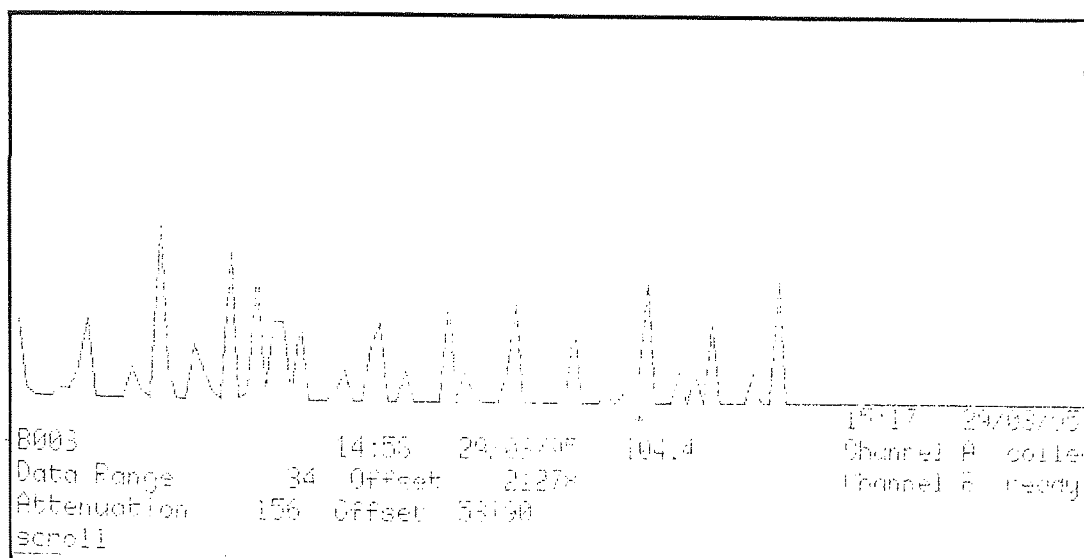


Figure 3.8 The isotachogram of 10 μl of the tear sample with 2 μl of spacer pH 3.5-10.

Chapter 4

Principles of Protein Absorption Applied to Hydrogel Polymers

Chapter 4

Principles of Protein Absorption Applied to Hydrogel Polymers

4.1. Introduction

The pioneering work of Landsteiner and Uhliz [96] in 1905 demonstrated that various inorganic powders absorb high quantities of horse serum protein. Since then other workers have reported the interaction of serum proteins with synthetic surfaces. In 1926, Hitchcock [97] reported that the absorption of egg albumin onto collodion membranes followed a Langmuir isotherm with a maximum absorption occurring near the isoelectric point of the protein. In 1932, Palmer [98] found that protein absorption was a function of pH at low protein concentration, but independent of pH at higher protein concentrations.

In summary, many variables associated with the absorption of proteins onto synthetic surfaces have been studied over the past 80 years. These variables include pH, protein type and concentration, time and type of the surface. In addition, several types of bonds have been reported to explain the absorption behaviour, namely, hydrogen bonds, hydrophobic bonds, ionic bonds, electrostatic bonds and a combination of these.

In this part of work the effect of protein concentration, pH and temperature have been studied for the absorption of different proteins onto hydrogel contact lenses. The nature of the bonds to explain the absorption have also been demonstrated.

4.2 Sequential and Competitive Absorption of Proteins on Acuvue™

4.2.1 Theory

The majority of the literature on sequential and competitive adsorption of proteins refers to blood proteins [99]. The blood or any other body fluid's proteins interact with any surface they encounter, generally leading to their adsorption. Protein adsorption is the overall result of various types of interaction between the different components present in the system, i.e. the sorbent surface, the protein molecules, the solvent (water) and any other solute such as low molecular mass ions. The mechanism of absorption is very complex, which involves attachment of different amino acid residues of the protein molecule to the sorbent surface.

Whatever the mechanism and kinetics of adsorption, the process (at constant pressure and temperature) can occur only if the Gibbs energy G of the system decreases.

$$\Delta_{abs} G = \Delta_{abs} H - T\Delta_{abs} < 0,$$

where, H, S and T refer to the enthalpy, entropy and absolute temperature, respectively, and Δ_{abs} indicates the change of the thermodynamic functions of state resulting from the adsorption.

As a result, the adsorbed proteins are extremely difficult to remove by diluting the solution. On the other hand, if the solution contains a displacer or other protein whose molecules with an affinity for absorbent, any desorbing segment can be replaced by another. Desorption of the molecule is now virtually an exchange process and, as $\Delta_{exchange} G \ll \Delta_{desorption} G$, this process is much more likely. Brash [100] reported examples of exchange between adsorbed and dissolve protein molecules where desorption upon dilution did not take place.

It can be suggested that the boundary between a solution containing different kinds of proteins and any other (solid) phase is a dynamic scene of protein adsorption, desorption and displacement.

4.2.2 Experimental Procedure

In this part of study the adsorption of lysozyme, ribonuclease and α-lactalbumin was studied. These proteins have similar size and shape, but their isoelectric points and structural stability differ. It was suggested that after the protein contacts the sorbent, the interface will initially accommodate the protein molecules that (i) have the highest rate of arrival, the largest diffusion coefficient and (ii) are most abundantly present in the solution. However, the adsorbed molecules may be gradually displaced by others that have a higher affinity. The final composition of the adsorbed layer at a given interface is determined by the concentration of various kinds of proteins in the solution, the intrinsic adsorption affinities, and the possibilities that the protein have to desorb.

The sorbent was Acuvue™ contact lens which is a negatively charged copolymer of HEMA and MAA. The spoilation procedure was similar to other *in-vitro* spoilation studies which were carried out previously and the quantity of the adsorbed proteins were monitored and measured after 5 days at room temperature spoilation with frequent shaking. All of the protein solutions were made with concentrations of 0.3 mg/ml and 3 ml of each was used for the spoliation process.

The total quantity of the first protein deposited on the lenses were measured by U.V. before subjection of the samples to further spoilation with the second protein. The spoiled lenses were further spoiled in the second protein solution for another 5 days. Reference experiments were carried out for comparison by leaving the spoiled lenses in the same protein solution for further spoilation during the second 5 days. The differences between the total protein after the second spoilation for the samples and the references must be due to the effect of the primary deposition.

4.2.3 Results and Discussions

Figure 4.1 shows that, after the absorption of lysozyme the quantity of ribonuclease absorbed decreases. However, it does not reach the value of singular ribonuclease absorption. On the other hand, the quantity of the lysozyme absorbed on Acuvue™ pre-coated with ribonuclease increases and attains values similar to those in singular lysozyme absorption (Figure 4.2). It can, therefore, be suggested that lysozyme can displace ribonuclease from the surface. Since lysozyme is more positively charged than ribonuclease, it is inferred that at the negatively charged surface of Acuvue™ the sequential and competitive absorption of these two proteins is dominated by electrostatic interactions. It can also be revealed from Figures 4.1 and 4.2 that positively charged lysozyme and ribonuclease displace small pre-absorbed negatively charged α-lactalbumin from the negative surface of Acuvue™. On the other hand, α-lactalbumin is not able to displace lysozyme or ribonuclease.

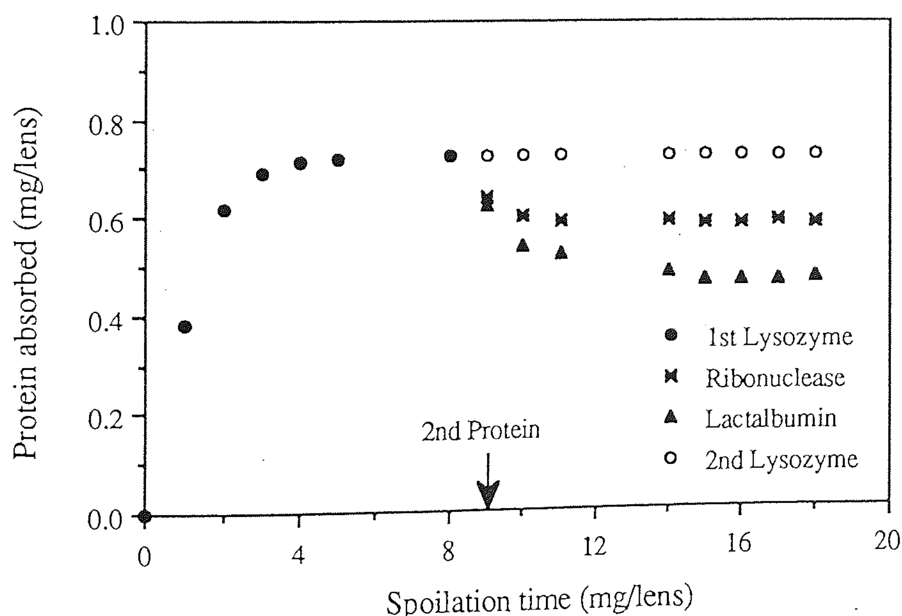


Figure 4.1 The build-up of lysozyme, ribonuclease and α-lactalbumin on Acuvue™ lens previously coated with lysozyme.

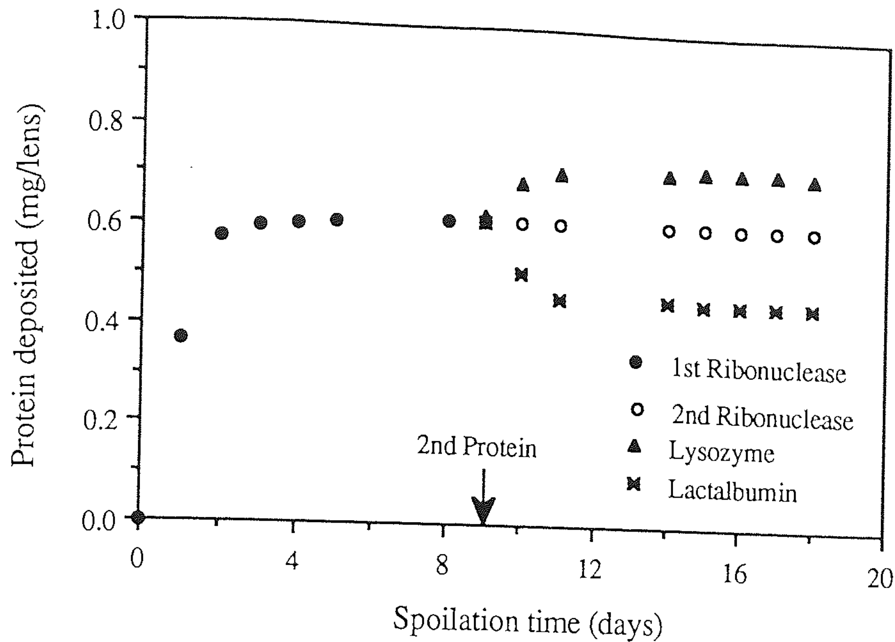


Figure 4.2 The build-up of lysozyme, ribonuclease and α-lactalbumin on Acuvue™ lens previously coated with ribonuclease.

4.2.4 Conclusions

The absorption of proteins on negatively charged Acuvue™ surface is largely determined by electrostatic interaction. This can be concluded from the following observations:

1. The amount absorbed from single protein solutions increases with increasing charge contrast between the protein and the surface.
2. Sequential absorption occurs only if the second protein has a more electrostatic interaction with the surface.
3. It is also suggested that the protein having the most favourable electrostatic interaction absorb preferentially from a mixture, so that the final composition of the absorbed layer essentially consists of this protein.

4.3 The Absorption Isotherms of Protein on Hydrogels

4.3.1 Introduction

The concentration of the protein solution used for the *in-vitro* spoilation of the lenses may have an effect on their absorption onto the hydrogels. This phenomenon has been known for the absorption of solid particles from the solution to a solid surface [100].

Understanding the relationship between the amount of protein in the solution and the amount absorbed gives good indication of the protein absorption in the patients with abnormalities in their tear fluid in terms of the protein content. This may happen in various diseases related to the eye of other symptoms which affect the tear fluid. If the absorbed amount (Γ) is plotted as a function of protein concentration in solution C, an absorption isotherm can be obtained. The objective of this part of the study was to establish the absorption isotherms for different proteins. The absorption isotherms were also constructed for the absorption of lysozyme on different FDA Groups I-IV contact lenses.

4.3.2 Experimental Procedure

A series of Acuvue™ contact lenses (Group IV) with similar powers were spoiled in lysozyme, albumin and lactoferrin solutions with concentrations ranging from 0.2-0.5 mg/ml for 5 days at room temperature (22 ± 2°C). The volume of each spoiling solution was 3 ml the lenses were shaken frequently in the protein solutions. The quantity of the deposited protein was measured after a rinse with distilled water using the U. V. absorption at 280 nm. A blank of the same Acuvue™ lens was used as reference.

4.3.3 Results and Discussion

The quantities of proteins deposited on Acuvue™ and lysozyme on different group lenses are shown in Tables 4.1 and 4.2 respectively. The results presented here are the mean value of at least three similar measurements. The error in the protein quantity measured by U. V. was less than 5% in most cases. The absorption isotherms are obtained by plotting the quantity absorbed against the concentration of the protein used for spoilation studies (Figures 4.3 and 4.4). It can be seen that the plateau-values for absorption of all proteins on different lens materials is reached at around 0.4 mg/ml. It was also found in previous studies that the time required to reach this value is about 5 days. Therefore most of the *in-vitro* spoilation experiments were carried out under these conditions.

Table 4.1 The quantities of different proteins on Acuvue™ lenses.

Concentration of solution (mg/ml)	Protein absorbed (mg/lens) (± 0.002)		
	Lysozyme	Lactoferrin	Albumin
0.10	0.616	0.124	0.015
0.20	0.638	0.212	0.027
0.30	0.726	0.244	0.032
0.40	0.816	0.245	0.038
0.50	0.902	0.245	0.042
0.60	0.916	0.246	0.042

Table 4.2 The quantities of lysozyme deposited from solution on different lenses.

Concentration (mg/ml)	Lysozyme deposited on (mg/lens) (± 0.002)			
	Group I	Group II	Group III	Group IV
0.1	0.042	0.048	0.112	0.616
0.2	0.045	0.053	0.211	0.638
0.3	0.055	0.058	0.260	0.726
0.4	0.055	0.060	0.302	0.816
0.5	0.055	0.061	0.314	0.902
0.6	0.056	0.062	0.322	0.916

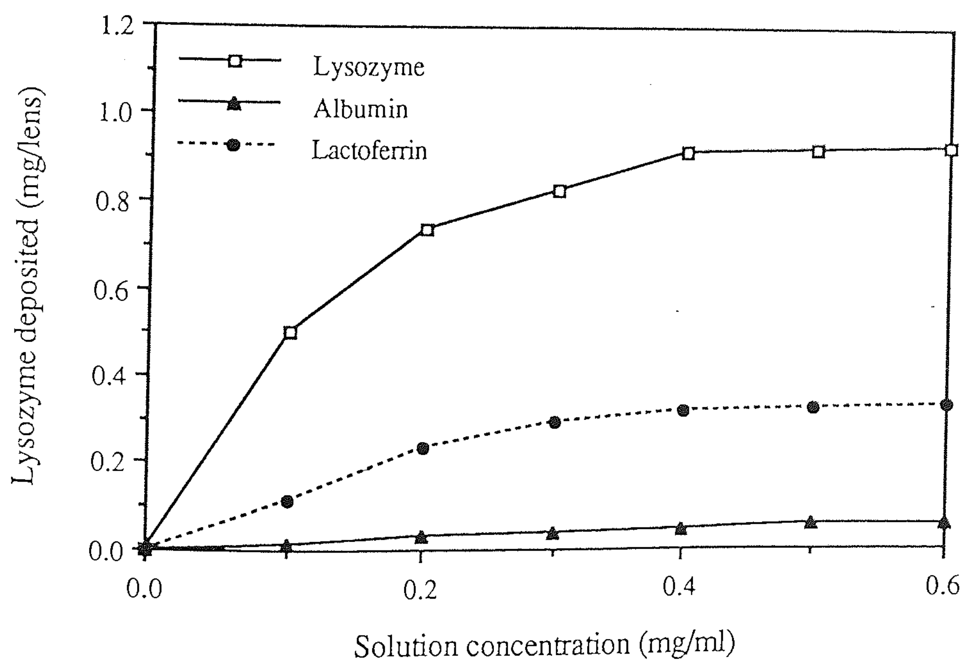


Figure 4.3 The absorption isotherms of different proteins on Acuvue™ at 25°C.

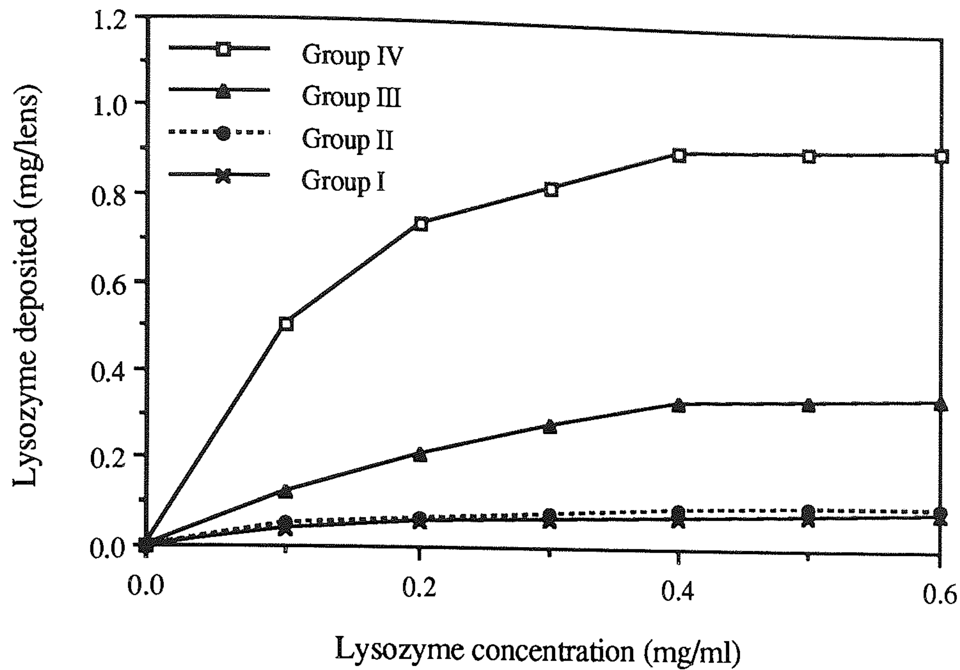


Figure 4.4 The absorption isotherms for lysozyme on different lenses at 22°C.

4.4 The Effect of pH and Temperature on the Absorption Isotherms

4.4.1 Introduction

In this part of study the effect of some factors such as temperature and pH of the medium on the absorption of proteins on Acuvue™ was investigated. The proteins used in this study were all small proteins with similar molecular masses, globular size, and consequently, diffusion coefficient. Accordingly, the effects of molecular size and diffusion coefficient on the absorption are particularly negligible. They, do however, differ in hydrophobicity, stability of native structure and isoelectric point, so that at a given pH their charge densities are different. The effect of pH is, therefore, easier to study in this set of proteins. Some physico-chemical properties of these proteins are given in Table 4.3.

4.4.2 The Effect of pH

One of the most obvious factors affecting protein absorption onto hydrogel polymers is the pH of the environment. pH can affect both the protein structure in terms of its surface charge and the ionicity of the polymer material. This phenomena can be very important in the spoilation of hydrogel contact lenses with tear proteins. In this part of the work the *in-vitro* spoliation of some contact lenses by different protein solutions in a range of pH was studied.

Table 4.3 Some physico-chemical properties of the proteins [101].

Properties	Lysozyme	Ribonuclease	Myoglobin
Molar mass (Daltons)	14,000	13,600	17,800
Dimensions (nm^3)	4.5 x 3.0 x 3.0	3.8 x 2.8 x 2.2	4.5 x 3.5 x 2.5
Diffusion coefficient (m^2s^{-1})	1.04×10^{-10}	1.26×10^{-10}	1.31×10^{-10}
Isoelectric point (pH units)	11.1	9.4	7.0
Overall hydrophobicity ($J\ g^{-1}$)	- 7.6	- 8.7	- 4.1
Gibbs energy of heat denaturation (Jg^{-1}) [7]	- 4.1	- 3.2	- 2.8

4.4.2.1 Materials and solutions

The following buffer solutions were prepared, the protein solutions (0.1-0.6 mg/ml) were then made using these buffers as solvents. The composition and pH values of the buffers are shown in Table 4.4.

1. Sodium acetate/acetic acid solution (0.01 M, pH 4.7)
   0.082 grams of sodium acetate was dissolved in distilled water, 0.06 grams of glacial acetic acid (FSA laboratory Supplies, Code: A/0360) was added and the final volume was made up to 1000 ml. The pH was checked with pH meter.
2. Tris (hydroxymethyl) aminomethane solution (pH 6.8)
   6.0 grams of tris (hydroxymethyl) aminoethane (tris) (Sigma T-1378) was dissolved in 100 ml of distilled water and the pH was adjusted with a pH meter.
3. Tris solution (pH 8.8)
   36.3 grams of tris was dissolved in distilled water and the final volume was made up to 200 ml. The pH of the solution was checked with pH meter.
4. Tris-buffered saline (0.0M tris, 0.05 M NaCl, pH 7.5)
   2.42 grams of tris and 29.24 grams of sodium chloride (BDH, 1024) were dissolved in one liter of distilled water and the pH was adjusted using pH meter.
5. Borate-Tween buffer solution (0.05 mM $Na_2B_4O_7$, 10 H_2O, 0.2 % Tween, pH 9.3)
   14.07 grams of sodium tetraborate (Sigma, B-9876) was dissolved in distilled water, 0.2 ml of Tween 20 "polyoxyethylene sorbitan monolaurate" (Bio-Rad, 170-6531) was added and the final volume was made up to one liter with distilled water.

6. Protein solutions (1.0 mg/ml)

0.1 grams of each individual protein, lysozyme, lactoferrin, ribonuclease, myoglobin and albumin were dissolved in 100 ml of distilled water and 0.1-0.6 mg/ml solutions were then prepared by diluting the appropriate volume with distilled water. The stock protein solution could be kept frozen for one month and diluted samples were made as required.

Table 4.4 The compositions and pH values of the buffers used for the preparation of the protein solutions.

Number	pH	Buffer Composition
1	4.7	0.01 M Sodium acetate/Acetic acid
2	6.8	0.01 % Tris
3	7.5	0.02 M Tris
4	8.8	0.05 M Tris
5	9.3	0.05 mM Sodium tetraborate

4.4.2.2 Experimental procedure

The lenses were spoiled in 3 ml of individual protein solutions for 5 days with regular mixing at room temperature (about 22°C). The quantities of the proteins deposited were then measured using U. V. at 280 nm. The lenses were rinsed with distilled water once before the measurements and a blank contact lens of the same type was used as reference.

4.4.2.3 Results and Discussions

It can be seen from Table 4.4 that the overall hydrophobicity which is based on the hydrophobicities of the constituting amino acids increases in the order ribonuclease, lysozyme and myoglobin. The values for Gibbs energy of denaturation is an indication of the stability of the native globular structure. This stability decreases in the order lysozyme, ribonuclease and myoglobin. Figures 4.5 and 4.6 show the absorption isotherms of lysozyme and ribonuclease at two different pH values. Both solutions have pH values lower than the isoelectric point of lysozyme, at lower pH the intensity of the positive charge on the protein is higher and, therefore, the absorption on negative surface of Acuvue™ has a higher value. Figures 4.7 and 4.8 show the plateau values for the absorption of the 3 proteins at several pH values. The shape of the curve in Figure 4.7 also proves that at pH values lower than their isoelectric points all three

proteins have higher charge densities and absorb more on the negative charged surface. It can be suggested that proteins with high structural stability are hard proteins which do not change their structure due to absorption. Their absorption is governed by electrostatic interactions.

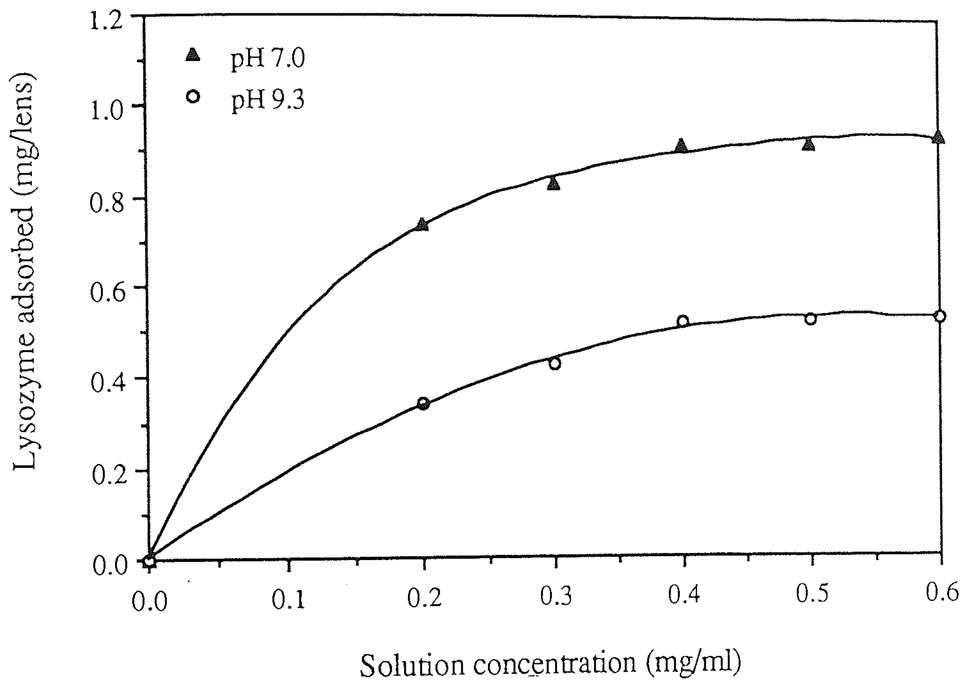


Figure 4.5 The effect of pH on the absorption isotherms of lysozyme on Acuvue™.

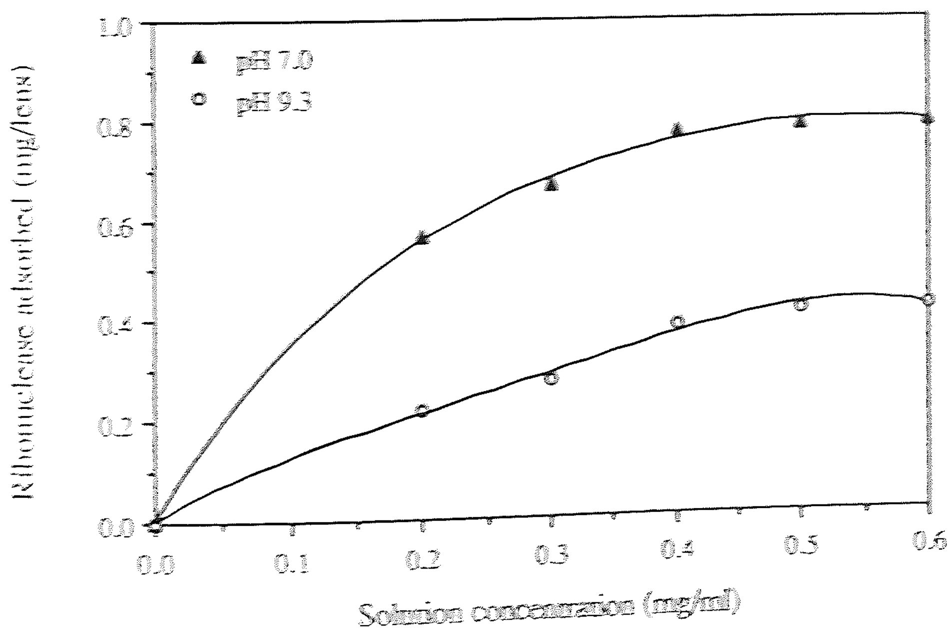


Figure 4.6 The absorption isotherms of ribonuclease at pH values lower than its isoelectric point.

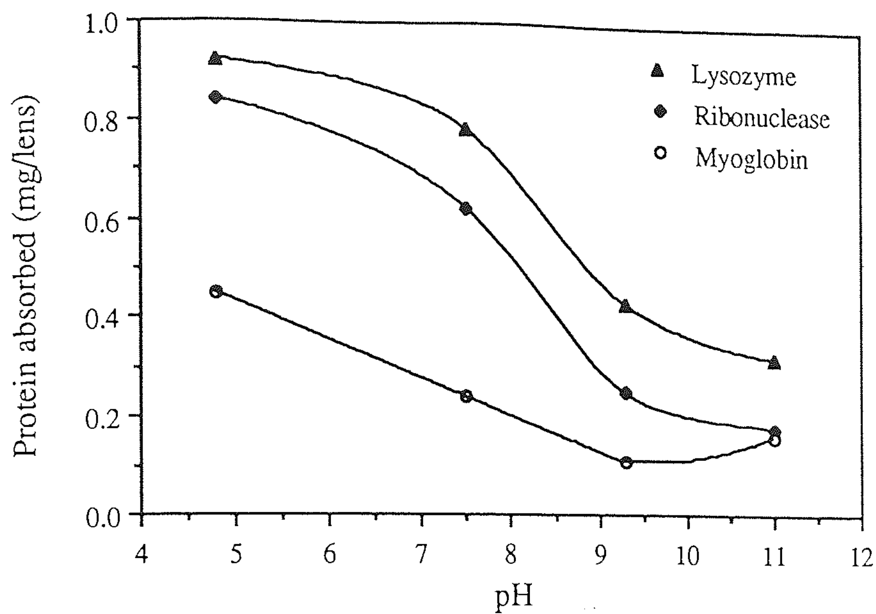


Figure 4.7 Plateau-values for the absorption of different positively charged proteins from solutions with different pH on Acuvue™.

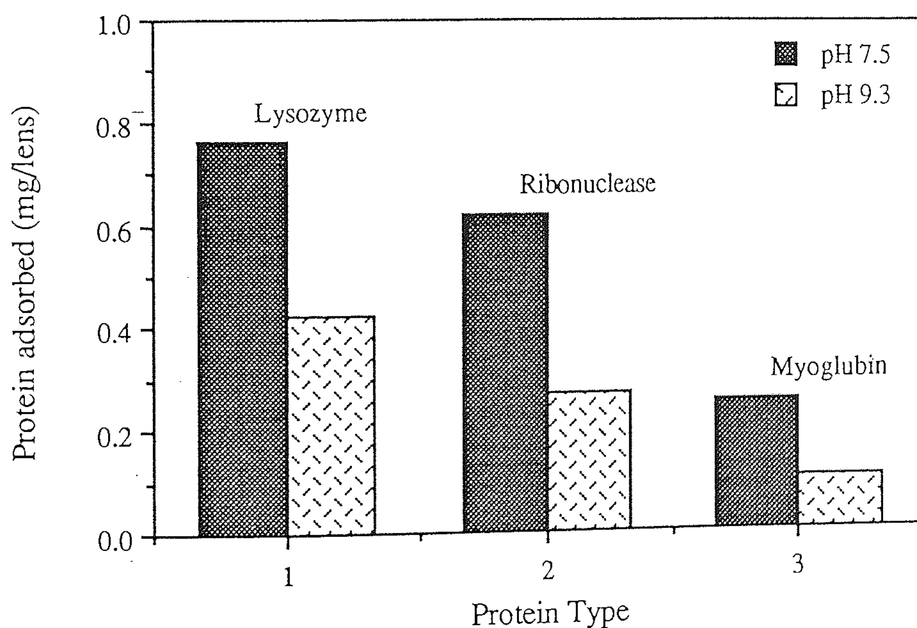


Figure 4.8 The plateau-values for the absorption of proteins at pH values above their isoelectric points on negatively charged Acuvue™.

In Figure 4.9 plateau absorption of albumin as a function of pH is shown at negatively charged Acuvue™. It can be revealed from this figure that albumin has the highest absorption at around its isoelectric point (4.3). Albumin is known for its high conformational adaptability towards changing environmental conditions. Norde *et al.* [101] have studied the adsorption of serum albumin on different positively and negatively charged surfaces. They have proved that the reduction in the absorption at

either side of the isoelectric point is due to the structural rearrangements in the adsorbing molecule (albumin).

The possible rearrangement in the structure of a hypothetical protein molecule is shown in Figure 4.9. Although the absorption is strongly dependent upon structural stability of albumin, the influence of the interaction between the charge of the protein and the charge on the surface can also be observed. Figure 4.10 shows that albumin absorbed is higher below its isoelectric point than above the isoelectric point.

The absorption isotherms shown in Figure 4.11 for albumin absorption at different pH values also are in agreement with this argument. All the pH values used in this part of the experiment are above the isoelectric point of albumin and the protein is negatively charged. Therefore, the density of negative charge on the albumin increases with the increase in pH, as a result the absorption isotherms on negative surface are at lower levels at higher pH values.

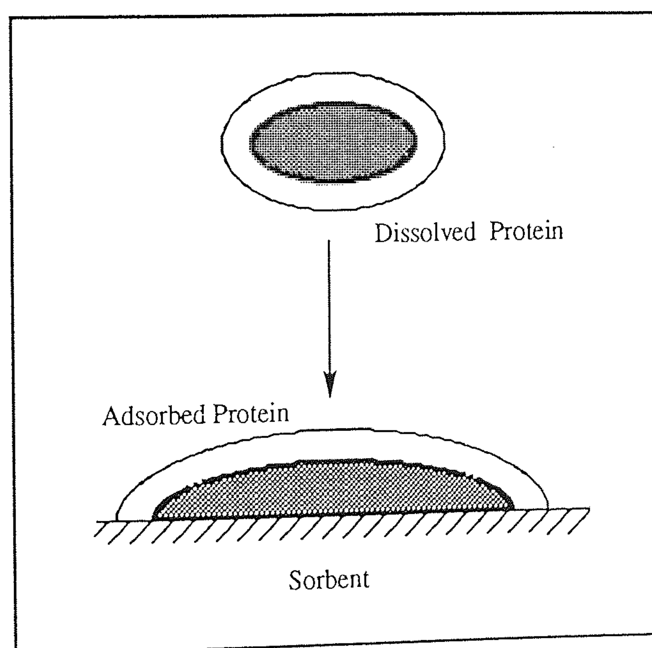


Figure 4.9 The rearrangement of the structure of a protein due to its adsorption. Shaded areas indicate hydrophobic regions.

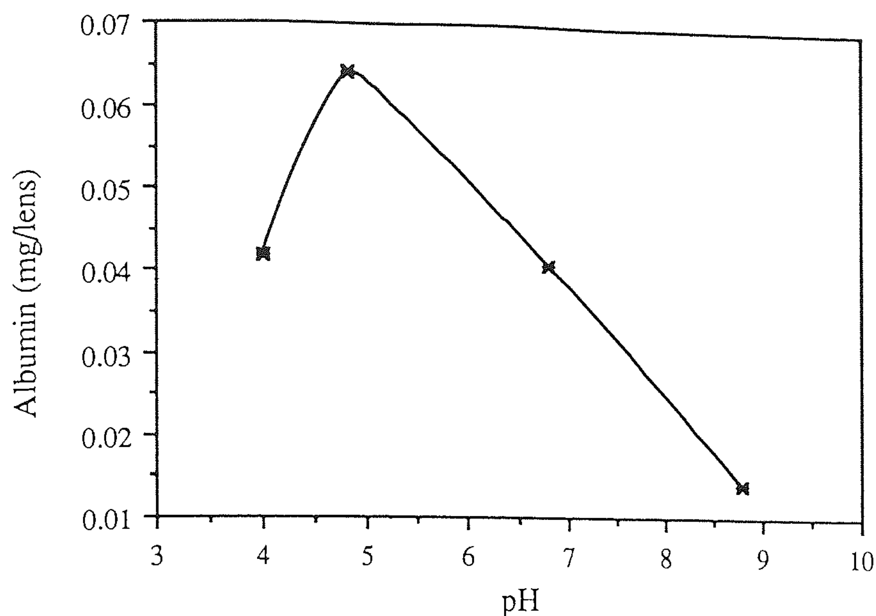


Figure 4.10 Plateau-values for the absorption of bovine albumin from solutions with different pH on Acuvue™.

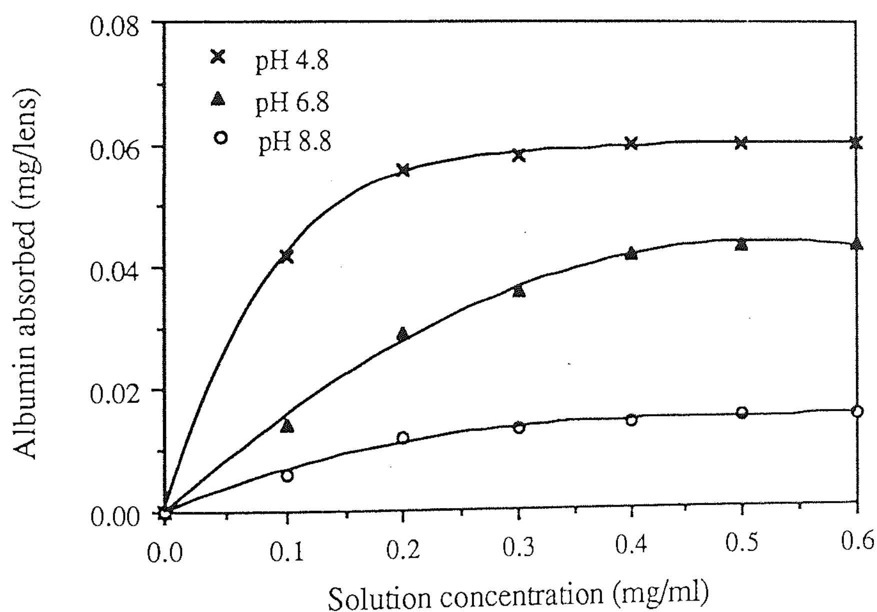


Figure 4.11 The effect of pH on the absorption isotherms of albumin on Acuvue™.

In Figure 4.12 the absorption of lysozyme after 5 days from solutions of lysozyme with different pH values on Acuvue™ and HEMA are shown. In this case the protein is kept constant and the effect of pH on its absorption at different surfaces is studied. HEMA contain only a very low negative charge which is due to the presence of MAA impurities. It can be seen that absorption increases with both increasing pH and the amount of negative charge on the sorbent surface. This again proves previous

conclusion of the effect of electrostatic interactions on the absorption of a hard compact structure protein as lysozyme.

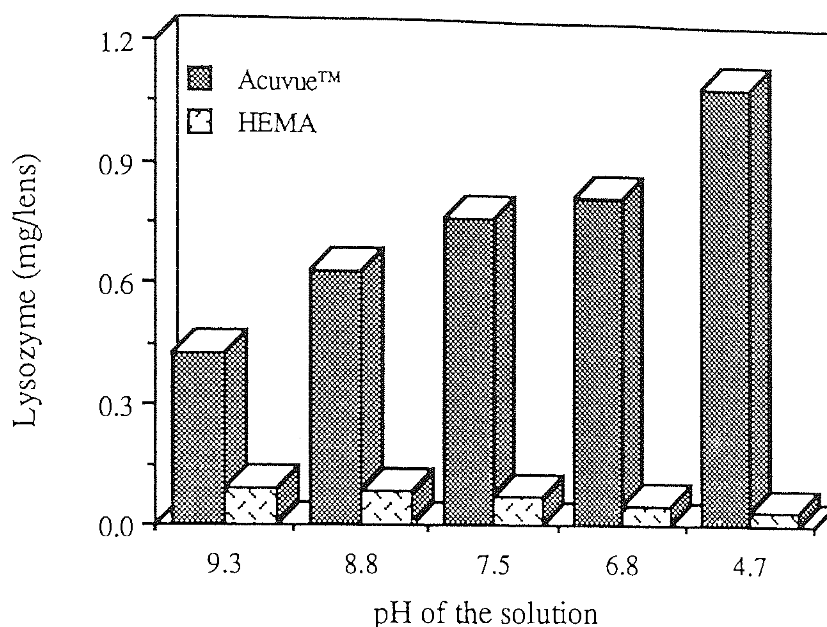


Figure 4.12 Comparison between the effect of pH on the up-take of lysozyme by HEMA and Acuvue™ lenses (Groups I and IV).

4.4.3 The Effect of Temperature

4.4.3.1 Introduction

Almost all protein absorption experiments carried out so far were carried out at room temperature (20-22°C). However, only a few studies have been undertaken to show the effect of temperature on protein absorption. The effect of temperature is different for different system studied. Norde [103] has reported that the absorption increases with a rise in temperature. He observed that the influence of temperature on the adsorption of human plasma albumin to polystyrene surfaces depends on the pH of adsorption. Other authors [104, 105] reported a decreased adsorption on raising the temperature.

In this study, the effect of temperature on the absorption of lysozyme and lactoferrin at pH 7 on HEMA contact lenses was studied.

4.4.3.2 Experimental procedure

The HEMA lenses were incubated in 3 ml of 0.3 mg/ml of individual solutions of lysozyme and lactoferrin at four different temperatures. The constant temperature was provided by the use of thermostatically controlled oven, the accuracy of which was ±

2°C. The spoilation was carried out for 5 days during which time the solutions were shaken occasionally using the IKA-Vibrax-VXR, electronic vibrator on 150 speed. The quantity of the protein deposited was measured using direct U. V. spectrophotometry on the spoiled lenses at 280 nm. The lenses were rinsed once with distilled water before measuring their U. V.

4.4.3.3 Results and discussions

The spoilation results are shown in Figure 4.13. It can be seen that the two proteins show different behaviour due to the increase in the temperature of the spoiling solution. The pH and other conditions such as concentrations and physical factors are kept constant. Both lysozyme and lactoferrin are absorbed more at 37°C, with the lowest absorption at very high and very low temperatures.

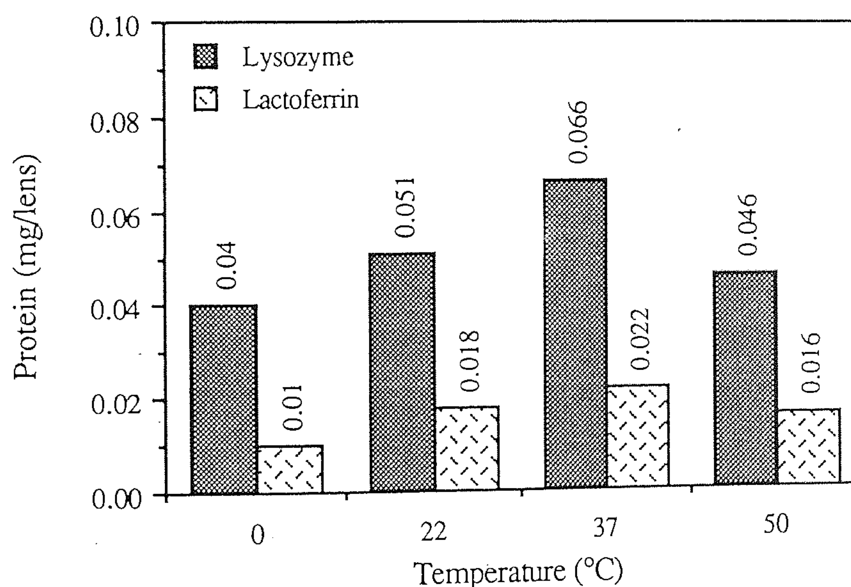


Figure 4.13 The effect of temperature on the absorption of lysozyme and lactoferrin on HEMA contact lenses.

4.4.3.4 Conclusions

According to the results obtained in this study, the temperature has not a straight forward effect on the protein absorption into contact lenses. At low and very high temperatures the absorption decreases while the highest absorption takes place around physiological temperature. This phenomena makes the tear protein absorption on contact lenses even more considerable. Mitra et al [105] found that the temperature has a different effect on the absorption isotherm of bovine serum albumin (BSA) on

alumina powder. They showed that the amount of albumin adsorbed increases with the decrease in temperature.

The prediction of contact lens spoilation by proteins at different temperature is almost impossible and it may depend on different factors such as the pH of the solution, protein type and protein concentration.

There are many factors which may cause error in these types of experiments. The results obtained are, however very reliable and reproducible, as the sum errors caused from different sources is less than 5% in all cases. The absorption experiments were carried out on at least three contact lenses at the same time and under similar condition. In each case the standard deviation was calculated for the results. The error measurements and the standard deviations are presented in Appendix IV.

4.5 General Conclusions on Protein Absorption

The absorption of tear proteins to hydrogel contact lenses is a complex phenomena which includes the diffusion of the protein particle through the aqueous solution and the collision and interaction of the protein at the interface. In the case of small proteins with charges opposite to the high water content hydrogels the primary absorption is followed by the penetration of the protein into the hydrogel matrix. The protein absorption depends on many factors such as the protein concentration, protein type, size and charge, the chemical structure of the sorbent surface. Temperature and the pH of the spoilation solution also influence the absorption, as the pH affects the charge of the protein and the hydrogel surface.

The interaction of proteins with hydrogel polymers rarely seem to be adsorption alone. Even when the protein does not penetrate to an appreciable depth into the hydrogel matrix, its interaction with surface of the polymer suggests that the term absorption should be used when defining protein-hydrogel interaction.

Chapter 5

Structure, Activity and Mobility

Chapter 5

Structure, Activity and Mobility

5.1. Introduction

Proteins constitute an important group of tear components and a major source of soft contact lens spoilation. The process of protein adsorption onto the surface takes place almost instantaneously when a biomaterial comes into contact with a biological environment [35]. Once adsorbed, the protein is very difficult to completely detach and it is known that the important tear protein lysozyme can diffuse into the matrix of some high water content lenses making it even more difficult to remove.

The protein sorption process is very complicated and involves the primary migration of the protein from the tear fluid onto the lens material, which leads to further surface deposition of protein coupled with diffusion into the lens matrix. This part of work describes the *in-vitro* surface deposition of individual proteins onto different lens materials. The materials have been used to study the effect of hydrogel structure on the nature and quantity of protein absorbed into the lens matrix and the biological activity of the protein which, subsequently leaches out into, for example, storage solutions from *in-vitro* and *in-vivo* spoiled lenses.

5.2 Materials and Methods

The major tear proteins, albumin, lactoferrin and lysozyme were used together with a group of other proteins (ribonuclease, myoglobin, insulin and ferredoxin which enable the effect of size and charge to be studied, Table 5.1). 0.1 grams of each individual protein were dissolved in 100 ml of distilled water (1.0 mg/ml) and 0.5 mg/ml working solutions were then prepared using the stock protein solution.

A group of representative contact lenses from FDA Groups I-IV were also used to compare different lens materials (Table 5.2).

5.3 Spoilation Studies

The lenses were soaked individually in 2.0 ml of 0.5 mg/ml solutions of each protein at room temperature (20 ± 2). They were constantly shaken on a shaker with a low speed for 5 days. The deposited lenses were then rinsed carefully with distilled water and

the quantity of the protein deposited into them was calculated using an unworn lens of the same type and power as background.

Table 5.1 Characteristics of some proteins used for *in-vitro* spoilation.

Name	Source	Molecular Weight	Relative Charge
Lysozyme	Chicken Egg	12600	(+++)
Albumin	Human Serum	65000	(- - -)
Lactoferrin	Bovine Colostrum	74000	(++)
Ribonuclease	Bovine Pancreas	13000	(+++)
Myoglobin	Horse Heart	17000	(+)
Insulin	Bovine Pancreas	6000	(0)
Ferredoxin	Spinach	12000	(- - -)

Table 5.2 The Characteristics of some commercial contact lenses.

USAN Name	Lens Group	% EWC	Chemical Composition
Polymacon	I	38	HEMA
Surfilcon A	II	74	MMA-VP
Bufilcon A	III	45	HEMA-DA-MAA
Etafilcon A	IV	58	HEMA-MA
Vifilcon A	IV	55	HEMA-PVP

Abrevations: HEMA(2-Hydroxyethyl methacrylate), VP (Vinyl pyrolidone), MAA (Methacrylic acid), DA (Diacetone acrylamide) and PVP (Poly vinyl pyrolidone).

5.4 The Activity Measurement

The different contact lenses which were spoiled with lysozyme and ribonuclease were cut into quarters and their enzymes were extracted by vigorously shaking the lens pieces in 1ml ReNu™ solution for 24 hours.

The activity of lysozyme in the extraction solutions was measured using a method based on the decrease in the turbidity of a *Micrococcus lysodietikus* at 450 nm (Section 2.4.2). The absorption of the mixture of substrate and the lysozyme sample was measured at room temperature using U.V. spectrophotometry at 450 nm. The

measurement of the absorption was repeated after three minutes and the difference in the absorption was calculated (ΔA). The activity of lysozyme was calculated as discussed in Section 2.4.2.

The activity of ribonuclease was measured by a modification of Kuntiz spectrophotometric assay (Section 2.4.4). The method is based on the reactivity of the enzyme on yeast ribonucleic acid in a buffer environment.

The effects of the equilibrium water content and the surface charge of the contact lens material were studied using some poly 2-hydroxyethyl methacrylate poly(HEMA) lenses with varying cross-link density i.e. water content and methacrylic acid (MAA) content. These were the new biomaterials which were synthesized within the research group (Section 6.2). Increasing the proportion of methacrylic acid (1-5%) caused an increase in the concentration of negative charges in the polymer (Chapter 6).

Group IV lenses, such as Etafilcon and Vifilcon which are high water content ionic lenses that contain a significant negative charge are well known to absorb relatively high quantities of protein. Therefore, they were used for the *in-vitro* spoilation to show the effect of size and charge of the protein on its absorption.

5.5 The Effect of pH on the Mobility of Protein

The mobility of the protein is very important especially when considering the enzymatic activity and trying to clean the lens from proteins by soaking over-night in the care solution. A mobile enzyme is more easily leached out of the lens matrix into the tear fluid and function normally.

The pH has a pronounced effect on the mobility of the proteins in and out of the matrices of the high water content ionic lenses [106]. To show the effect of pH, an Acuvue™ lens previously spoiled *in-vitro* in lysozyme solution was soaked in 1 ml of 15 % H_2O_2 for two minutes and then stored in 2% H_2O_2 for three days. The quantity of the lysozyme leached into the solution was monitored during the storage in slightly acidic solution. This was compared to the amount of lysozyme leached from a similar Acuvue™ spoiled lens into ReNu™ which is slightly alkaline (pH 7.2)

5.6 Results and Discussions

In order to establish the relative uptake of the three main tear proteins, the uptake profile for lysozyme, lactoferrin and albumin was developed as a function of time. The results are shown in Figure 5.1.

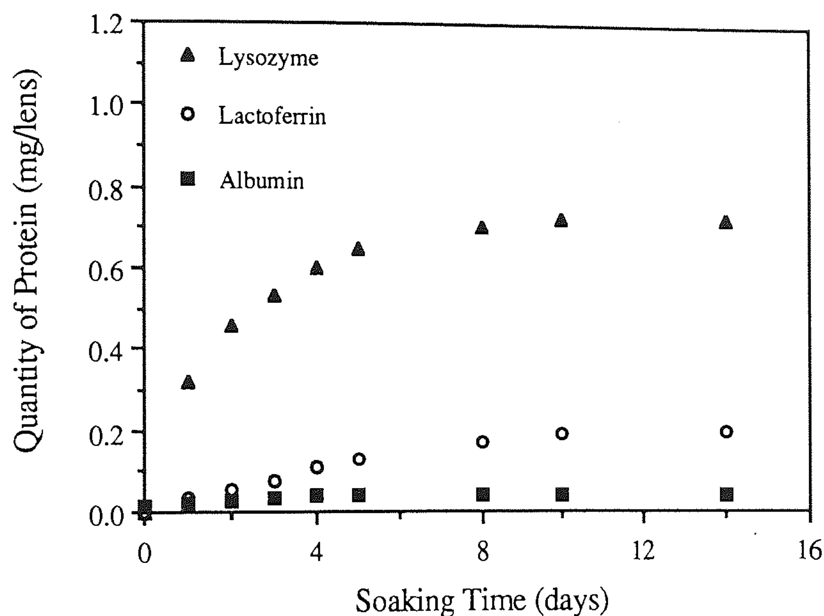


Figure 5.1 The effect of size and charge of the protein on its up-take by a Group IV lens material.

From a comparison of size and charge of the protein (Table 5.1), it is apparent that both size and charge may be influential. Since lactoferrin is both larger and less charged than lysozyme, its lower level of uptake may be affected by either or both. In order to investigate these factors separately, the uptake of a series of proteins having similar size to lysozyme but with different charges were studied. Figure 5.2 shows the relative deposition rates of lysozyme, ribonuclease, myoglobin, ferredoxin together with insulin which has no charge but is significantly smaller than lysozyme. This figure clearly demonstrates that it is the charge rather than the size of lysozyme that produces its high level of uptake. This was previously repoted by us for the *in-vitro* spoiled different lenses [107, 108] .

Some further deductions may be made. First of all, tear specific pre-albumin, which is a significant tear component of similar size to lysozyme but without the positive charge, is unlikely to be significantly absorbed. This important tear component is not available as an isolated protein. It is only by studies shown in Figure 5.2 that informations relating to its deposition behaviour may be deduced. Secondly, both the shape and the size of the protein appear to be influenced in governing its up-take by different lens

materials. As a result, the more compact lysozyme structure, illustrated in Figure 5.3, permits a more efficient interaction with the material than is the case with ribonuclease which has similar size and charge.

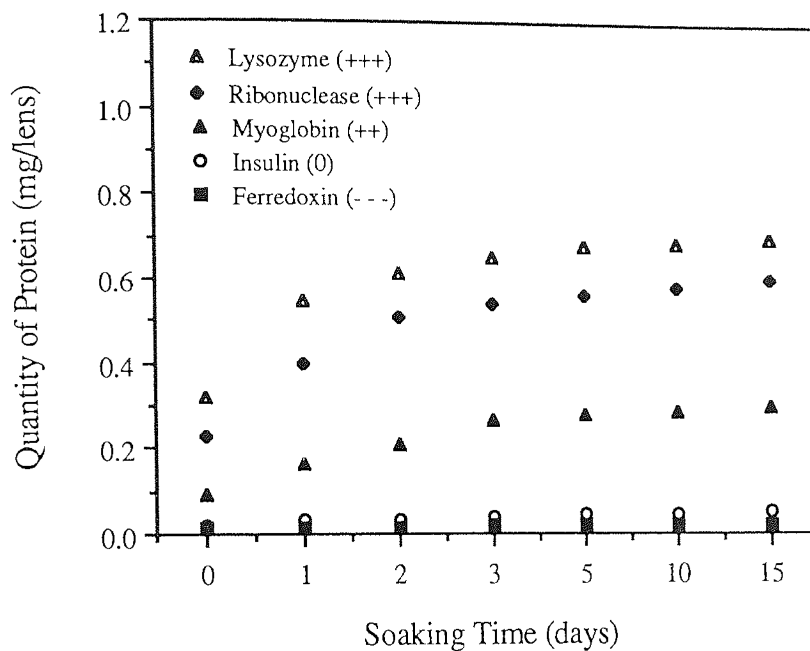


Figure 5.2 The effect of the charge of proteins on their *in-vitro* deposition onto a Group IV contact lens.

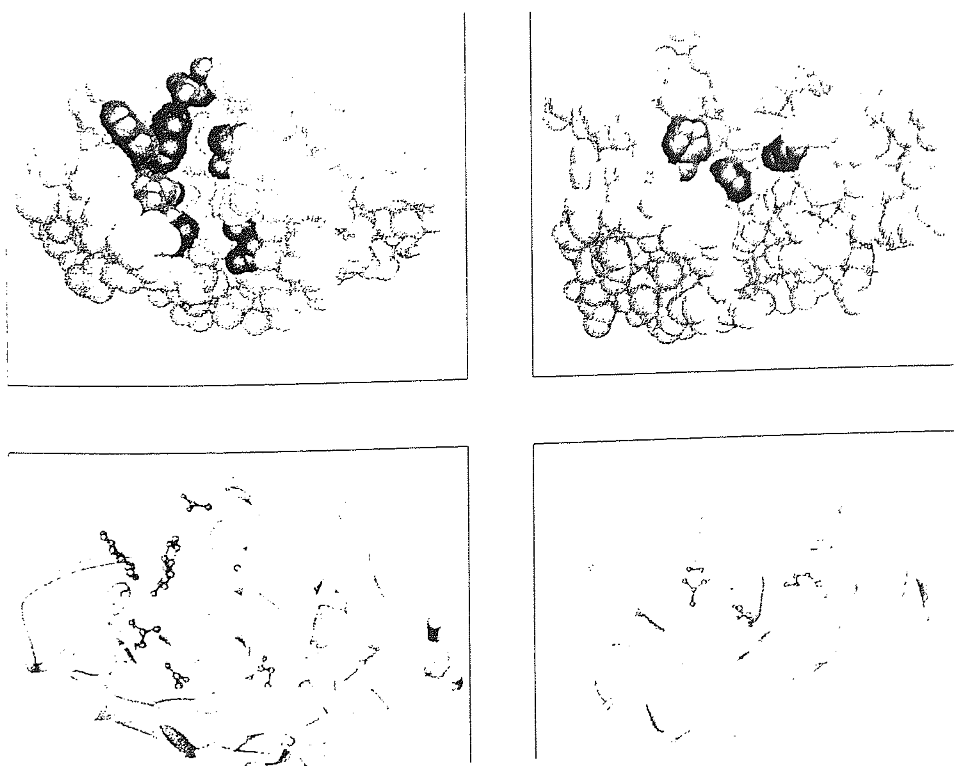


Figure 5.3 The three dimensional strucures of lysozyme (left) and ribonuclease (right).

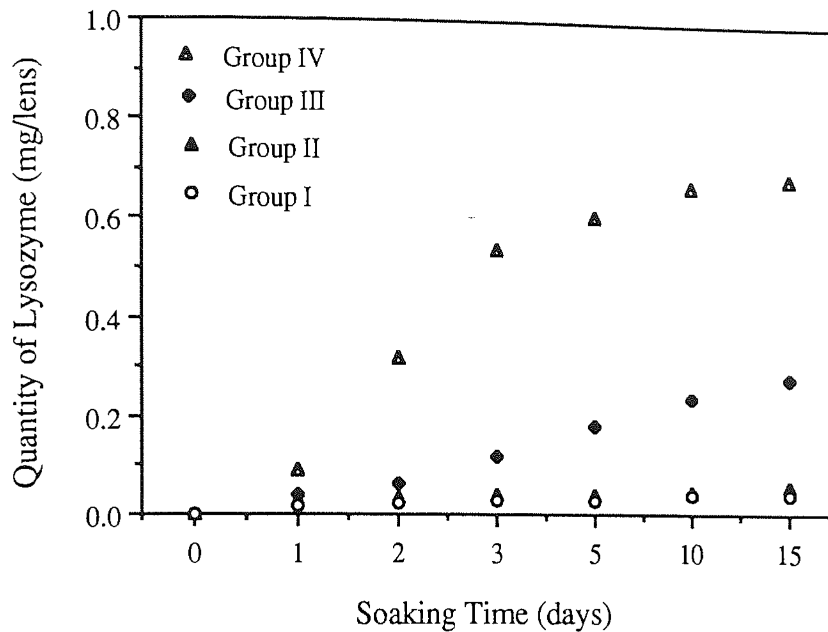


Figure 5.4 The effect of lens material on the deposition of lysozyme.

Because of its charge, size and compact structure, lysozyme is a useful member of the family of proteins used for studying the interaction of tear proteins with different contact lens materials.

Group I-IV lens materials are defined as low water content non-ionic (I), high water content non-ionic (II), low water content ionic (III) and high water content ionic lenses (IV) respectively. A typical member of each group (Polymacon, Perfilcon, Bufilcon and Etafilcon) were used as substrates to study the effect of lens material on lysozyme uptake. The results are shown in Figure 5.4. Here again the message is clear. It is charge not water content that dominates the interaction.

In order to distinguish between surface adsorption and absorption into the lens material of ionic lenses, two families of material were used. One consisted of hydroxyethyl methacrylate-methacrylic acid copolymers in which the normal (~1%) level of cross-link was used, producing a family of materials where water content rose from 42% at 1% MAA to 67% at 5% MAA. The second family was prepared with the same percentages of MAA, but with a very high (10%) cross-link density (Chapter 6, Section 6.2).

This was designed to prevent the network from expanding and thus to inhibit lysozyme uptake into the matrix. The equilibrium water content (EWC) of this family varied only

between 42-48%. The results of lysozyme and lactoferrin uptake over a 14 days period are shown in Figure 5.5.

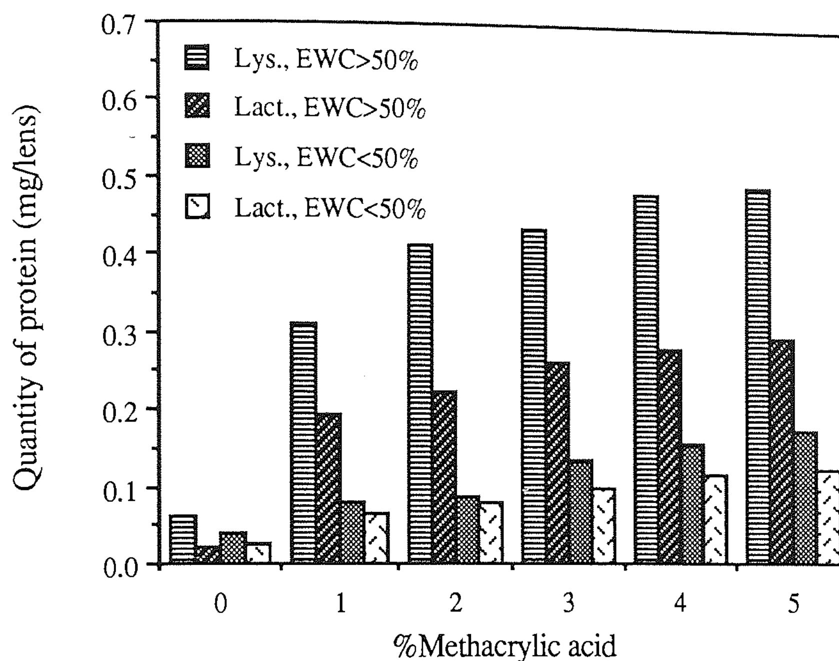


Figure 5.5 The effect of concentration of methacrylic acid in hydroxyethyl methacrylate copolymers on the absorption of some tear proteins.

Two clear points emerge from Figure 5.5. In the case of high cross-link density materials, the lysozyme and lactoferrin penetration into the lens matrix will be negligible enabling the surface concentration of these proteins on negatively charged ionic polymers to be assessed. In the case of Group IV lens materials, such as Etafilcon and Vifilcon, the figure will be in the region of 150 micrograms per lens. The second point that emerges is that lactoferrin (as well as lysozyme) is both adsorbed onto the surface of Group IV lens material and absorbed into the bulk of the materials although much more slowly. This explains the slow, but progressive rise in lactoferrin uptake into Etafilcon shown in Figure 5.1.

The question that must now be addressed is the mobility and activity of protein deposited onto the surface and into the matrix of ionic materials. By exposing the lenses to lysozyme (or using ex *in-vivo* lenses), measuring surface protein, extracting with conventional care systems (such as ReNu™) and measuring the protein concentration and destruction of activity (if any) can be monitored.

Figure 5.6 compares the extraction of protein from *in-vivo* and *in-vitro* lenses. Each lens was extracted into 1 ml of solution. These lenses contained very similar gross level of protein initially and, as can be seen, the protein leaches steadily from both *in-*

vivo and *in-vitro* spoiled lenses. This suggests that there is no apparent difference in extraction of protein from worn lenses and it is clear that the mobility of protein diffusing from the lenses is of the same order as that diffusing into the lens (Figure 5.1 cf Figure 5.6).

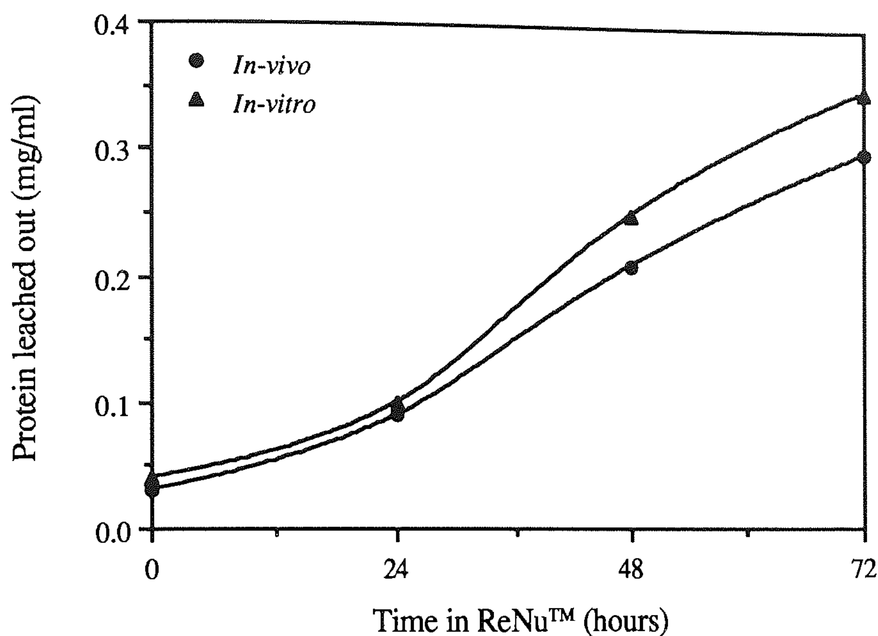


Figure 5.6 The Quantity of protein leached out into ReNu™ from *in-vitro* and *in-vivo* deposited lenses.

By measuring the biological activity of protein desorbed from the surface or the bulk of a lens the extent to which its uptake and removal have produced denaturation may be established.

Figure 5.7 shows the power and sensitivity of this technique. This figure relates to lysozyme adsorbed onto the surface of a poly(HEMA) lens produced from a commercial lens blank material. The material contained appreciable residual methacrylic acid than high quality poly(HEMA) lenses and accumulated over 100 µg onto the lens surface. The protein was progressively extracted into ReNu™ multi-purpose solution and both concentration and biological activity determined.

It is clear from Figure 5.7 that, within experimental errors, the figures were identical. This period of deposition and studying of the material followed by analysis were of the order of four weeks, indicating that surface adsorption of lysozyme over this period does not affect the activity of the protein.

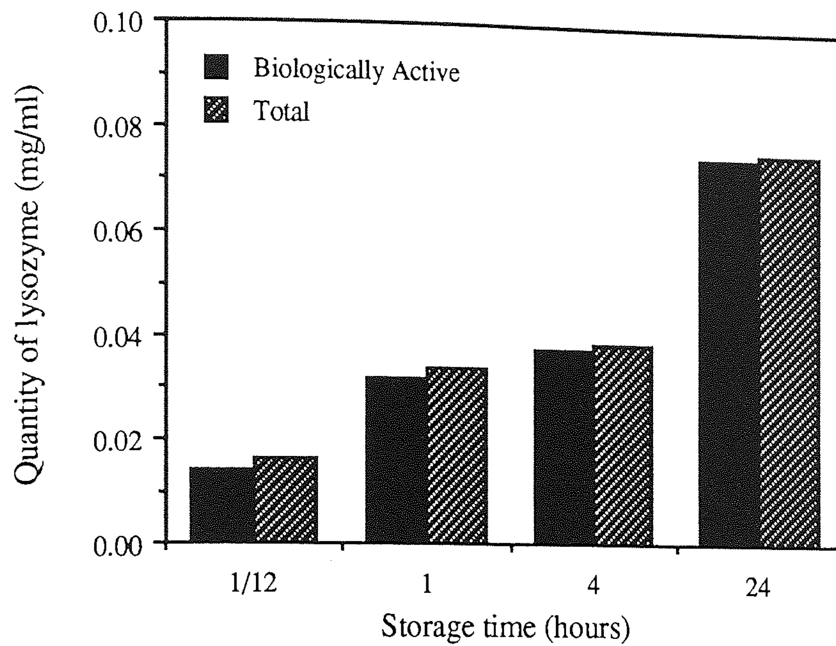


Figure 5.7 The quantity and biological activity of lysozyme extracted from a HEMA (containing abnormally high MAA levels) into ReNu™.

A similar experiment (Figure 5.8) was carried out with a Group IV (Etafilcon) lens using lysozyme and ribonuclease which both penetrate the lens matrix. The experiment was again carried out over a period of few weeks and demonstrated that absorption into the Etafilcon material does not, within experimental errors, cause any lysozyme or ribonuclease denaturation.

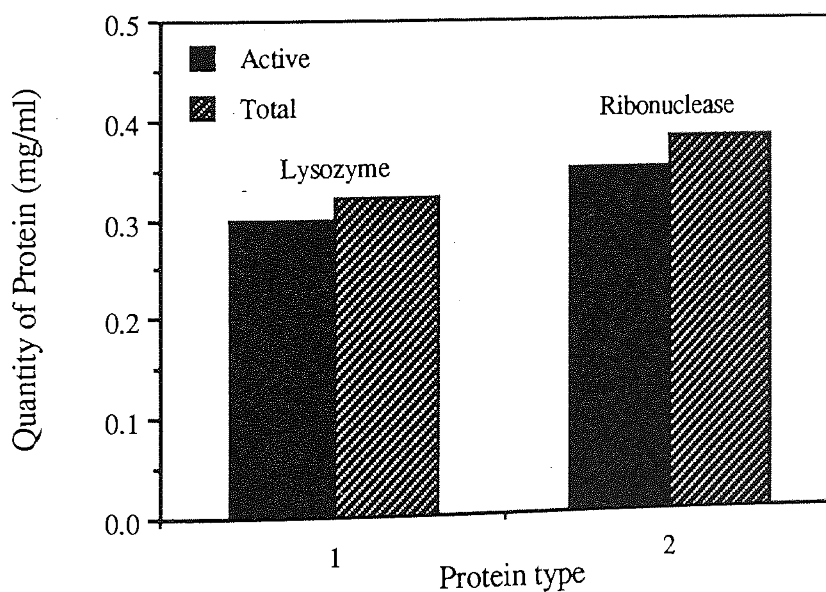


Figure 5.8 The activities of lysozyme and ribonuclease that leached into ReNu™ from an Etafilcon lens.

The application of this technique to protein extracts of *in-vitro* and *in-vivo* lenses (Figure 5.6) shows no detectable difference with activity of the lysozyme associated with *in-vitro* and *in-vivo* spoiled lenses.

The effect of pH of the storage solution on the leachinng of lysozyme from the matrix of Acuvue™ in-vitro spoiled lenses is shown in Figure 5.9. This figure shows that lysozyme is mobile in alkaline solutions and the quatity of the protein increases in the storage solution while it decreases on the lens. The acidic solution contains virtually no lysozyme during the storage time and it can be seen that the quantity of lysozyme on the lens has remained constant in acidic environment. Primary soaking the lens in a high concentration of hydrogen peroxide for two minutes neutralises the negative charges on the surface of the ionic lens and the positively charged lysozyme is then trapped into the matrix. This can be the reason for the lack of mobility and therefore, lysozyme can not leach into the storage solution.

It can be suggested that, it may be useful to soak the unworn high water content ionic lens into a slightly acidic solution before use to increase its spoilation resistance from positively charged lysozyme and lactoferrin. In this case care should be taken to rinse the lens throughly with distilled water after the acid treatment.

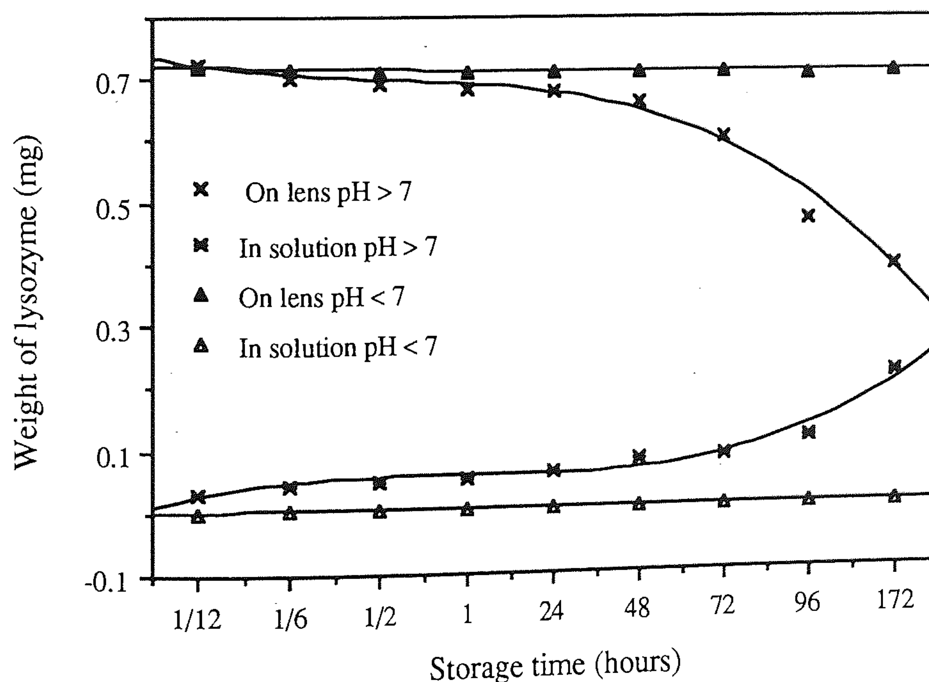


Figure 5.9 The effect of pH on the leaching of lysozyme from an Acuvue™ *in-vitro* spoiled lens into the storage solution.

5.4 Conclusions

1. Both size and charge of proteins are influential in their deposition onto contact lenses.

2. The uptake of different proteins into the matrices of Group IV lenses is more influenced by the charge of proteins than their size.

3. Lysozyme and lactoferrin adsorb onto the surface of Group IV lenses to a high degree, whereas albumin and tear specific pre-albumin are less adsorbed.

4. Lactoferrin, as well as lysozyme, penetrates into the matrix of Group IV lenses despite its greater size.

5. Lysozyme is mobile and can be leached out of the matrices of Group IV lenses stored at pH > 7 in, for example, ReNu™.

6. The mobility of lysozyme from the matrices of high water content ionic lenses depend on pH of the storage solution. In acidic pH the negative charges on the lens surface are neutralised and the protein is trapped into the matrix. On the other hand, lysozyme is quite mobile in slightly alkaline storage solutions.

7. The biological activity of lysozyme is not detectably changed by the absorption and desorption processes.

8. The biological activity of ribonuclease which is an enzyme with a similar weight, but slightly different compact strcture has not been affected due to its absorption to hydrogel contact lens followed by its extraction from the matrix of the lens.

Chapter 6

Protein Interactions with Novel Biomaterials

Chapter 6

Protein Interactions with Novel Biomaterials

6.1 Introduction

A biomaterial is a material designed to fulfil a purpose and to exist at a physiological interface without being rejected. A large number of biomaterials have been developed for the progressive use as biomedical devices, especially in recent years. Table 1.7 shows some common biomaterials and the problems related to their use. One of the major growth areas is the design and development of polymers to fulfil the growing number of biomedical requirements.

It is apparent that the most important problem, when in the use of biomaterials is their biocompatibility. It is essential for an implanted device to be able to avoid physiological rejection at the biological interface to which it applied. Rejection can be manifested in many ways depending on the biological environment of the implanted material. For example, a material rejected at a blood interface may cause thrombosis in a patient, whilst an inadequate contact lens material may promote tear protein and lipid deposition that will impair the quality of the lens and also give discomfort to the wearer. The term "spoilation" used in biomaterial studies refer to interaction which leads to deposition of the interacting material.

A number of polymers are known which have a wide range of properties ranging from hard and glassy plastics, through hydrophobic rubbery materials, to soft water containing hydrogel matrices.

Poly(HEMA) has a number of limitations when used as a biomaterial. Even a highly cross-linked poly(HEMA) matrix has relatively poor mechanical properties. Its uses are further restricted by its limited biocompatibility. This is illustrated in its use as soft contact lenses where, even though it is found to be mechanically adequate, ocular incompatibility is observed leading ultimately to the formation of "white spot deposits" on the lens surface.

Despite some disadvantages of hydrogel lens materials, poly(HEMA) is still the most commonly used material for contact lenses. It has the advantage of high oxygen permeability together which is related to its adequate water content. Care must be taken when polymerising hydroxyethyl methacrylate as residual methacrylic acid (MAA)

produced during the polymerisation may remain in the polymer. Excess MAA in the polymer causes some negative charge on the surface of the material, making it susceptible to spoilation especially with positively charged proteins.

The main advantage of working in an interdisciplinary research group consisting of polymer chemists, polymer technologists, cell biologists, biochemists and opticians is that the research can be carried out as the result of a collaboration. Therefore, in this chapter a joint work was performed with the kind help of some of my colleagues. A group of HEMA and other copolymers were synthesised some by the writer, some by J. Ma, some by K. French and some by M. Smith. These copolymers contained various comonomers including methacrylic acid to establish the resistance to the biological environment. The cell culture studies on the copolymers were carried out by H. Fitton.

6.2 The Effect of Methacrylic Acid (MAA) Copolymerised with HEMA on Protein Up-take

This investigation was designed to establish the effect of different amounts of MAA and cross-linking agent on protein deposition on polymers produced.

A series of HEMA polymers with increasing weight percent (1-5%) of methacrylic acid (MAA) were used to study the effect of negative charge on the material surface on the deposition properties. The copolymers were cross-linked by the use of varying percentage (1 and 10%) of ethylene glycol dimethacrylate (EGDM). The quantity of cross-linker used would affect the equilibrium water content of the copolymer. The equilibrium water contents of the copolymers were lower (18.1-39.3%) in the case of the copolymers with 10% cross-linker, while they were higher (35.1-67.3%) for the copolymers with 1% cross-linker.

6.2.1 Materials and Solutions

The synthesised copolymers were used as they were produced in the laboratory and a uniform section of each sample was selected for the spoilation experiment. The stock protein solutions were made by dissolving 0.1 gram of each protein in saline. The working solutions of proteins were then prepared by diluting the stock solution to the required concentration.

6.2.2 Spoilation Studies

The polymers were cut into disks of a similar size as contact lenses (about 1 cm^2). They were spoiled at room temperature in 2 ml of 0.5 mg/ml of individual protein solutions for 5 days with regular vibrating on a slow speed vibrator. The quantity of the deposited protein was measured by the use of U.V. spectrophotometry at 280 nm. The spoiled copolymer disks were placed right at the bottom of the U. V. cell (Figure 2.21) and the absorption was measured against distilled water. The absorption of the blank was measured before the sample was spoiled and the value was deducted from the absorption of the spoiled sample. In this way the possibility of getting different section which may have different absorption was reduced. Three samples of each type of copolymer were spoiled at the same time with similar spoilation conditions and the absorption value was taken as the mean value obtained for each sample.

The amount of EGDM used is very important, as it directly affects the water content of the polymer, and in the case of higher percentage of cross-linker the water content is lower as the cross-linker replaces the water in the polymer matrix.

6.2.3 Results and Discussions

The build up of lysozyme and lactoferrin on the copolymers produced are shown in Figure 6.1-6.4 for both high and low water content polymers. These show that lysozyme (a small, positively charged protein) absorbs very rapidly, especially on the polymers with higher MAA contents (Figures 6.1 and 6.2). Comparison of Figures 6.1 and 6.2, shows that the absorption is highly dependent on the water content of the polymer. In the case of polymers with 1% cross-linker, the water contents are high and the amount of lysozyme absorbed is about 2.5 times higher than the low water content polymers containing 10% cross-linker (Figure 6.2).

absorbed is about 2.5 times higher than the low water content polymers containing 10% cross-linker (Figure 6.2).

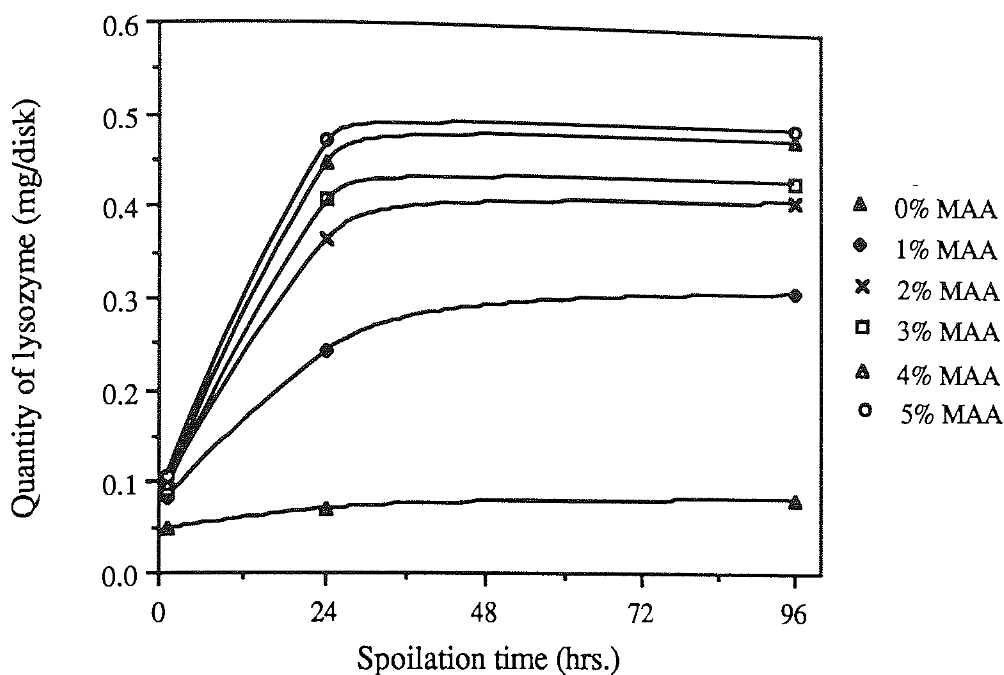


Figure 6.1 The build up of lysozyme on HEMA copolymers with different MAA % and 1% cross-linking agent.

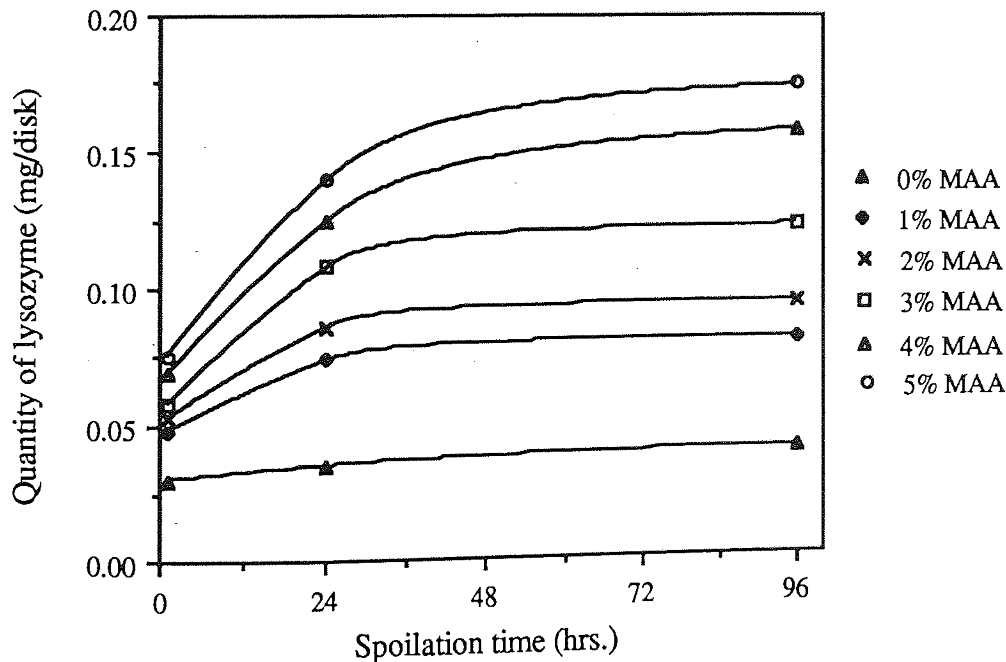


Figure 6.2 The build up of lysozyme on HEMA copolymers with different MAA % and 10% cross-linking agent.

In the case of lactoferrin (a positively charged, larger protein), the increase in the absorption of protein with the increase of MAA and water content is less

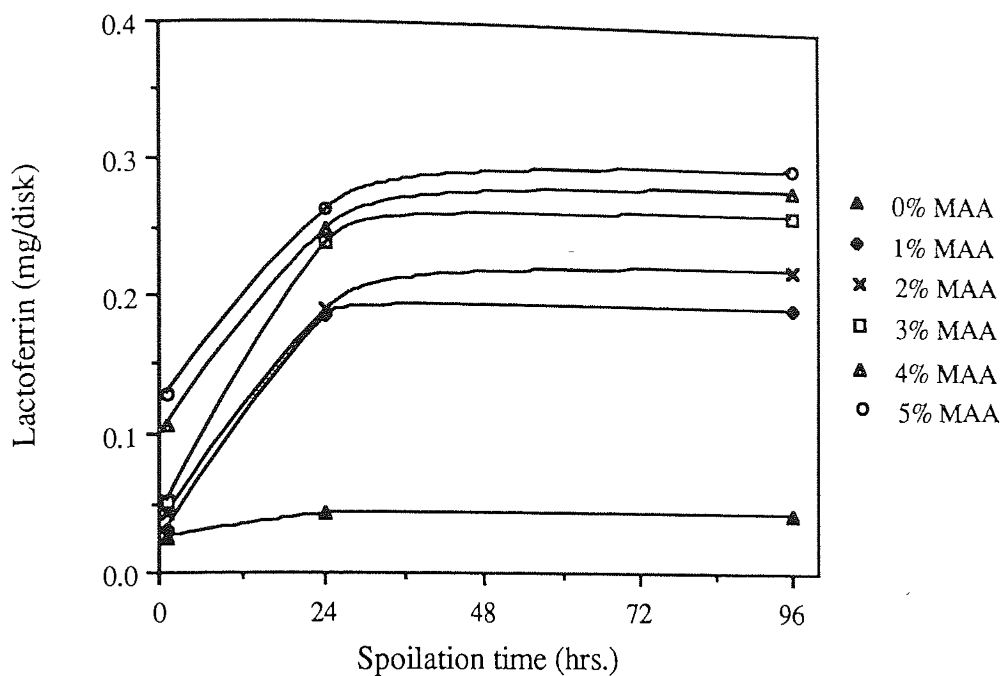


Figure 6.3 The build up of lactoferrin on HEMA copolymers with different MAA % and 1% cross-linking agent.

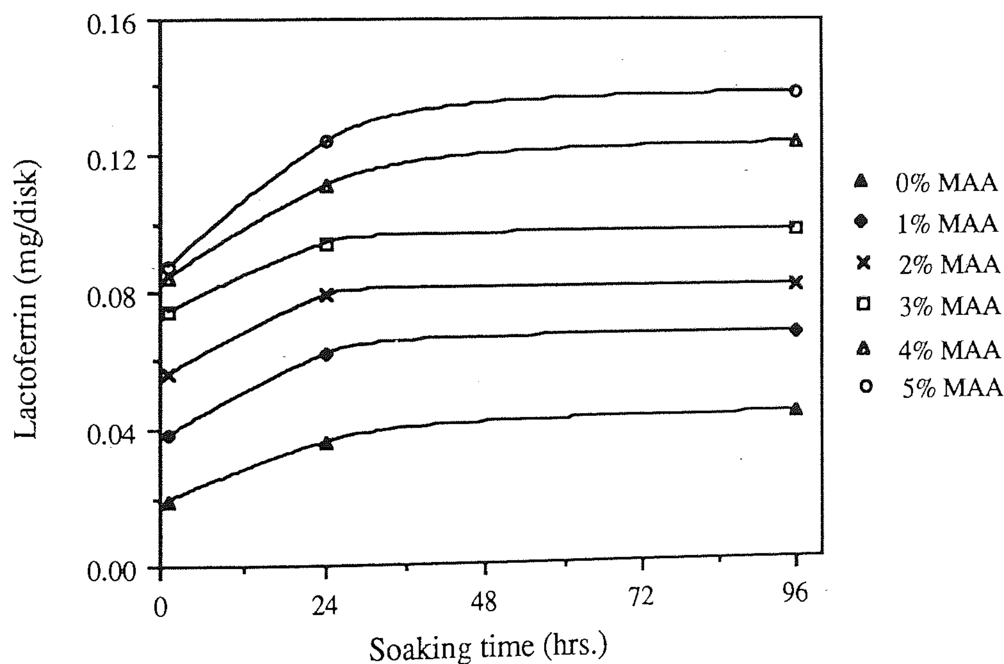


Figure 6.4 The build up of lactoferrin on HEMA copolymers with different MAA % and 10% cross-linking agent.

Figure 6.5 shows the effect of water content of polymers with similar MAA content. The quantities of lysozyme absorbed are higher than that of lactoferrin which would be expected from their sizes. Tables 6.1 and 6.2 together with Figures 6.6 and 6.7 show the quantities of different proteins deposited *in-vitro* during 5 days on the different

copolymers. The characteristics of the copolymer and their water contents are also included in Tables 6.1 and 6.2.

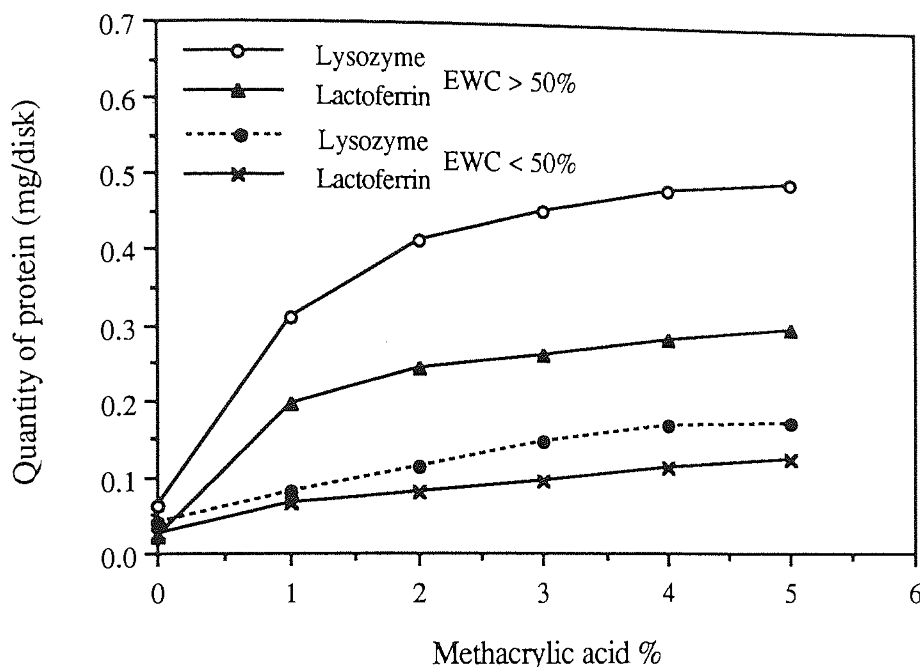


Figure 6.5 The effect of MAA % and water content on the protein uptake by HEMA copolymers.

The informations obtained are summarised in the Tables 6.1 and 6.2 and were used to compare the effect of the polymer stucture in terms of water content and the amount of the negative charge on its surface on the protein absorption. The various proteins used in this study were selected so that a range of size and charges were included. The properties, molecular sizes, charges and shapes of these proteins have been shown in the previous chapters.

Table 6.1 The quantities of proteins deposited on HEMA/MAA copolymers with 10% cross-linker.

% MAA	% EWC	Protein deposited (mg/disk) (± 0.002)				
		Lysozyme	Albumin	Lactoferrin	Ferredoxin	Lactalbumin
0	18.1	0.040	0.021	0.025	0.035	0.028
1	30.1	0.081	0.019	0.065	0.032	0.026
2	33.4	0.089	0.017	0.081	0.031	0.025
3	35.4	0.135	0.016	0.097	0.028	0.025
4	37.2	0.157	0.016	0.115	0.025	0.022
5	39.3	0.174	0.015	0.125	0.024	0.021

Table 6.2 The quantities of proteins deposited on HEMA/MAA copolymers with 1% cross-linker.

% MAA	% EWC	Protein deposited (mg/disk) (± 0.002)				
		Lysozyme	Albumin	Lactoferrin	Ferredoxin	Lactalbumin
0	35.1	0.062	0.032	0.021	0.029	0.025
1	42.3	0.311	0.034	0.194	0.032	0.027
2	52.3	0.412	0.038	0.223	0.033	0.028
3	59.7	0.435	0.043	0.263	0.034	0.028
4	63.2	0.482	0.046	0.282	0.035	0.028
5	67.3	0.493	0.046	0.297	0.036	0.029

6.2.4 Conclusions

The results obtained lead to the conclusion that in the case of highly positive charged proteins such as lysozyme and lactoferrin, the absorption increases with increases in the % of MAA, i.e., the amount of the negative charge on the surface of the polymer.

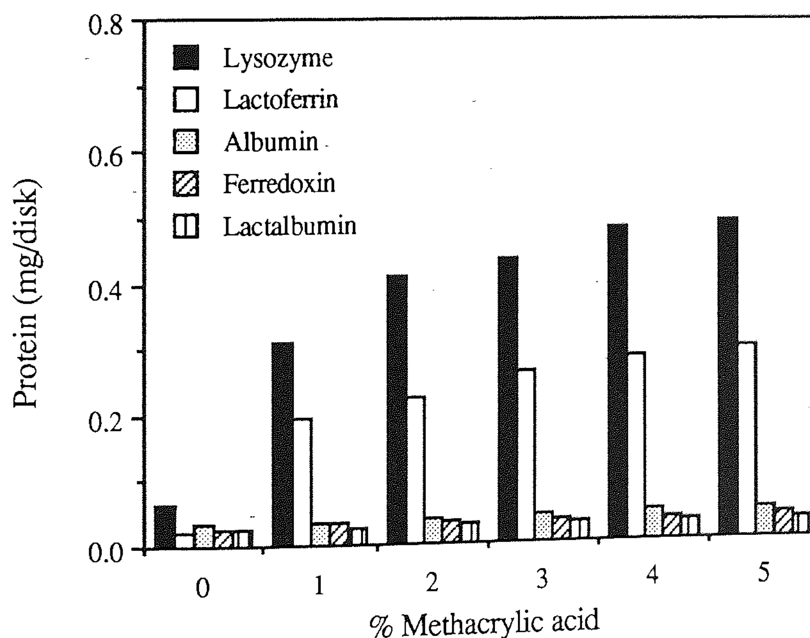


Figure 6.6 The proteins deposited on a HEMA/MAA copolymer with 1% cross-linker in 5 days.

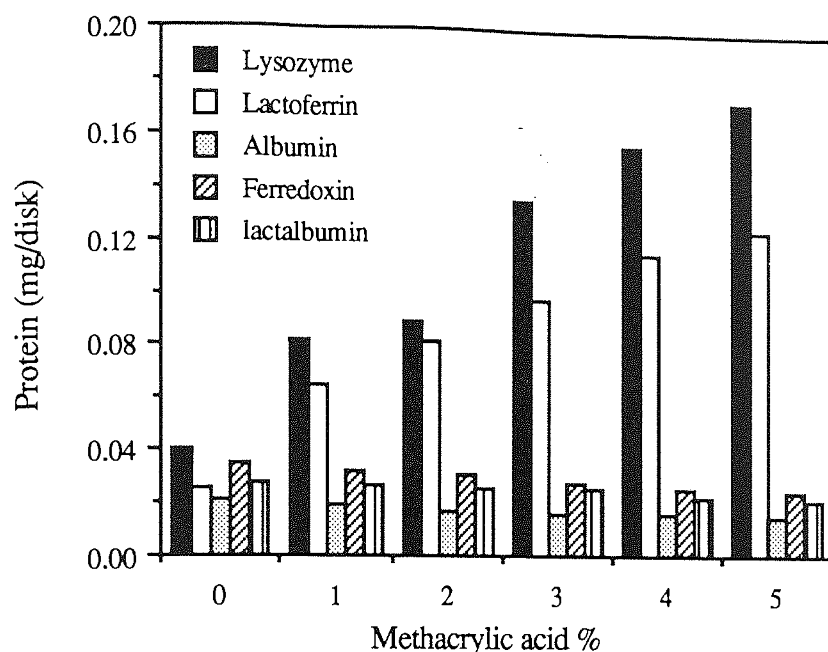


Figure 6.7 The proteins deposited on a HEMA/MAA copolymer with 10% cross-linker in 5 days.

However, the increase in the quantity of the negative charge in terms of MAA % have a slightly opposite effect on the negatively charged proteins (such as ferredoxin and lactalbumin) uptake by the polymer material. This is more pronounced in the case of polymers in Table 6.1 (10% cross-linker) as their water content is lower and does not affect the absorption, while in polymers with 1% cross-linking agent (Table 6.2) the increase in water contents slows down the effect of negative charge on the surface of the negatively charged proteins.

6.3 Protein Absorption on New HEMA Copolymers

6.3.1 Introduction

A group of copolymers were synthesized using HEMA as the major monomer and a range of comonomers to investigate their effect on the biological behaviour of pure poly(HEMA). Ethylene glycol dimethacrylate (EGDM) with a concentration of 1% was used as cross-linker. The equilibrium water contents of the copolymers produced were between 35-70%. The comonomers were loaded with 1-5% weight into the polymer.

The selection of comonomers were based on their charge and hydrophobicity. The chemical structures of itaconic acid (ITC), N-vinylimidazol (NVI) and N-(3-

sulphopropyl)-N-methacryloxyethyl-N, N-dimethyl ammonium betaine (SPE) are given in Figure 6.8.

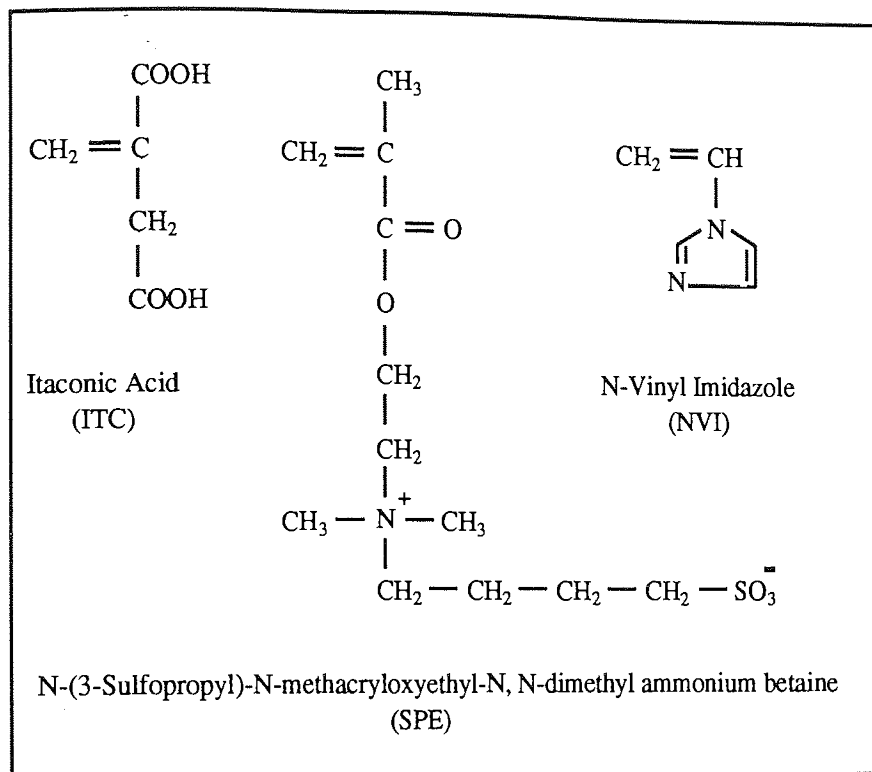


Figure 6.8 The chemical structures of the monomers used for the copolymerisation with HEMA.

6.3.2 Materials and Solutions

The protein solutions were made as stock solutions with concentrations of 1-2 mg/ml. The working solutions were then prepared as required by diluting these solutions. The synthesised polymers were cut into disks from a uniform part of the material.

6.3.3 Methodology

The produced hydrogels were cut into disks of similar sizes to contact lenses for the study of their protein spoilation. They were spoiled in 2 ml individual solutions of different proteins (0.5 mg/ml) for 5 days at room temperature (22 ± 2°C) with constant shaking on a slow speed shaker. The quantities of proteins on their surfaces were measured after a rinse with distilled water using the direct U.V. absorption at 280 nm (Figure 2.21). The absorptions of each blank material was measured before the spoilation to avoid error due to the difference in the absorption of different polymer section. Each spoilation process was carried out on three samples separately and the results were taken as a mean value of the three readings.

6.3.4 Results and Discussion

The different proteins with a range of charge and sizes have deposited to different degrees on the copolymers of various chemical compositions. Table 6.3 shows the quantities of the deposited proteins together with the type and amount of comonomer used for the production of copolymer.

Figures 6.9 - 6.11 show the quantities of different proteins deposited on each copolymer. Comparing Figure 6.9 with 6.10 and 6.11, proves that the chemical structure of the monomer used for the copolymerisation process has a distinct effect on the behaviour of the proteins in terms of spoilations by proteins.

Table 6.3 The Quantities of different proteins absorbed on HEMA copolymers.

Polymer	Monomer Wt.(%)	% EWC	Protein deposited (mg/disk) (± 0.002)				
			Lysozyme	Albumin	Lactoferrin	Ferredoxin	Lactalbumin
A	ITC (0.0)	35-70	0.060	0.034	0.021	0.026	0.026
B	ITC (1.0)		0.145	0.042	0.098	0.023	0.024
C	ITC (2.0)		0.342	0.049	0.264	0.021	0.022
D	ITC (3.0)		0.446	0.054	0.286	0.019	0.020
E	ITC (4.0)		0.496	0.067	0.298	0.018	0.018
F	ITC (5.0)		0.562	0.069	0.301	0.017	0.019
G	NVI (0.0)	35-40	0.065	0.030	0.021	0.025	0.024
H	NVI (1.0)		0.068	0.032	0.021	0.028	0.028
J	NVI (2.0)		0.054	0.031	0.018	0.029	0.029
K	NVI (3.0)		0.046	0.028	0.017	0.034	0.031
L	NVI (4.0)		0.042	0.026	0.012	0.038	0.041
M	NVI (5.0)		0.040	0.026	0.009	0.041	0.042
N	SPE (0.0)	35-44	0.063	0.032	0.023	0.024	0.024
P	SPE (1.0)		0.065	0.032	0.045	0.026	0.027
Q	SPE (2.0)		0.062	0.033	0.047	0.027	0.029
R	SPE (3.0)		0.057	0.035	0.049	0.027	0.029
S	SPE (4.0)		0.054	0.036	0.052	0.029	0.030
T	SPE (5.0)		0.054	0.036	0.055	0.031	0.032

The deposition of positively charged proteins increases with the increase in the content of itaconic acid (ITC) in the copolymer. On the other hand, the negatively charged (such as

ferredoxin and lactalbumin) deposite less on the copolymers with higher ITC contents (Figure 6.9). The chemical structure of ITC shown in Figure 6.8 reveals that there are more negatively charged groups present than in the structure of MAA. This point is even more proved in Figure 6.10 and 6.11 which show a decrease in the absorption of positively charged proteins with increasing the NVI and SPE contents of the copolymers, while a slight increase is shown in the case of negatively charged proteins. The structures of these monomers are also shown in Figure 6.8.

The higher degree of positive charge on SPE, which is due to the presence of a quaternary nitrogen, causes a larger decrease in the absorption of positively charged proteins to the copolymer made by using monomer. Figures 6.12 and 6.13 compare the deposition of a positively and a negatively charged protein on the three copolymers.

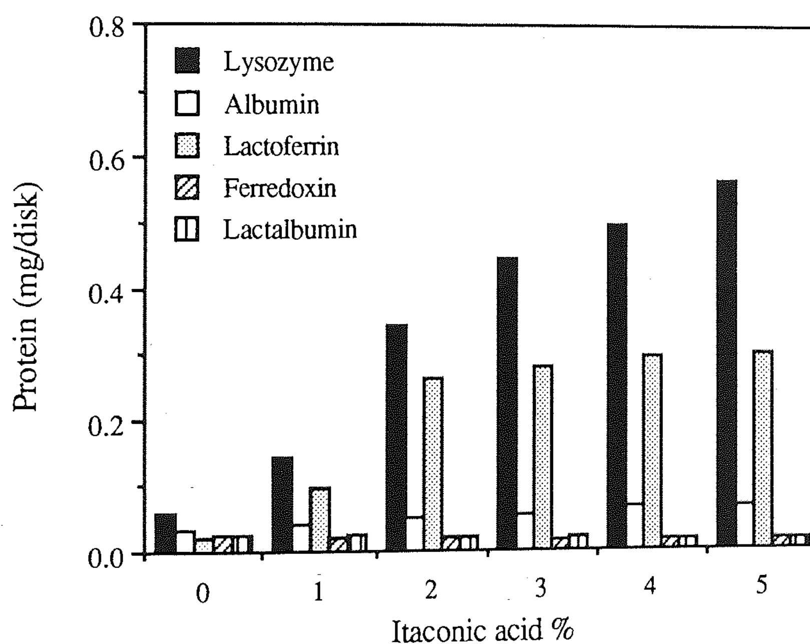


Figure 6.9 The effect of itaconic acid copolymerised with HEMA on the up-take of different proteins.

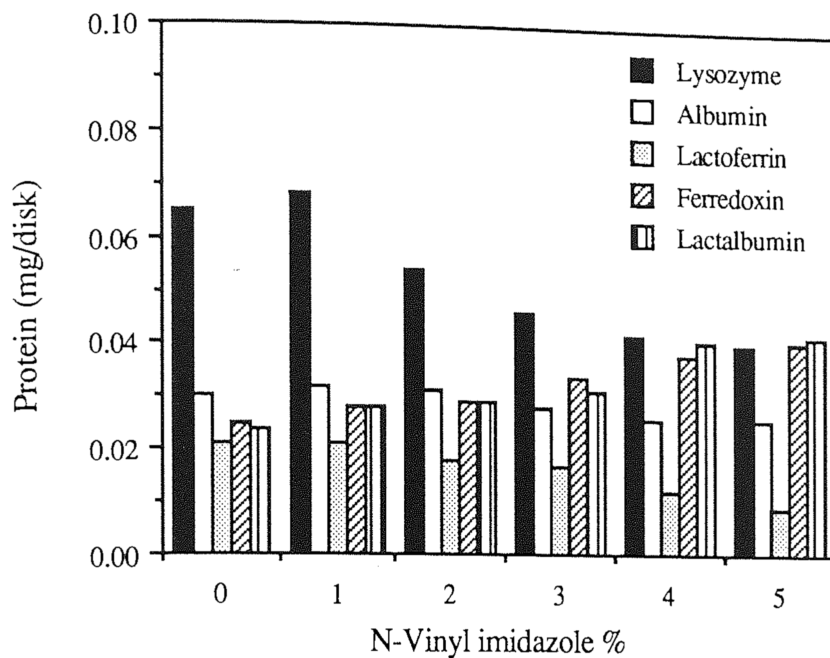


Figure 6.10 The effect of NVI % on the quantity of the protein adsorbed on HEMA/NVI co-polymers.

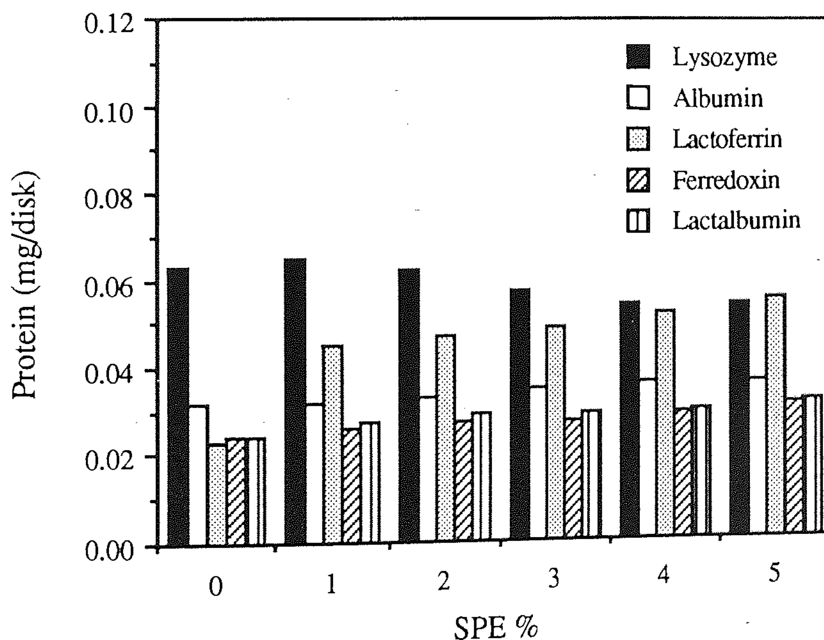


Figure 6.11 The quantity of different proteins adsorbed on HEMA/SPE co-polymers with various % of co-monomer.

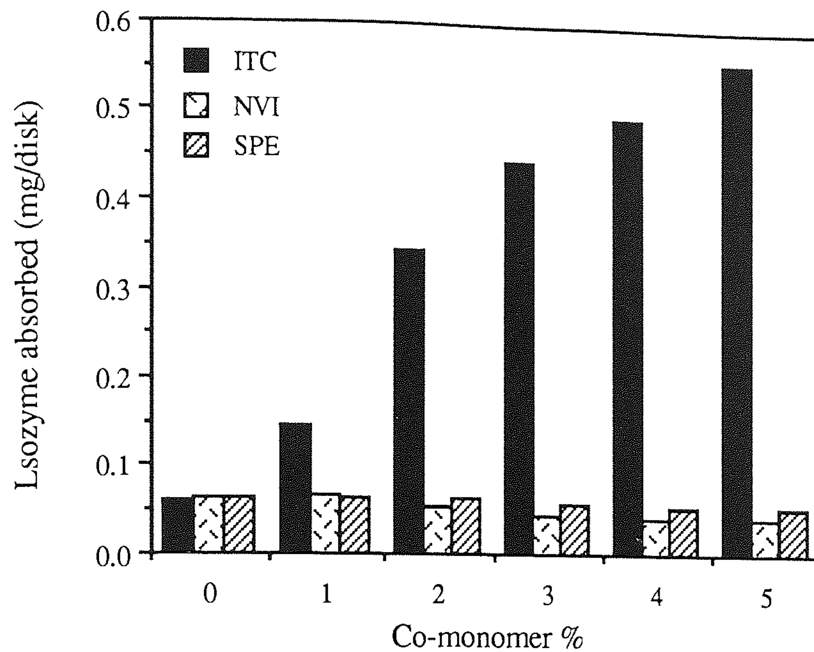


Figure 6.12 The quantity of a positively charged protein absorbed on different HEMA co-polymers.

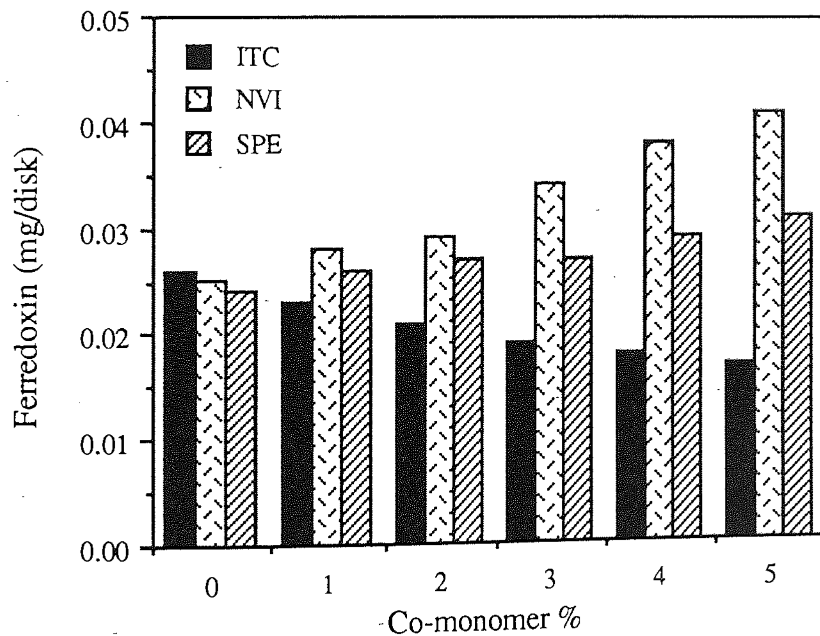


Figure 6.13 The quantity of a negatively charged protein absorbed on different HEMA co-polymers.

6.3.5 Conclusions

The structure of HEMA biomaterials can be modified by the co-polymerisation of small amounts of some other monomers. The co-monomers used have different effects on the absorption of different proteins. Normally, the presence of methacrylic acid residues in

the monomer structure creates more negative charges on the copolymer causing positively charged proteins to be absorbed to a higher degree.

On the other hand, a decrease in the absorption of positively charged proteins (e.g. lysozyme and lactoferrin), is acieved by copolymerising HEMA using monomers with slightly hydrophobic character or containing quaternary nitrogen, i.e. higher positive charges. This is also supported by the use of negatively charged proteins which absorb less on hydroxy containing and more on the positively charged and hydrophobic polymer materials.

6.4 The Effect of N-Containing Monomers on the Spoilation of HEMA

6.4.1 Introduction

In the previous section the effect of different comonomers polymerised with HEMA on the stucture of polymer produced, i.e. on the protein deposition were studied. It is the object of this part of the work to measure the effect of nitrogen containing monomers and, therefore, be able to comment on the role of nitrogen on the structure of the copolymer produced in terms of its interaction with different proteins.

6.4.2 Experimental Procedure

Three different monomers all containing nitrogen were used for copolymerisation with HEMA. The polymerisation was carried out as bulk polymerisation at 60° C for three days. The produced polymers were hydrated in distilled water with frequent changes for a period of two weeks. The cross-linker for all these membranes was 1% ethylene glycol dimethacrylate (EGDM). The copolymer composition was between 90:10 and 50:50. The resulted copolymer was cut into disks of about contact lens size (1 cm^2), which were then spoiled in solutions of 0.5 mg/ml of different proteins for five days at room temperature. The spoilation solutions with the disks in them were shaken constantly on a slow speed shaker. The quantity of protein which deposited on the materials was measured by U.V. at 280 nm and the value for the absorption of the same blank was deducted from the absorption obtained for the spoiled samples. The monomers used were acrylomorpholine (AMO), N-Vinylpyrolidone, (NVP) and N, N dimethyl acrylamide (NNDMA), the sructures of which are shown in Figure 6.14.

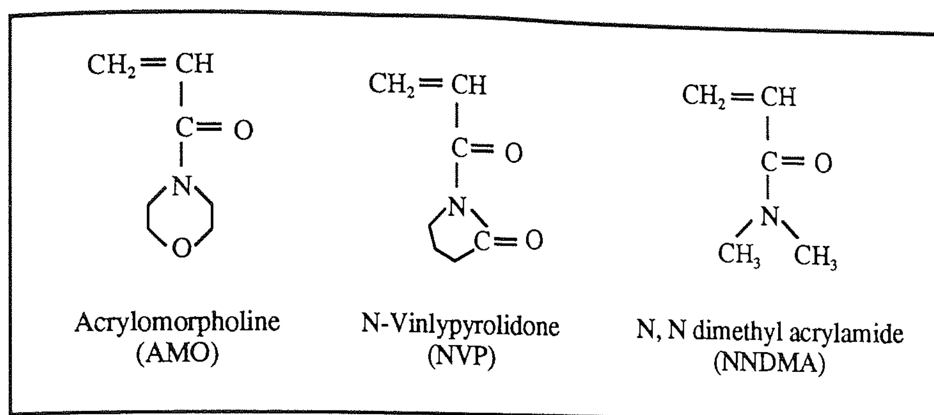


Figure 6.14 The structures of monomers used for copolymerisation with HEMA.

6.4.3 Results and Discussions

The compositions of the copolymers and the results of the protein spoilations can be seen in Table 6.4. It can be shown from these information that the N-containing monomers have caused different effect on the absorption of the proteins on the copolymers produced. This effect is slightly dependent on the structure of the proteins in terms of their charge and the chemical structure of the comonomer.

Figures 6.15, 6.16 and 6.17 show the build up of different proteins on copolymers of increasing percentage of the comonomer. The conclusions from these figures are shown in Figure 5.18 in which the protein absorption is plotted against the comonomer type with the same weight percent (50 %). It can be seen from the results that in this case, NVP is the preferred monomer for the copolymerisation with HEMA to produce a copolymer that will resist the biological environment, i.e. deposition of lysozyme and lactoferrin. Looking at the structures of three comonomers proves that NVP carries a high positive charge, while NNDMA has a slightly positive charge due to the mesomeric effect of the methyl groups on the nitrogen. On the other hand, AMO is a hydrophilic monomer with low positive charge. The protein absorption, however, is slightly variable and not consistent which can match these structures. The reason can be due to the very low quantities of protein absorbed which shows very close results. The results presented here are the mean values of three similar spoilation experiment carried out under the same conditions (± SD). The causes of error and calculation of the standard deviations (SD) are shown in Appendix IV.

Table 6.4 The composition of the N-containing copolymers and their spoilation results.

HEMA (%)	Co-monomer (%)	Protein deposited (mg/disk) (± 0.002)				
		Lysozyme	Albumin	Lactoferrin	Ferredoxin	Lactalbumin
90	NVP 10	0.030	0.026	0.025	0.034	0.030
80	NVP 20	0.025	0.029	0.022	0.036	0.036
70	NVP 30	0.022	0.036	0.018	0.044	0.041
60	NVP 40	0.018	0.038	0.012	0.046	0.046
50	NVP 50	0.012	0.039	0.010	0.049	0.048
90	AMO 10	0.052	0.027	0.033	0.035	0.028
80	AMO 20	0.066	0.031	0.036	0.033	0.025
70	AMO 30	0.073	0.033	0.051	0.032	0.022
60	AMO 40	0.076	0.035	0.055	0.032	0.018
50	AMO 50	0.082	0.041	0.054	0.029	0.01
90	NNDMA 10	0.033	0.023	0.024	0.033	0.031
80	NNDMA 20	0.036	0.026	0.022	0.036	0.033
70	NNDMA 30	0.030	0.029	0.018	0.040	0.036
60	NNDMA 40	0.030	0.028	0.022	0.042	0.038
50	NNDMA 50	0.027	0.031	0.016	0.046	0.038
100	--- --	0.032	0.024	0.023	0.035	0.028

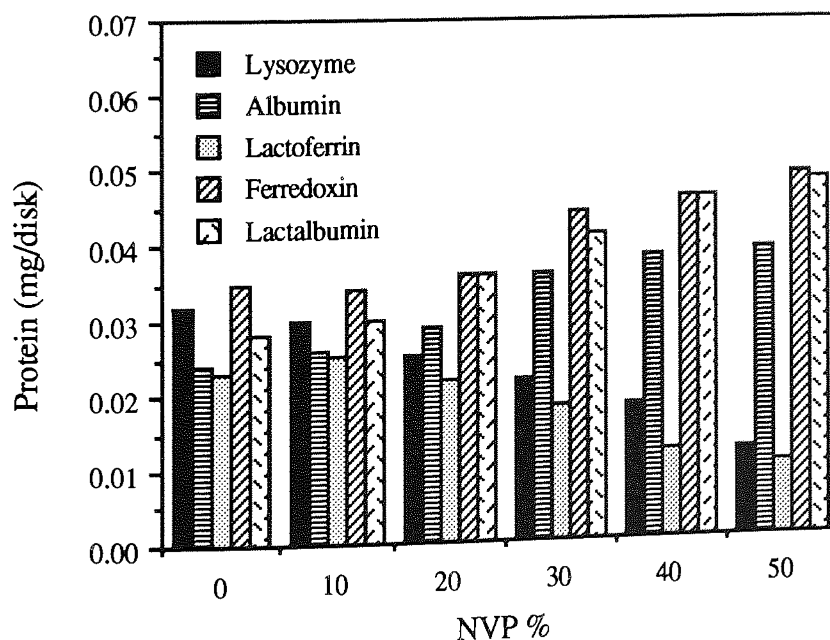


Figure 6.15 The deposition of different proteins on HEMA/NVP copolymers.

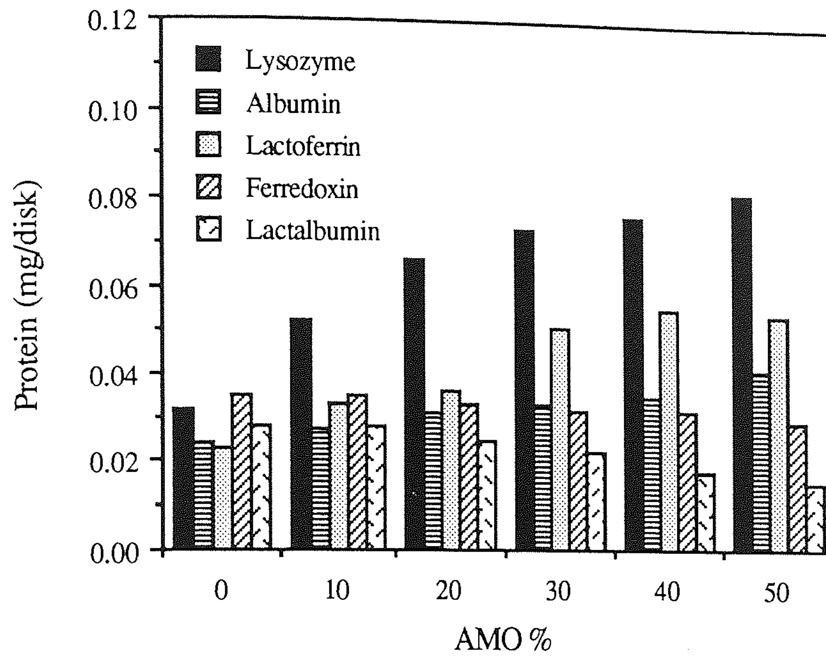


Figure 6.16 The absorption of different proteins on HEMA/AMO co-polymers.

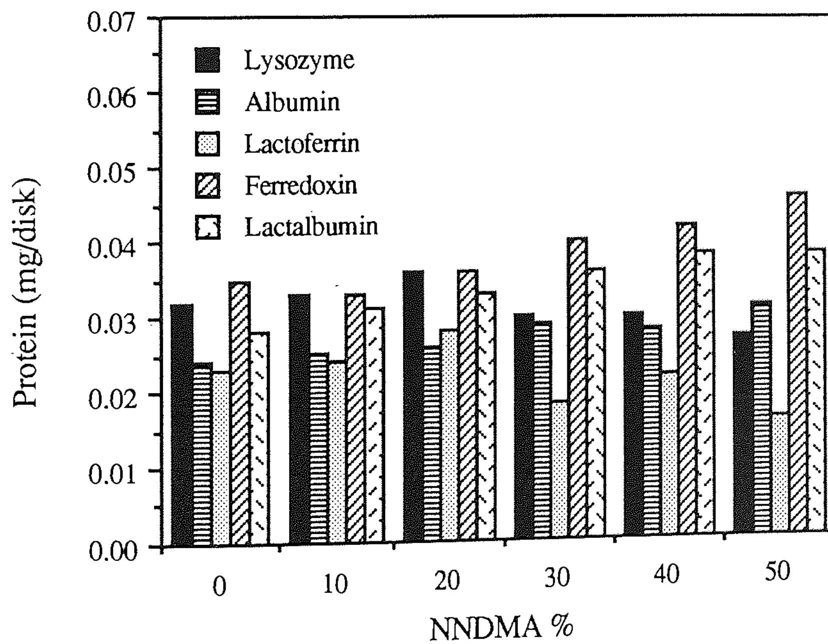


Figure 6.17 The absorption of different proteins on HEMA/NNDMA co-polymers.

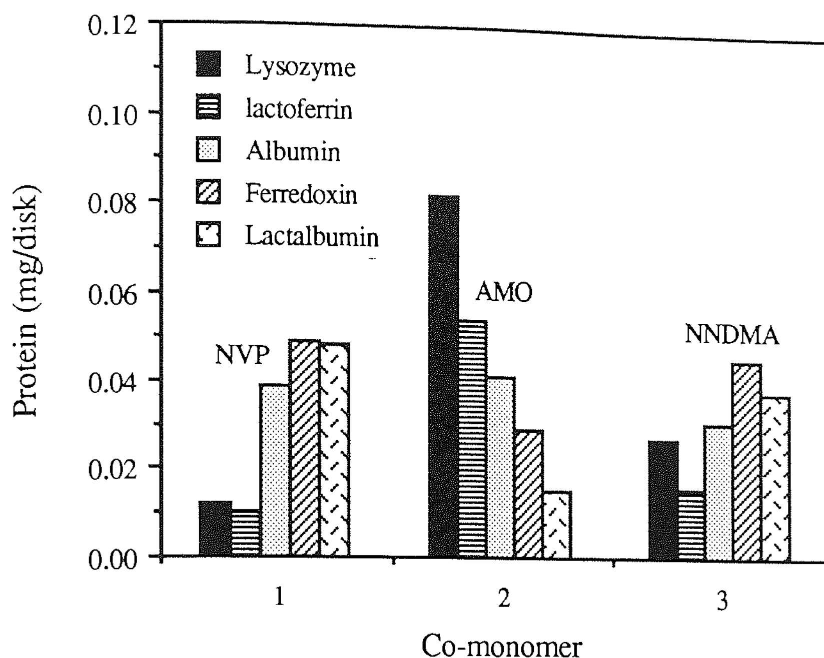


Figure 6.18 Comparison between the absorption of different proteins by HEMA co-polymers composed of 50% co-monomer.

6.5 The Protein Spoilation of Positively Charged Co-polymers

6.5.1 Introduction

Two sets of co-polymers were synthesised with the aim of positively charged surfaces. Their equlibrium water contents varied in each series ranging from 18.4 to 70.7%. These monomers were N, N-dimethyl acrylamide (NNDMA), acrylomorpholine (AMO), cyclohexylmethacrylate (CHMA) and N-vinyl imidazole (NVI) the structures of which are shown in Figure 5.19.

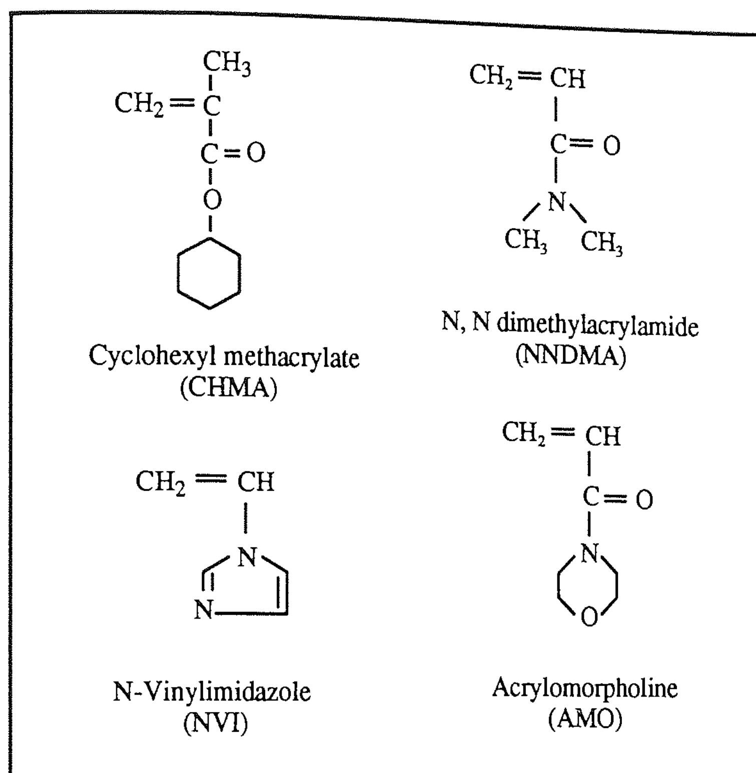


Figure 6.19 The chemical structures of positively charged monomers.

6.5.2 Spoilation Methodology

The copolymers produced were cut into disks of about $1cm^2$. The disks were spoiled in 2ml of 0.3 mg/ml individual protein solutions for 5 days at room temperature. The quantity of the protein deposited on each was measured by the use of direct U.V. at 280 nm on the disks after a rinse with distilled water. A blank of the same copolymer material was used in each case and its absorption was measured before spoilation. In each case three samples of the same copolymer were soiled in protein solution at the same conditions and the results were calculated as the mean value of the three similar measurements.

6.5.3 Results and Discussions

The quantities of different proteins deposited on different copolymers are presented in Tables 6.5 and 6.6. The values given in these tables are the mean values obtained from the spoilation of the same copolymer in the same protein solution. In most of the cases the values obtained were similar with variations of around 5 %. The U. V. absorption readings were very accurate and only varied in the third figure with a standard deviation of $\pm$ 0.002 mg/disk as indicated in Tables 6.5 and 6.6.

The equilibrium water contents of the co-polymers containing NNDMA are higher than those containing AMO. In both groups of the copolymers, NVI and CHMA are present which will makes the comparison between the effects of CHMA and AMO more reasonable.

Figures 6.20 and 6.21 compare the effect of quantities of NVI used in the co-polymerisation mixture on the spoilation behaviour of the NNDMA/CHMA co-polymer.

Table 6.5 Properties and spoilation behaviour of NNDMA / CHMA / NVI co-polymers.

Monomer	NVI %	EWC %	Protein deposited (mg/disk) (± 0.002)				
			Lysozyme	Insulin	Lactoferrin	Ferredoxin	Ribonuclease
80 %	0	64.1	0.051	0.030	0.039	0.037	0.052
NNDMA	5	61.8	0.051	0.034	0.031	0.088	0.047
+	10	66.3	0.039	0.024	0.035	0.066	0.038
20 %	15	65.5	0.025	0.016	0.022	0.072	0.021
CHMA	20	70.7	0.043	0.048	0.043	0.071	0.042
70 %	0	48.1	0.011	0.005	0.008	0.032	0.010
NNDMA	5	49.6	0.013	0.007	0.009	0.039	0.012
+	10	51.2	0.016	0.008	0.010	0.046	0.013
30 %	15	52.0	0.019	0.009	0.011	0.052	0.015
CHMA	20	54.9	0.021	0.010	0.012	0.063	0.018

Table 6.6 The compositions and the spoilations of NNDMA / AMO/ NVI co-polymers.

Monomer	NVI %	EWC %	Protein deposited (mg/disk) (± 0.002)				
			Lysozyme	Insulin	Lactoferrin	Ferredoxin	Ribonuclease
80 %	0	35.5	0.016	0.009	0.010	0.029	0.011
AMO	5	41.2	0.017	0.011	0.012	0.035	0.013
+	10	44.2	0.021	0.013	0.013	0.042	0.016
20%	15	45.4	0.024	0.015	0.014	0.048	0.018
CHMA	20	46.0	0.026	0.018	0.016	0.054	0.020
70 %	0	18.4	0.006	0.005	0.004	0.019	0.006
AMO	5	23.0	0.007	0.005	0.004	0.021	0.007
+	10	28.7	0.007	0.006	0.006	0.029	0.008
30 %	15	28.6	0.009	0.008	0.007	0.033	0.009
CHMA	20	24.8	0.012	0.009	0.008	0.038	0.010

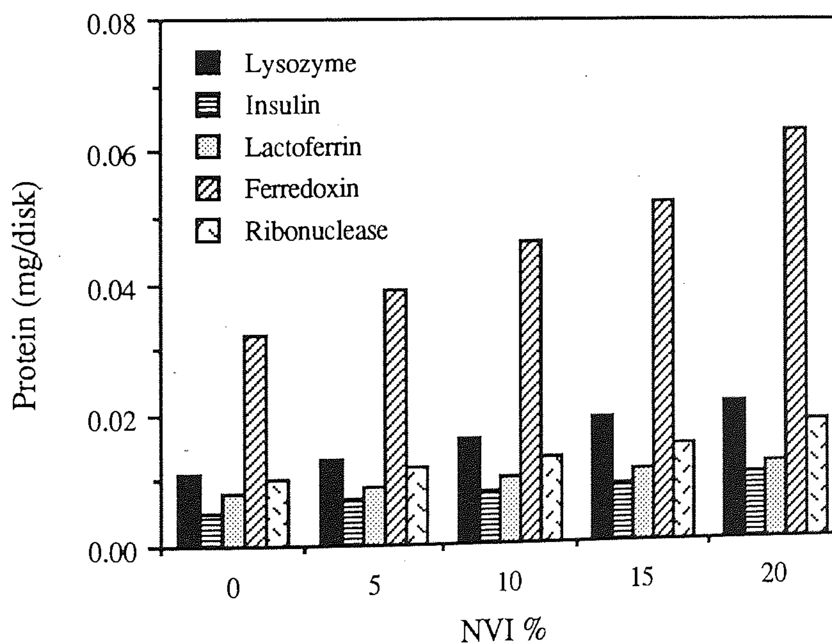


Figure 6.20 The effect of NVI % on the protein absorption by co-polymers composed of 70 % NNDMA and 30 % CHMA.

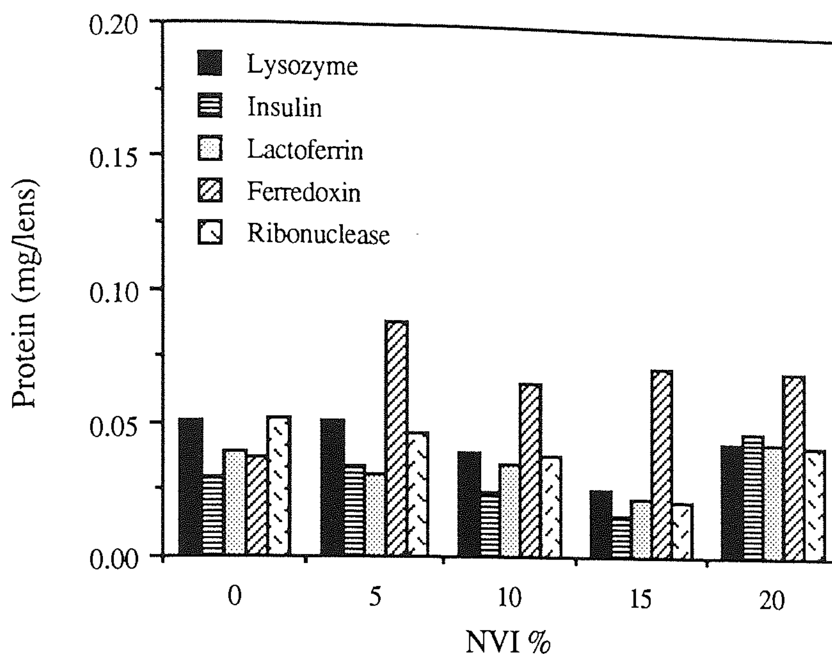


Figure 6.21 The effect of NVI% on the protein absorption by co-polymers composed of 80% NNDMA and 20% CHMA.

Figures 6.22 and 6.23 compare the quantities of proteins deposited on the co-polymers of AMO/CHMA. The differences in the equilibrium water contents must be considered when interperating these results. The increase in the quantity of NVI causes a slight increase on the absorption of positively charged proteins in all cases, with a more pronounced effect on the up-take of negatively charged proteins.

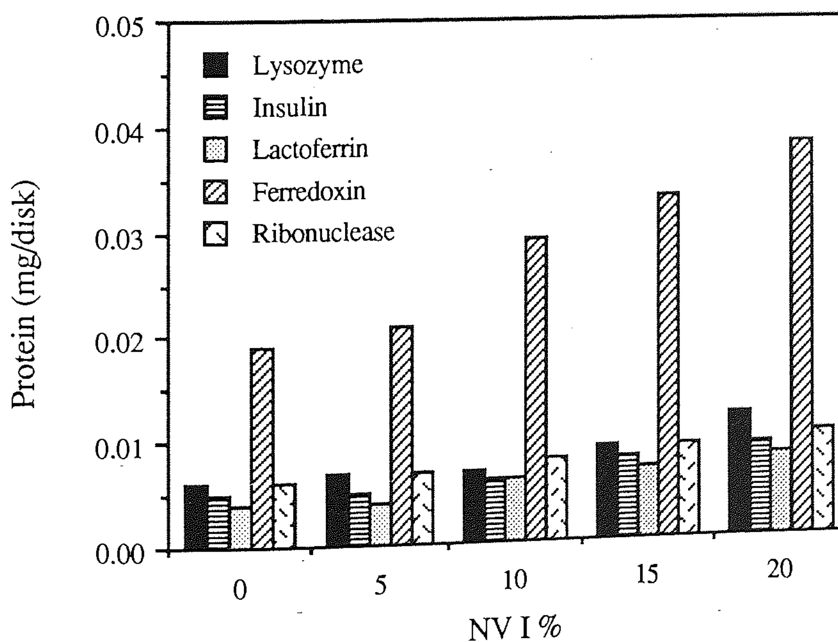


Figure 6.22 The effect of NVI% on the protein absorption by co-polymers composed of 70% AMO and 30% CHMA.

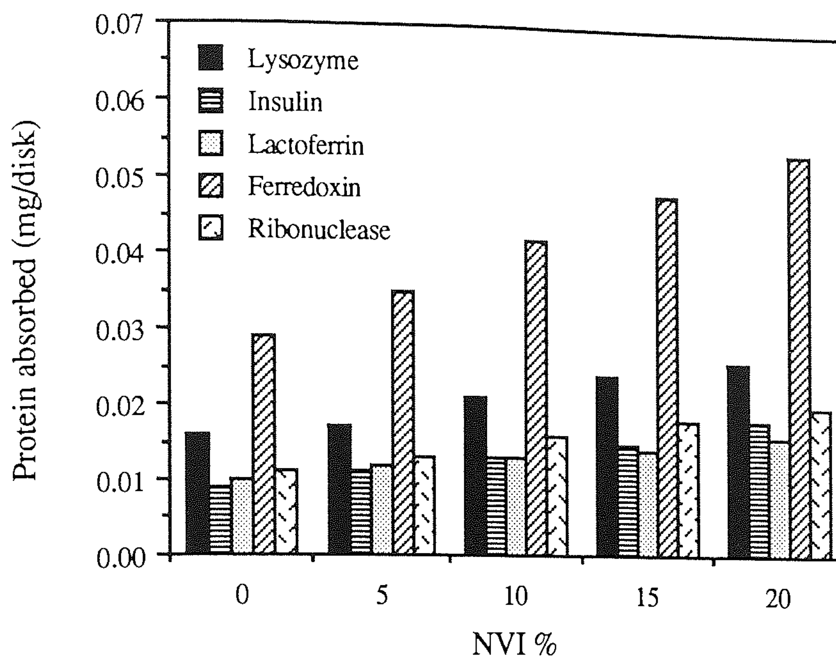


Figure 6.23 The effect of NVI% on the protein absorption by co-polymers composed of 80% AMO and 20% CHMA.

6.6 Novel Positively Charged Co-polymers as a New Group of Biomaterials

6.6.1 Introduction

In continuing the research on the spoilation properties of positively charged biomaterials, four new sets of co-polymers were synthesized. The co-polymer compositions were very similar to the previous set (Section 6.5), but a new monomer, dimethylamino ethylmethacrylate (DMAEMA) was used in place of NVI. The effect of increasing the weight of this new monomer was studied as before. Figure 6.24 shows the chemical structure of the monomers used in this part of the study.

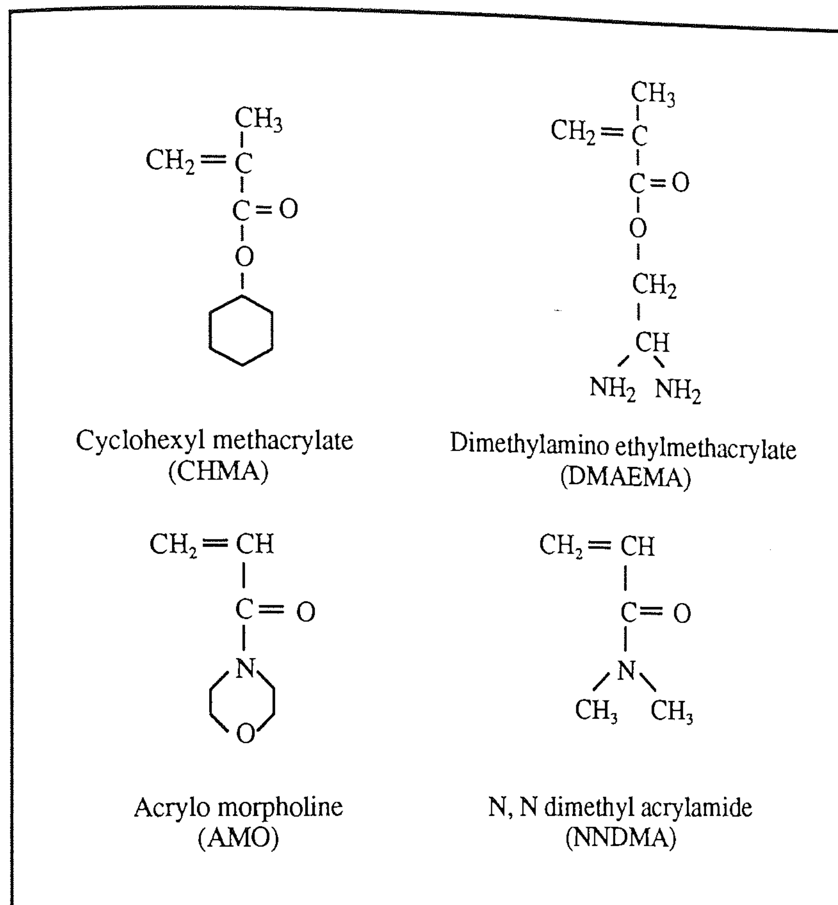


Figure 6.24 The monomers used for the co-polymerisation of novel positively charged biomaterials.

6.6.2 In-vitro Spoilation Procedure

The copolymers which were synthesied within the research group, were cut into identical disks of 1cm diameter. They were spoiled individually in 3.0ml of 0.3-0.5 mg/ml of different protein solutions. The disks were left to spoil for 5 days at room temperature with occasional vibration. The quantities of the protein deposited on each disk was measured using U. V. absorption at 280 nm. The absorption of the blank samples was measured prior to their spoliation to avoid the inconsistency error. The surface of the material was rinsed once with distilled water before the measurements to wash off the loosely deposited protein from the surface.

6.6.3 Results and Discussions

The composition of the copolymers used in this study together with the results of their spoilation resistance to different protein solutions are shown in Table 6.7.

Table 6.7 The spoilage properties of NNDMA/CHMA/DMAEMA copolymers.

Monomer	DMAEMA %	EWC %	Protein deposited (mg/disk) (± 0.002)				
			Lysozyme	Insulin	Lactoferrin	Ferredoxin	Ribonuclease
80 %	0	57.5	0.020	0.013	0.014	0.033	0.015
NNDMA	5	61.5	0.020	0.013	0.014	0.031	0.014
+	10	65.8	0.019	0.012	0.016	0.028	0.014
20%	15	64.7	0.016	0.011	0.015	0.026	0.012
CHMA	20	67.9	0.014	0.009	0.014	0.022	0.011
70 %	0	52.9	0.010	0.009	0.008	0.023	0.010
NNDMA	5	52.3	0.009	0.009	0.008	0.021	0.010
+	10	52.9	0.009	0.008	0.009	0.019	0.009
30 %	15	52.2	0.008	0.008	0.009	0.018	0.008
CHMA	20	54.4	0.008	0.007	0.008	0.018	0.008

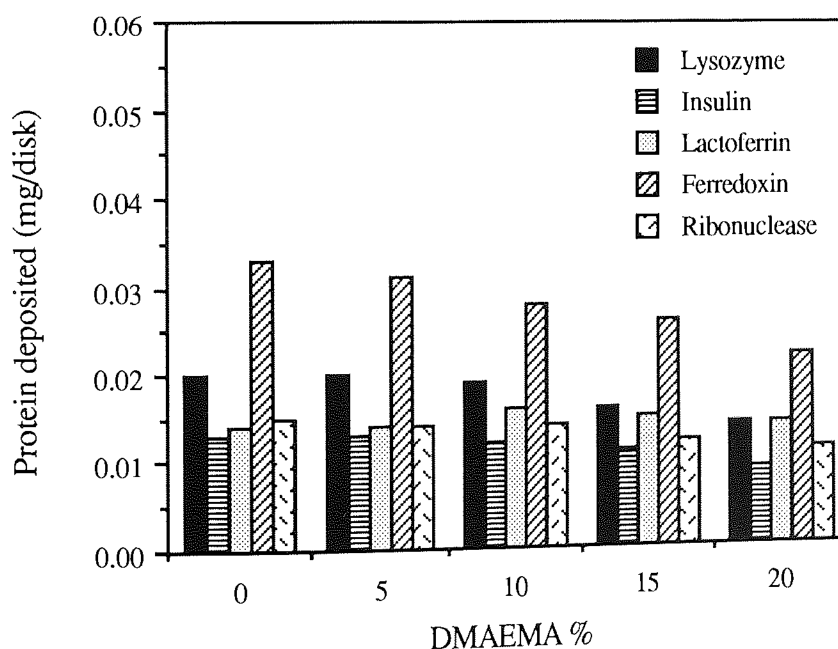


Figure 6.25 The effect of DMAEMA content on the protein up-take by 80% NNDMA and 20% CHMA copolymers.

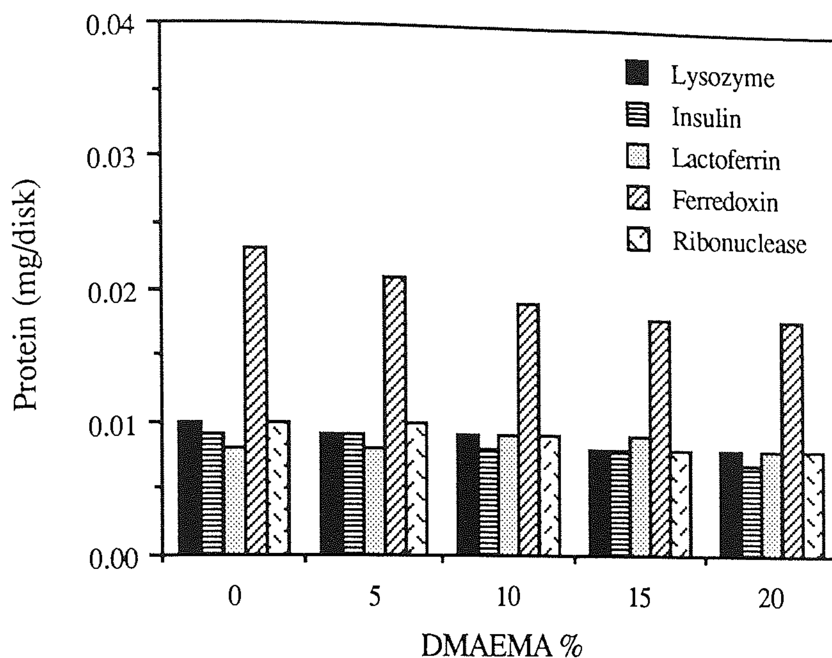


Figure 6.26 The effect of DMAEMA content on the protein up-take by 70% NNDMA and 30% CHMA co-polymers.

Table 6.8 The spoilage properties of AMO/CHMA/DMAEMA co-polymers.

Monomer	DMAEMA %	EWC %	Protein deposited (mg/disk) (± 0.002)				
			Lysozyme	Insulin	Lactoferrin	Ferredoxin	Ribonuclease
80 % AMO + 20% CHMA	0	35.2	0.019	0.012	0.013	0.032	0.014
	5	42.4	0.018	0.011	0.012	0.029	0.013
	10	45.5	0.016	0.011	0.011	0.025	0.012
	15	46.9	0.013	0.010	0.010	0.023	0.011
	20	51.9	0.013	0.008	0.010	0.022	0.010
70 % AMO + 30 % CHMA	0	24.9	0.009	0.008	0.007	0.021	0.009
	5	26.1	0.009	0.008	0.007	0.020	0.009
	10	30.7	0.008	0.009	0.006	0.018	0.010
	15	30.8	0.009	0.009	0.005	0.017	0.007
	20	34.6	0.008	0.008	0.005	0.014	0.007

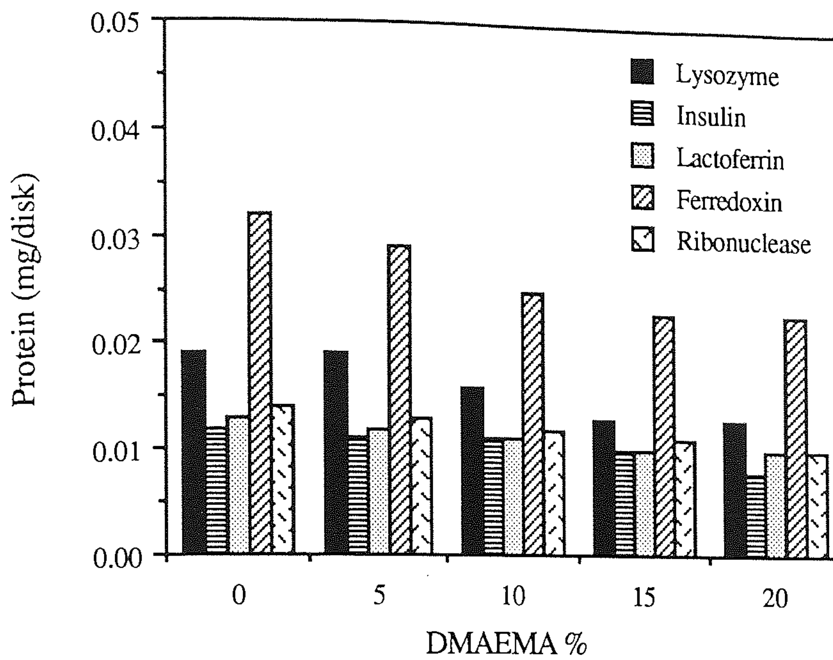


Figure 6.27 The effect of DMAEMA content on the protein up-take by 80% AMO and 20 % CHMA co-polymers.

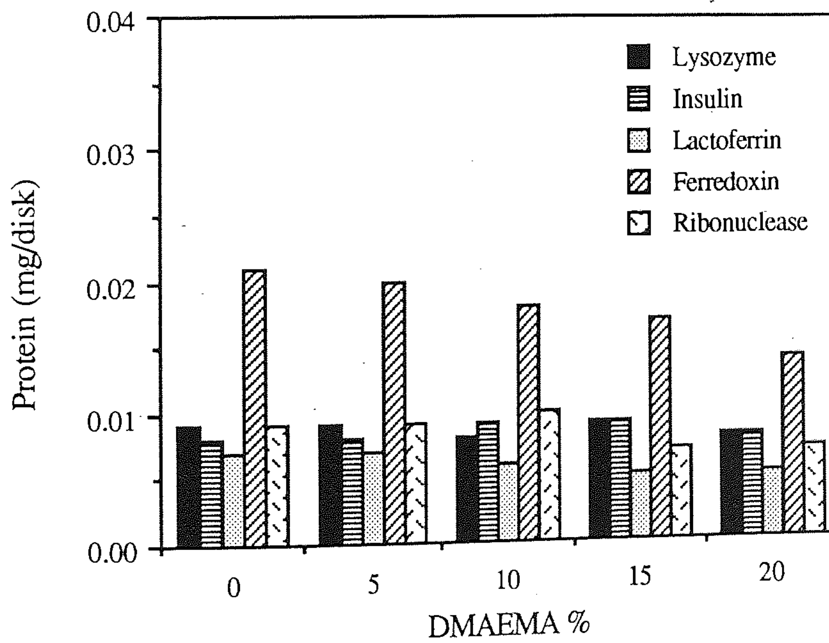


Figure 6.28 The effect of DMAEMA content on the protein up-take by 70% AMO and 30% CHMA co-polymers.

6.7 Protein Interactions with New Hydrogel Biomaterials

6.7.1 Introduction

In this section, a group of novel hydrogels were synthesized to resist physiological spoilation and the resulting co-polymers were tested for this behaviour using cell culture and *in-vitro* protein spoilation. These were new polyether-based vinylic monomers which were incorporated into poly(HEMA) based hydrogels, which are of interest to the contact lens industry.

It has been shown that polyethylene oxide plays a significant role in reducing the absorption of biological species to substrates [109]. Hydrogels examined during this study included polyethylene oxide (PEO) modified poly(HEMA) based gels. The structures of HEMA and a PEO methacrylate (PEGMA) are compared in Figure 6.29. The HEMA pendant group is OCH_2CH_2OH while, it is a multiple (OCH_2CH_2) ether linked structure that terminates in either OH or OCH_3 (hydroxy or methoxy terminated) on a polyethylene glycol methacrylte (PEGMA).

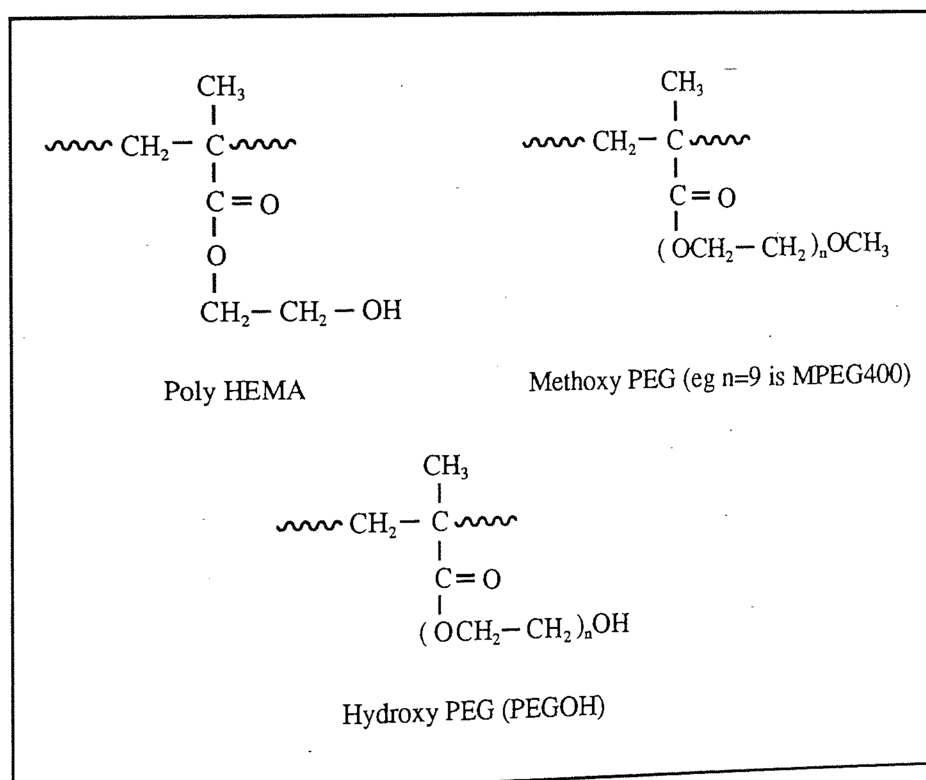


Figure 6.29 The chemical structures of PEG and MPEG.

6.7.2 *Experimental Procedure*

The effect of polyethers in reducing the protein absorption onto hydrogels is known and the following experimental studies were carried out.

1. Synthesis of various molecular weight polyethylene glycols (PEGOHs).
2. Synthesis of methoxy polyethylene glycols (MPEGs) of various molecular weights.
3. Incorporation of the various adducts into poly(HEMA) based hydrogels.
4. Study of some bulk and surface properties of hydrogels produced, these included a series of cell culture and *in-vitro* protein spoilation.

6.7.2.1 *Protein spoilations*

A series of poly(HEMA) based hydrogels (50% HEMA+50%MMA) with different concentrations of MPEGs and PEGOHs were first cut into disks of 1 cm^2. Their absorption by ultraviolet absorption spectroscopy between 200 and 400nm and the readings at 280nm were recorded as blanks. Each sample was placed right in the bottom of a one centimetre cuvette to ensure that the measurements were consistent (Figure 2.21).

The disks were spoiled in 3ml solutions of 0.5 mg/ml lysozyme and lactofferin with frequent shaking at the room temperature (22 ± 2° C) for 72 hours. They were removed from the protein solutions and rinsed once with distilled water to remove any loose surface protein. The U.V. absorbances at 280 nm were then measured by placing the disks into the U.V. cuvette containing distilled water. The amount of protein adsorbed on each disk was calculated by the use of a standard line obtained for a series of appropriate protein solutions taking into account the values for each blank.

6.7.2.2 *Cell adhesions studies*

These studies were based on the fact that when the hydrogels interact with a biological interface for a period of time, the gels will interact with the proteins in that medium either reversibly or irreversibly (deposition). The washing of the hydrogel is only able to remove the loosely bound proteins leaving the permanently deposited proteins. Specific cells can recognize the special sites on the deposited protein, in cultures, under controlled conditions and adhere to them. The adhered cells can then be stained and counted under a microscope. The cell count is proportional to the the degree of protein

deposition that has occured on the hydrogel surface. The results of cell adhesion studies with the discussion is given elsewhere [110].

5.7.3 Results

The results of protein absorption are presented graphically in Figures 6.30 - 6.33. The 0% refers to the pure HEMA: MMA co-polymers (50:50). The MPEG is the methoxy terminated whilst the PEGOH is hydroxy terminated. 5-20% refers to the percentage by weight, of the polyether used in the composition and the numbers 400, 550, 1000 and 2000 are the molecular weight rather than the number of PEG monomers.

Figures 6.30 - 6.33 show that the addition of both MPEGs and PEGOHs have some effects on the protein absorption by hydrogels relative to a pure poly(HEMA:MMA). These observations suggest that the incorporation of methoxy terminated polyethylene glycols (MPEGs) into poly(HEMA:MAA) based hydrogels cause a significant decrease in the deposition of both lysozyme and lactoferrin at the hydrogel surface (Figures 6.30 and 6.31). On the other hand, the incorporation of hydroxy terminated PEGs (PEGOHs) has a rather different effect and both proteins used for these *in-vitro* studies deposited to a greater extent on these hydrogels (Figures 6.32 and 6.33).

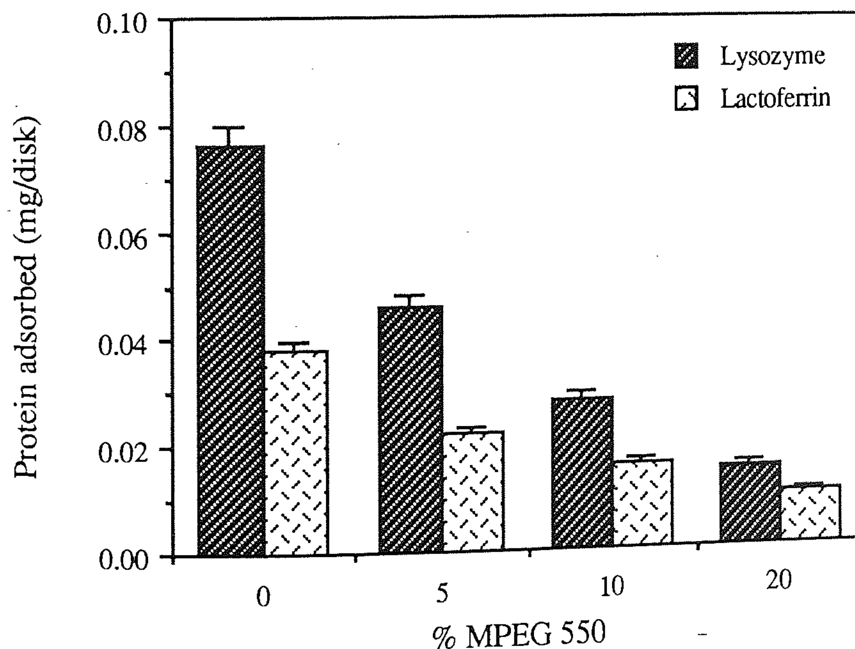


Figure 6.30 The adsorption of proteins to MPEG (low molecular weight) modified HEMA/MMA co-polymers.

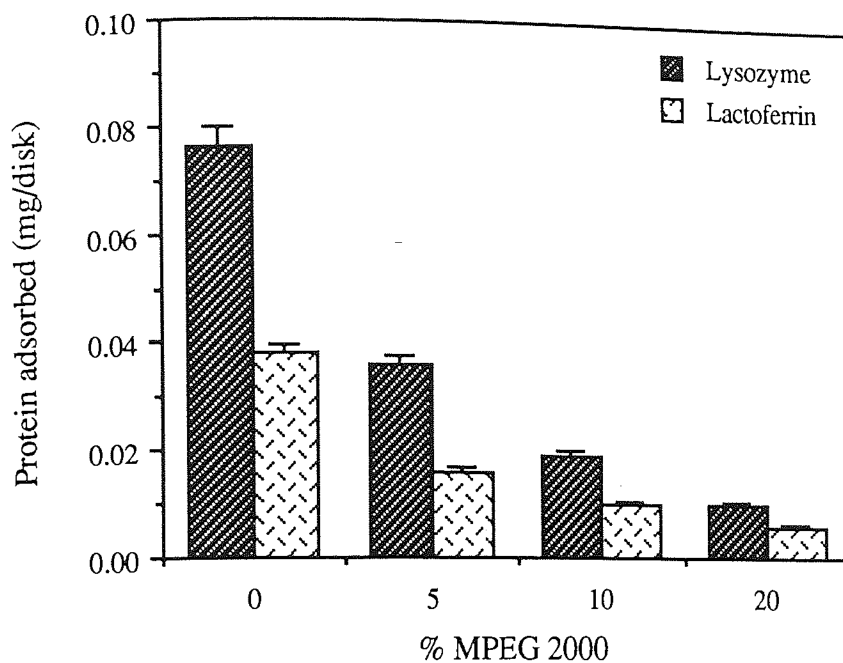


Figure 6.31 The adsorption of lysozyme and lactoferrin to MPEG (high molecular weight) modified HEMA/MMA co-polymers.

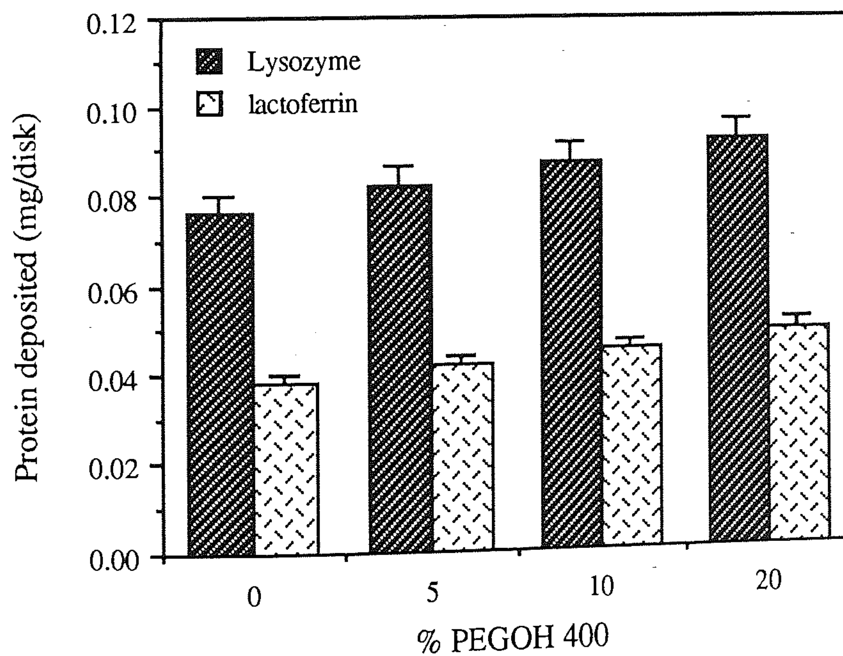


Figure 6.32 The adsorption of lysozyme and lactoferrin to PEGOH (low molecular weight) modified HEMA/MMA co-polymers.

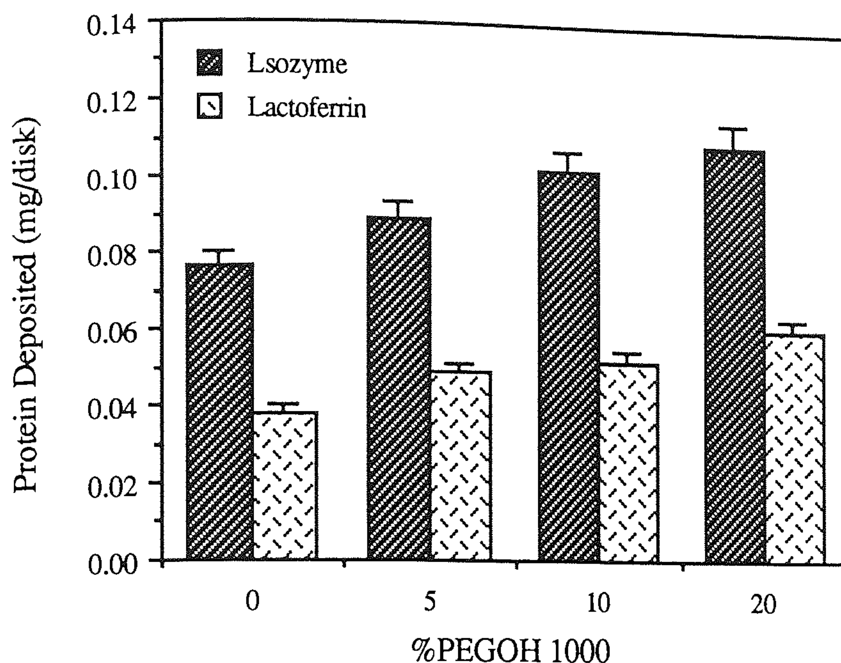


Figure 6.33 The adsorption of lysozyme and lactoferrin to PEGOH (high molecular weight) modified HEMA/MMA copolymers.

6.7.4 Discussions

The inclusion of long PEO chains in a HEMA based copolymer has two effects. It increases the water content of a copolymer, and also offers an unsuitable surface for cell adhesion. One reason for the decreased cell and protein adsorption is that the long chains act as a carpet of molecular cilia preventing the adsoprtion of adhesion proteins. Adhesion proteins adsorb irreversibly to the surface of hydrogels. They have binding regions for other molecules, and even adsorption of a tiny amount of adhesion proteins, may form the base for further deposition of other serum or tear constituents. A similar theory to explain the preventation of thrombogenic reactions, is that the poly(ethylene glycol) chains provide an excluded volume which prevents protein absorption at the polymer surface.

In general, the treatment and understanding of protein adsorption requires a knowledge of protein structure and the solid surface. Figure 6.34 is a schematic and idealised view of a single protein interacting with a single well-characterised solid surface [111]. It is clear from this figure that the protein can interact with the solid surface in a variety of different ways, depending on the particular orientation by which it approaches and the overall binding energetics.

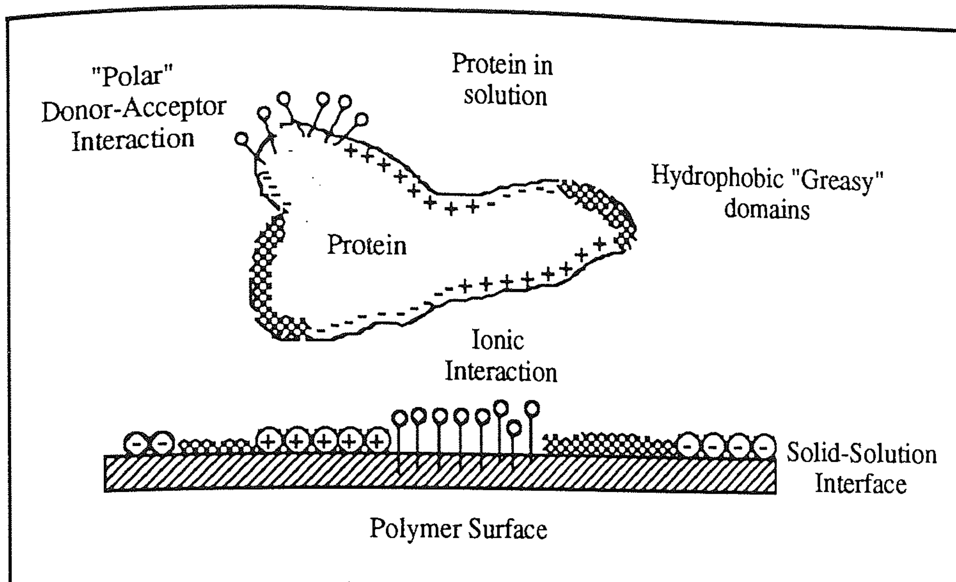


Figure 6.34 A schematic view of a protein interacting with a polymer surface. The protein has a number of surface domains with hydrophobic, charged, and polar character. The polymer surface has a similar domain-like character.

6.7.5 Conclusions

From the results obtained by protein deposition and cell adhesion the following conclusions can be made:

1. The inclusion of MPEGs increases the resistance of hydrogels to protein absorption.
2. Increasing the concentration of a particular MPEG in the terpolymer causes a decrease in the protein absorption.
3. The higher the molecular weight of the methoxy polyether derivative, the greater the decrease in the protein absorption, i.e. MPEG-2000 is more effective than MPEG-550 in reducing the protein deposition by the hydrogel.
4. In the case of hydroxy terminated polyether derivatives the protein absorption increases with the incorporation of higher concentrations with similar molecular weights.
5. The longer the hydroxy terminated PEG used in terpolymer synthesis, the higher is the protein absorbed into it at a similar concentration.
6. The effect of the polyether derivatives is, however, more pronounced in the case of methoxy terminated PEGs.
7. The cell adhesion studies also confirm these results, but in the case of PEGOHs the protein deposition does not follow a pattern. However, in the case of PEGOH-1000 the protein absorption is greater or similar to the obtained for a pure poly(HEMA:MMA).

6.8 General Conclusion

The effect of various monomers used to be co-polymerised with HEMA have been shown in this study. It can be concluded that the positively charged co-monomers can cause a lower level of protein (especially lysozyme and lactoferrin) spoilations. There are many factors which may cause error in this type of *in-vitro* spoilation studies. As the quantities of the protein absorbed are in some cases very low the percent of error may increase. However, to reduce the error in experiment and calculations, a list of posiible error were considered as shown in Appendix IV and the experiments were repeated three times under similar coditions in order to reduce the error. In this sort of experiments, generally, it was found that the error was always less that 7-8 % and the mean error taken into account was 5 %.

Chapter 7

Extrinsic Factors Affecting Protein Absorption on Contact Lenses

Chapter 7

Extrinsic Factors Affecting Protein Absorption

7.1 The Effect of the Dye used in Coloured Lenses on Protein Absorption

7.1.1 Introduction

The use of soft contact lenses as a cosmetic tool to change the colour of the eye has been introduced since the discovery of these sorts of lenses. These coloured lenses are frequently referred to as "opaque colours", and they can change the colour of even the darkest eye to a variety of available colours. This colour change is derived from the colourant applied to an intermittent pattern on to the front surface [112]. These lenses are to be distinguished from transparent tinted lenses which can not change the apparent colour of the dark eyes.

The problems associated with these lenses would be similar to those lenses used for vision correction. However, the spoilation behaviour of coloured lenses may be varied from uncoloured lenses due to the presence and interference of the dye which has been introduced to the polymer. The aim of this part of study was to compare the protein uptake by coloured and uncoloured lenses from the same group and the same manufacturer, and then evaluate the effect of the dye, if any, as well as its quantity and type used for lens colouration.

7.1.2 Materials and Solutions

The following coloured and uncoloured lenses were kindly provided by their manufacturers. A 0.3 mg/ml lysozyme solution was prepared freshly and used for the *in-vitro* spoilation. The power of each lens was kept constant (-3.0) in order to minimise thickness variations.

1. Focus™ from Ciba vision (Vifilcon A polymer, 55 % water),
   Royal Blue
   Aqua
   Vistint (uncoloured)

2. Optima 38™ from Bausch & Lomb (Polymacon tinted lenses, 38.6% water),
   Green

Blue
SAG (uncoloured)

3. Z6™ from Hydron (polymacon, 38% water)
Sapphire 20 %
Emerald 20%
Colourless Z6
Topaz 5, 10, 20 and 40 %
Coral 5, 10, 20 and 40 %
Amber 5, 10, 20 and 40 %
Amethyst 5, 10, 20 and 40 %

7.1.3 Spoilation Technique

As it has been discussed so far, the protein most associated with lens spoilation is lysozyme, this study was carried out using only lysozyme for the *in-vitro* spoilations. The lenses were spoiled in 3 ml of lysozyme solution with a concentration of 0.5 mg/ml for at least 5 days with frequent shaking at room temperature (20 ± 2). They were rinsed once with distilled water and the quantity of the protein deposited on them was measured against a blank of the same lens material and same colour using U.V. at 280 nm. The spoilation procedure was repeated for three samples of each type at the same time and the mean value was taken as the result. The lenses were placed right down at the bottom of the U. V. quartz cuvette (Figure 2.21).

The effect of the colourant concentration was studied by the use of group of coloured Z6™ lenses with various concentrations of the colourant. The spoilation technique was similar to the above and the lysozyme absorption was measured by U. V. at 280 nm after five days room temperature spoilation.

7.1.4 Results and Discussions

The results of lysozyme spoilation on various coloured lenses are shown in Table 7.1 and Figure 7.1. In this experiment the concentration of the dye was kept constant (20 %) to compare different types of the colourant. The effect of the concentrations of various colourant materials on protein spoilation is also shown in Table 7.2 and Figures 7.2-7.4. The dyes used in coloured lenses had been supplied by different companies and they had been mixed to give 20 % colour and depth to the lens produced.

Table 7.1 Lysozyme spoilation of various coloured lenses.

Lens	Colour	Lysozyme (mg/lens) (± 0.002)
Z6™	Sapphire	0.020
Z6™	Emerald, green	0.034
Z6™	Non-coloured (tinted)	0.027
Z6™	Topaz, gray	0.022
Z6™	Coral, orange	0.038
Z6™	Amber, yellow	0.038
Z6™	Amethyst, purple	0.042
Focus™	Royal blue	0.472
Focus™	Aqua	0.492
Focus™	Vistint (non-coloured)	0.485
Optima 38™	Green	0.076
Optima 38™	Blue	0.058
Optima 38™	SAG (non-coloured)	0.065

It can be seen that different colours have different effect on the spoilation behaviour of various types of soft contact lenses. The most interesting point revealed from these results is that the colour compound used in contact lenses have affected the protein up-take, which in some cases is favourable. The effect of the dye is more pronounced in the case of low water content non-ionic lenses (Z6™ and Optima 38™) compared to higher water content ionic Group IV lens (Focus). Unfortunately, due to the confidentiality, it was not possible to get much information about the structures of the dye from their manufacturer. However, chemically, these dyes are polycyclic organic compounds with conjugated double bonds in most cases. The presence of conjugated double bonds causes electron displacement which may give rise to different behaviour of the hydrogel with protein interactions. Quinn and Jahnke [113] compared the protein up-take between clear and coloured Durasoft3™ lenses. They found that coloured Durasoft3™ lenses had the same resistance to lysozyme deposition as did clear, uncoloured Durasoft3™ lenses without going into much detail.

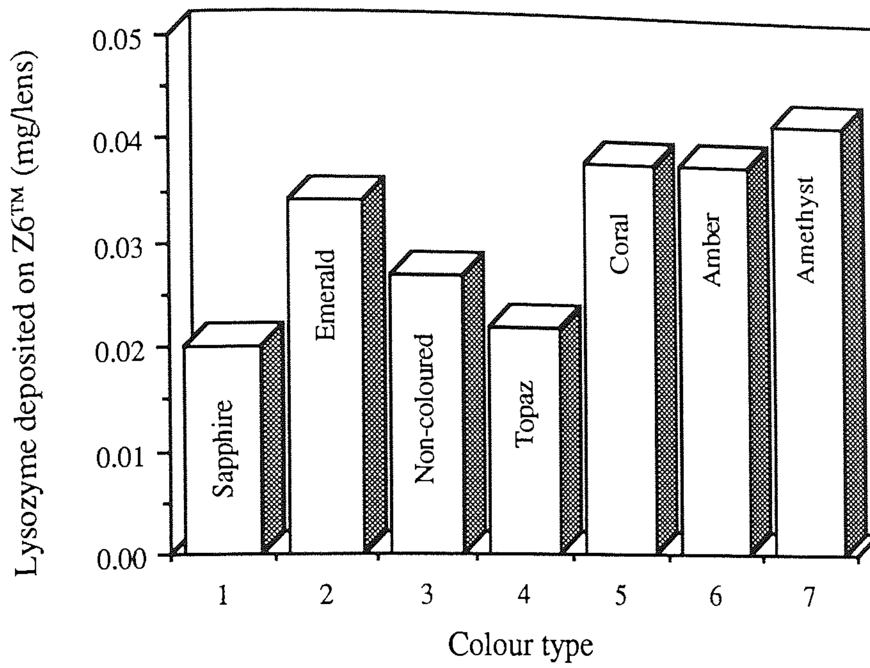


Figure 7.1 Lysozyme spoilation of a Group I lens with different colours.

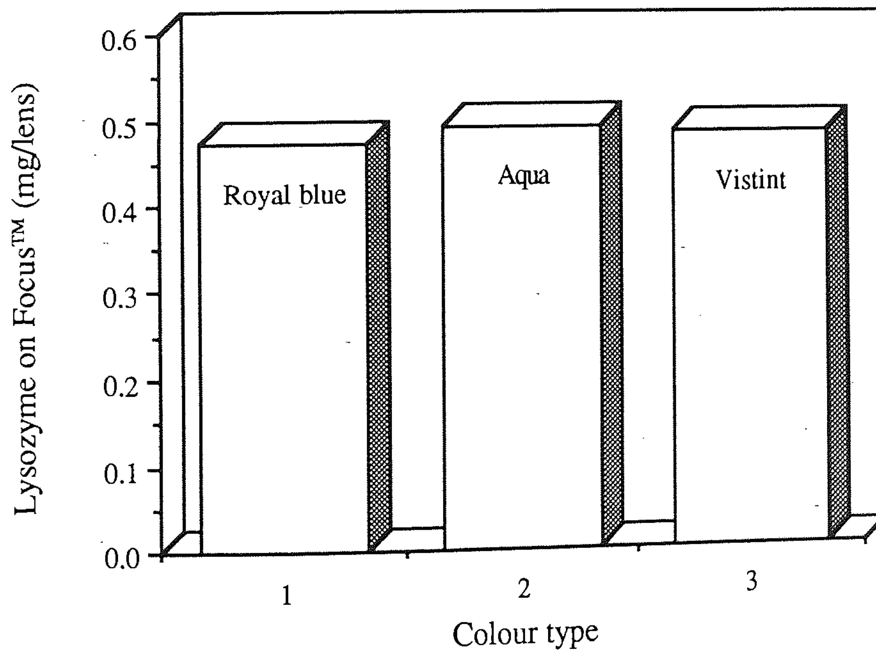


Figure 7.2 Comparison between the spoilation behaviour of coloured and non-coloured Focus™ (Group IV) lenses.

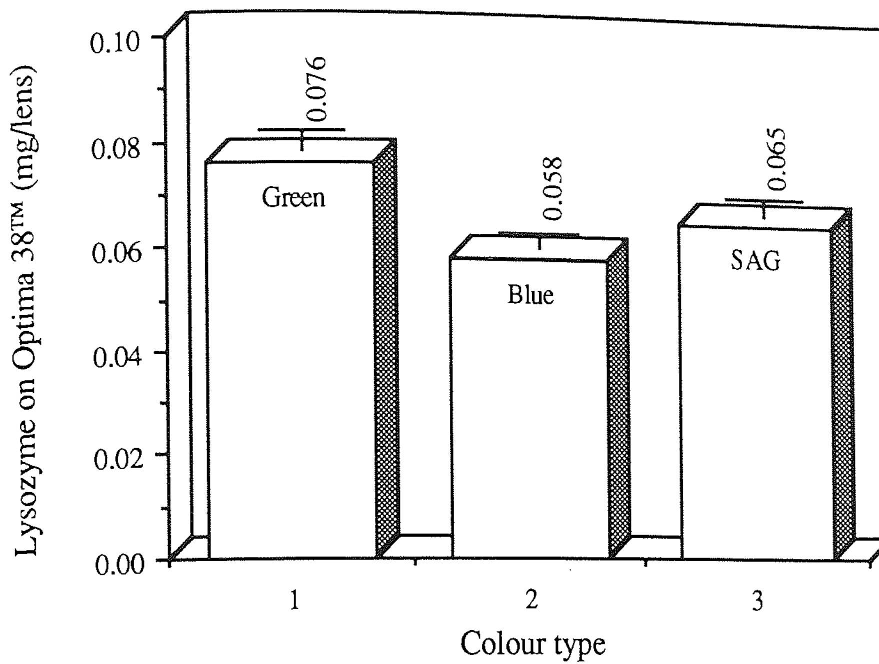


Figure 7.3 The effect of dye in coloured Optima 38™ (Group I) on its spoilation with lysozyme.

Table 7.2 The effect of dye concentration on the lysozyme up-take by Z6™ (Group I) coloured lenses.

Lens colour	Colour (concentration %)	Lysozyme (mg/lens ±0.002)
Topaz, gray	5	0.024
	10	0.024
	20	0.022
	40	0.016
Coral, orange	5	0.028
	10	0.032
	20	0.038
	40	0.044
Amber, yellow	5	0.030
	10	0.034
	20	0.038
	40	0.046
Amethyst, purple	5	0.032
	10	0.039
	20	0.042
	40	0.055

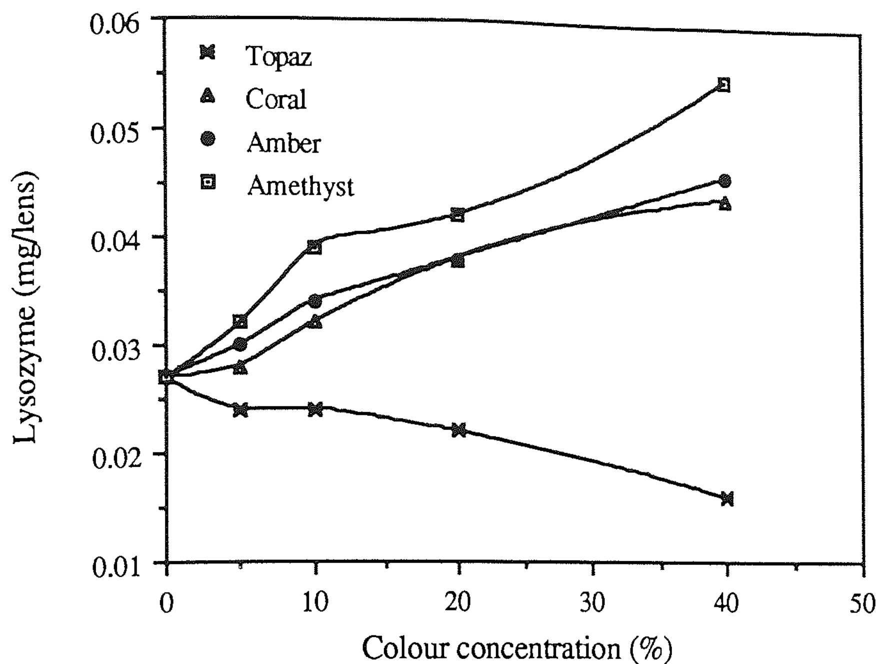


Figure 7.4 The effect of concentrations of various dyes used in Z6™ coloured lenses on their lysozyme spoilation.

7.1.5 Conclusions

Having considered the above findings , it can be concluded that lysozyme spoilation is different for coloured lenses compared to non-coloured lenses with similar properties and under the same spoilation conditions. This behaviour can be due to the introduction of dyes with conjugated double bonds. The concentration of the colourant used to colour lenses is important and the higher the concentration the more effect (positive or negative) it has on the spoilation of the lens. In the case of amber, amethyst and coral colours introduced to Z6™ lenses, the quantity of lysozyme uptake by the lens increases with the increase in the dye concentration. On the other hand, the increase in the concentration of topaz causes a decrease in the quantity of the lysozyme absorbed under similar conditions.

7.2 Acidic and Basic Impurities in HEMA

7.2.1 Introduction

One of the problems in the manufacture of poly(HEMA) lenses is that it is impossible to obtain the monomer in a state that is completely free of methacrylic acid. Previous studies (Chapter 5) have shown the importance of the surface and structural properties on protein interactions with soft contact lenses. Surface charge such as that produced

by methacrylic acid is particularly important in the absorption profiles obtained. In this part of the study the effect of residual methacrylic acid on the protein absorption on various HEMA lenses have been considered. It has been proved that only small quantities of charge are required to produce this type of deposition.

7.2.2 Experimental Procedure

A group of low water content non-ionic hydrogel contact lenses (FDA Group I) with poly(HEMA) structure were used for this part of study. The types and manufacturers are shown in Table 7.3. The lenses were spoiled in 3.0 ml of 0.6 mg/ml lysozyme solution for five days at room temperature (20 ± 2). The quantity of spoilation on each lens was measured after five days vibrating in the protein solution. A blank of the same material was used for the U. V. measurement and the lens was rinsed once with distilled water before placing in the cuvette.

Table 7.3 The characteristics of contact lenses used in the purity study.

Name	Company	EWC %	Monomer
Hydroflex™	Schonnkirchen	38	HEMA
Eurothin™	Pilkington	38.5	HEMA
Optima™38	Bausch % Lomb	38.6	HEMA
Silver 2™	Aspect vision care	38	HEMA
MCL 38™	Madden & Layman	38	HEMA
Medalist™	Bausch % Lomb	38.6	HEMA
Hydrofit 38™	Contor % Silver	38	HEMA
FHV-CLE™	MJS Contact lenses	38.6	HEMA
PLL™	---		HEMA
Z6™	Hydron	38	HEMA
Frequency disposables 38	Aspect vision care	38	HEMA
SeeQuence™	Bausch & Lomb	38.6	HEMA

7.2.3 Results and Discussions

To produce accurate results and show the reproducibility of the spoilation experiment, in all cases three similar lenses with the same power were spoiled under the same conditions. The results of *in-vitro* lysozyme spoilations are shown in Table 7.4 and Figure 7.5. The values given are the mean value of the three spoilation experiments.

By comparing the information given in Table 7.3 with the results obtained in Table 7.4 and Figure 7.5, it can be seen that although the properties of all the lenses are similar their lysozyme up-take is considerably different. It was found that the results were very consistent and reproducible and taking into account all the possible error sources the error range was ± 0.002 mg/lens.

It was discussed in previous studies [108] that lysozyme is a small positively charged protein and it is absorbed into negatively charged contact lenses. It was also showed in Chapter 6 that very small amounts of methacrylic acid (~1 %) produced negative charge on HEMA and causes considerable increase on the lysozyme uptake. This study shows that the so called contact lenses available in the market can be considerably impure and that the methacrylic acid content in some of these lenses is high enough to produce relatively high spoilation especially from positively charged proteins.

7.2.4 Conclusions

As discussed in Chapter 1, the deposition of the first layer of the protein can be a base layer for further deposition of other proteins and even lipids and some other biological species. It has been demonstrated [108] that lysozyme can penetrate into the matrices of the ionic lenses. We proved that various care and cleaning solutions can only remove up to 65 % of the deposited proteins (Chapter 9). Therefore, it can be predicted that failure in synthesising pure HEMA can cause a number of important problems in the contact lens use. Care must be taken when purifying the produced poly(HEMA) and the residual monomer be extracted from the product. The results of different HEMA lenses produced by various manufacturers show that, although all the lenses are HEMA, but depending on the procedure used for purification and the period between production/purchase of the hydrogel and distillation, the finish product contains some different negative charge. Comparing these results with those obtained in Section 6.2, it can be concluded that in some of HEMA lenses examined here, the methacrylic acid contents are as high as about 1 %. This is a very high value for the presence of impurities in such a delicate design which is used in one of the most valuable body sites. However, it seems that the lowest possible lysozyme value to deposit on a relatively good HEMA, is around 0.027 (± 0.002) mg/lens. Excelens™ from Ciba Vision is a higher water content (64 %) it was spoiled under the same conditions as the above HEMA lenses. The mean quantity of the lysozyme deposited on this neutral lens was 0.008 (± 0.002) mg/lens. This comparative experiment shows that the purest form of HEMA lenses available still contain traces of negative charge and, in fact, Group I contact lenses are not purely non-ionic as are classified by FDA.

An investigation about the prices of the commercially available poly(HEMA) lenses showed that the level of the impurity in some cases related to the finished cost of the contact lens. In general, the level of the impurities in HEMA lenses depends in part on the care taken in their synthesis and distillation of the finish product and also on the time spent between the production of the hydrogel and converting it into contact lenses.

It has been shown [114] that although N-vinyl pyrrolidone can be prepared in a pure state the problems associated with this monomer is that it polymerises relatively in effectively in conjunction HEMA with which it is combined in contact lens manufacture. Any unreacted monomer and soluble low molecular weight polymer is conveniently extracted at the hydration stage and these washings discarded before the lens is finally placed in storage solution. The residual N-vinyl pyrrolidone in HEMA also form another source of the impurities in the HEMA contact lenses. The levels of these types of impurities in various commercial products were also compared using HPLC [114].

It can be concluded that trace impurities in soft contact lens manufacture manifest themselves in different ways. Two of the most important are:

1. The presence of monomer impurities that become incorporated in the polymer.
2. The presence of residual un-polymerised monomer that is subsequently leached out of the lens.

The presence of impurities may cause many problems, the most important of which is the higher protein absorption by the hydrogel. It is, therefore, best for the manufacturers to keep the level of the impurities as low as possible in order to have a better market and more patient comfort.

Table 7.4 The spoilation behaviour of different poly(HEMA) Group I contact lenses compared to their market prices.

Contact lens Type	Manufacture	Lysozyme (mg/lens) (± 0.002)	Unit cost in £ (ex VAT)
Z6™	Lathed	0.027	9.98
Medalist ™	Spun/lathed	0.032	1.99
MCL 38™	Lathed	0.043	20.50
Aspect (disposable)™	Moulded	0.048	1.69
SeeQuence™	Spun-cast	0.052	1.59
PLL™	---	0.054	---
Hydroflex™	Lathed	0.065	16.85
Optima™	Spun/lathed	0.065	13.20
Silver 2™	Moulded	0.087	11.00
FH 42™	---	0.088	---
Eurothin™	Lathed	0.103	14.96
Hydrofit™	Lathed	0.112	10.18

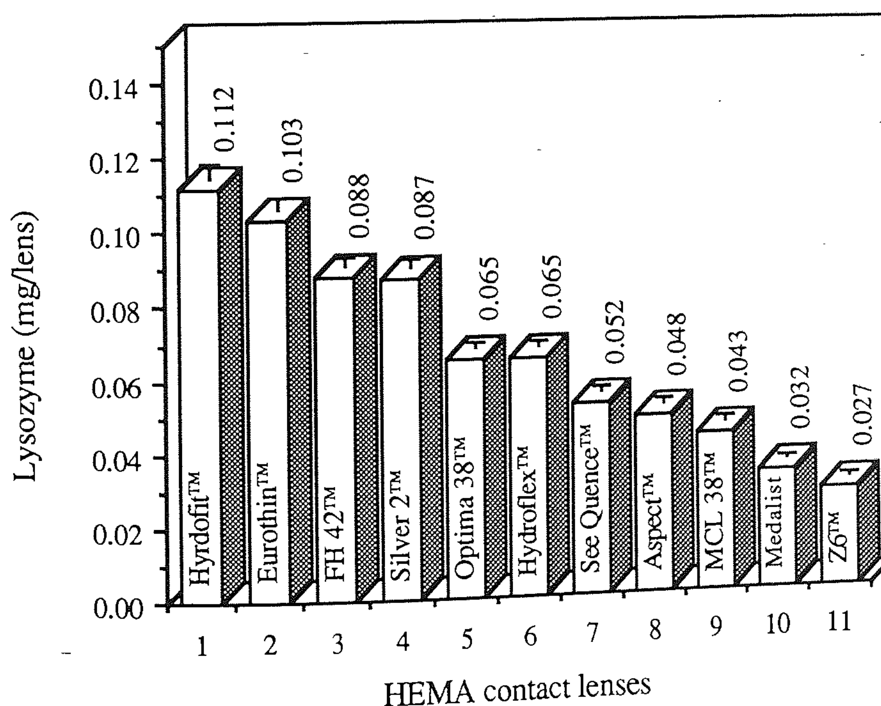


Figure 7.5 Comparison between the lysozyme up-take by different HEMA lenses.

7.3 The Spoilation Behaviour of Disposable and Non-disposable Lenses

7.3.1 Introduction

In an attempt to overcome the problems associated with spoilation of soft contact lenses, disposable lenses have been introduced. These hydrophilic lenses are regularly replaced, hopefully before they become too heavily deposited. An analysis of deposited lenses by Tripathi and colleagues in 1980 [115] led them to conclude that, for many patients a short-term answer may be an inexpensive, disposable lens which can be replaced weekly to bi-weekly. Standard disposable contact lenses already available are discarded every two weeks but still need daily cleaning.

The first disposable systems commercially available was 'Acuvue disposable system' which was released in 1987. The lenses are fabricated from Filcon 3b (Etafilcon), a mid-water content (58 %), ionic material. This has been followed by the introduction of a number of other disposable and frequent replacement systems, such that virtually every company manufacturing soft lenses now offers an option for planned replacement. Such lenses have also been used for therapeutic [116] and drug delivery [117] purposes.

An *in-vitro* study on the ability of disposable soft contact lenses to release drugs showed that they could provide an acceptable means of drug delivery [118]. In this study, the disposable lenses were soaked in the drug solution for 30 minutes and, depending on the lens use, the drugs were released continuously for a period of up to three hours. This indicates that the drug molecules which are smaller compared to the proteins are mobile and can be released from the matrix of the lens polymer. It is, therefore, possible to over-come the drawbacks associated with the use of non-disposable contact lenses as a drug delivery system.

It was shown that lysozyme bound to ionic lenses retained 85-90 % of its activity (Chapter 5). It is believed that the inflammatory complications induced by protein and lipid spoilation should be reduced as the lenses are discarded before they become highly deposited.

However, as their chemical structures are similar to the non-disposable contact lenses, they may behave similarly when come into contact with the tear proteins. This part of work was designed to compare the spoilation and the absorption isotherms of disposable with non-disposable lenses.

7.3.2 *Spoilation Studies*

The following three currently available disposable and non-disposable poly(HEMA) soft contact lenses with similar water contents (~38%) were used:

1. Aspect™ from Aspect Vision Care (Frequency disposables 38).
2. Optima 38™ from Bausch & Lomb (non-disposable).
3. SeeQuence™ from Bausch & Lomb (disposable).

The power of each lens was kept constant in order to minimise thickness variation. They were spoiled in the usual way in 3 ml of different concentrations of lysozyme solution (0.1-0.6 mg/ml). The quantity of deposited lysozyme was monitored during five days of spoilation at 280 nm against a blank of the same lens material. The quantity of the protein absorbed after five days was then plotted against the concentration of protein solution.

7.3.3 *Results and Discussion*

The absorption isotherms of a non-disposable and two disposable lenses are compared in Figure 7.6. It can be seen that, as predicted, the absorption behaviour for the lenses are similar and the difference in the quantity of the absorbed protein is not very significant, especially in the case of two disposable lenses. However, the quantity of lysozyme deposited on the disposable lenses is less than the extended wear lens under similar spoilation conditions. It can be suggested that the same behaviour can be seen in the case of lactoferrin and albumin spoilation. The higher quantity of lysozyme absorbed on non-disposable lens compared to the two disposable lenses can also be related to the MAA impurities in the lens material as was discussed in Section 7.2.

The *in-vivo* spoilation of the disposable Group II contact lenses was also measured (Section 8.6) using the Lowry micro-protein assay technique (Section 2.4.9) and was shown that the quantity of the total protein is dependent on the patient and the wear period and that 80-85% of lysozyme activity remained.

If the lenses are to be worn on a re-usable basis then an appropriate cleaning system is required. In the case of disposable contact lenses it can be afforded to make the cleaning system as simple as possible as they will be disposed before becoming highly deposited. This can be regarded as an advantage of the disposable lenses over the non-disposable contact lenses.

In a review for the ideal disposable contact lenses Jones [118] concluded that for majority of patients disposable contact lenses have the benefit of improved physiology, comfort, vision and convenience. The conclusion based on the protein spoilation behaviour is that in spite of a slightly lower spoilation isotherm for the disposable lens compared to the non-disposable SeeQuence™ lens, the difference is, within the experimental error, negligible. On the other hand, it was shown (Section 7.2) that the differences in the lysozyme spoilation of poly(HEMA) lenses is due to the manufacturers failure in producing pure biomaterial and that the traces of methacrylic acid in the finished product may cause high absorbed values for lysozyme and other positively charged proteins.

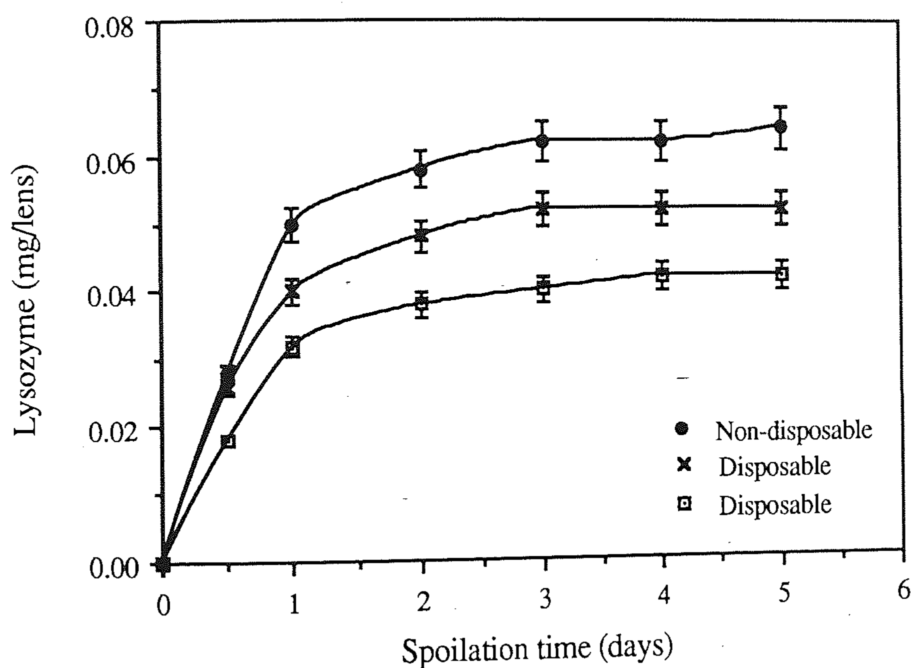


Figure 7.6 Lysozyme build-up on disposable and non-disposable lenses.

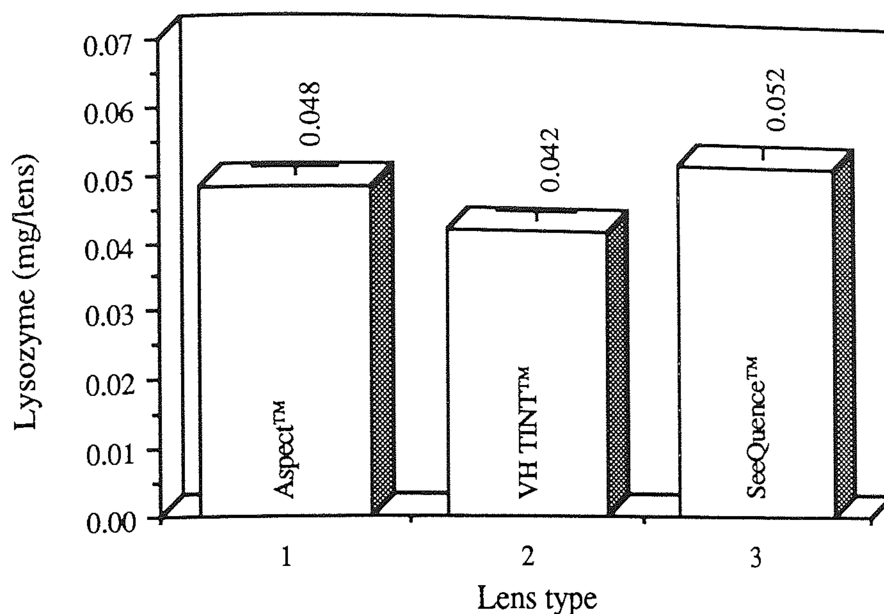


Figure 7.7 The plateau values for the *in-vitro* absorption of lysozyme on disposable and non-disposable lenses under the same spoilation conditions.

7.3.4 Conclusions

In spite of the fact that users of disposable contact lenses suffer fewer eye infections and are less prone to the microbe build-up affecting some wearers of conventional lenses, our results showed that the protein absorption is not much different between these two. As the disposable contact lenses are discarded every two weeks, the chance to get contaminated by handling is reduced so does their infection. However, their interaction with tear protein is not affected. Protein absorption takes place instantly when the lens is inserted into the eye and in a few hours the level of the deposited protein reaches nearly its maximum value. Therefore, disposable and non-disposable lenses behave similarly in terms of their interaction with proteins. It can be concluded that it is the type of the contact lens and its purity as well as the protein structure that dominates the spoilation behaviour of the lens. In similar spoilation conditions disposable and non-disposable can be deposited by proteins to the same extent.

Chapter 8

Clinical Studies

Chapter 8

Clinical Studies

8.1 The Spectroscopic Properties of Contact Lens Materials

The U.V. absorptions of a series of blank contact lenses from each type with different powers were examined in order to determine the effect of power on the spoilation assessments. This simple experiment was necessary as it gave a guide when testing different spoiled contact lenses.

8.1.1 The Effect of Handling on the U. V. Absorbance of Lens Materials

The spoilation of contact lenses from tear proteins was measured by comparison of the absorption at 280 nm. The U. V. technique is a quick and very accurate method for studying *in-vivo* spoilation. As the lens is handled many times during wear, the absorbance may be influenced and inaccuracy introduced into the results obtained. The following experiment which is a simple procedure to reduce this sort of error was carried out.

8.1.1.1 Procedure

The following 12 types of contact lenses were handled 10 times by different non-contact lens wearers. The power was kept constant (all types were -3.0) and their U. V. absorbances at 280 nm were measured against a blank lens material of the same type.

8.1.1.2 Results

The properties of the lenses used and the results of handling process are presented in Table 8.1 and Figure 8.1. The lenses which contain U. V. blocker can not be tested by this method (Precision™ U. V., in this case).

It can be seen that among all these lenses, the ones with thicker centres have resisted the handling better and their U.V. aborbances are not very high. The equilibrium water content of the lenses has also affected the change in absorbances due to handling. These results were then converted to the equivalent of the protein and taken into consideration for later spoilation studies.

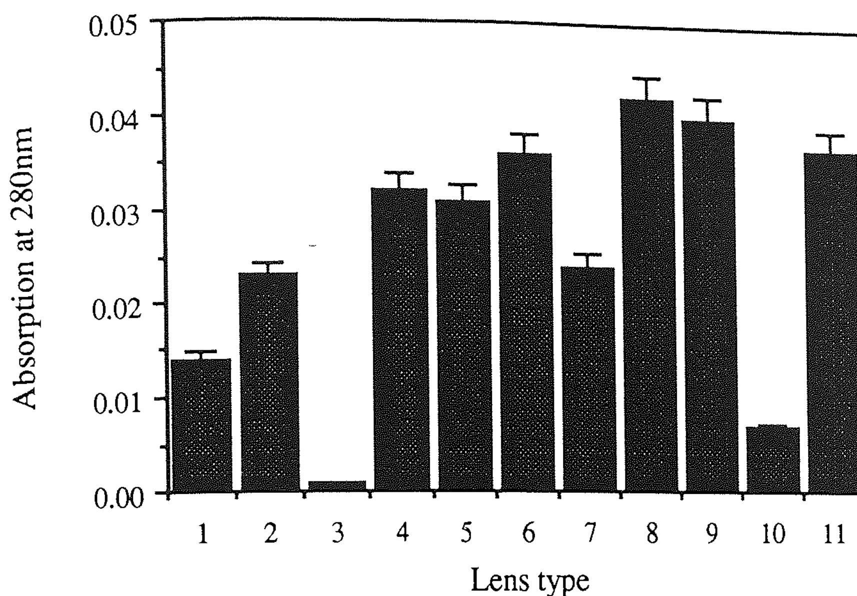


Figure 8.1 The effect of handling on the absorption of blank lenses (the numbers refer to the lenses listed in Table 8.1).

Table 8.1 The properties and effect of handling on the absorbances of different lenses.

Number	Lens type	Water content	Centre thickness (mm)	Absorption at 280 nm
1	Pilkington Precision™	74	0.140	---
2	J&J Surevue™	58	0.105	0.014
3	J&J Acuvue™	58	0.070	0.023
4	B&L SeeQuence™	38	0.038	0.001
5	Lunelle Rythmic™	73	0.150	0.032
6	Hydron Omniflex™	70	0.130	0.031
7	CIBA Excelens™	64	0.120	0.036
8	Lunelle ES70™	70	0.140	0.024
9	Hydron Z6™	38	0.060	0.042
10	Vista DR40™	40	0.060	0.040
11	CIBA Spectrum™	55	0.100	0.007
12	B&L Medalist66™	66	0.120	0.037

8.1.2 The Effect of Lens Power on Its U. V. Absorbance

A series of different lenses with powers ranging from - 6 to + 8 were selected and their absorption at 280 nm were measured. They were then blotted dry and weighed to study the effect of thickness on the different parts of the lens i. e. its positive or negative power

on the total U.V. absorption. They were also wavelength scanned to find out if they had absorption in any other U.V regions. Similar power of lenses with different power signs had similar absorptions and, therefore, the total weight of the polymer exposed to the U.V. light determines the total absorbance. A typical comparison between the weight and power of the lens and its absorbance in the U.V. is shown in Table 8.2. Figure 8.1 shows the power of different lenses against their corresponding absorbance at 280 nm. The data given in Table 8.3 was used in the following experiments using the *in-vivo* spoiled lenses without blanks.

Table 8.2 The U.V. absorbances of different power unworn Acuvue™ lenses.

Power	Absorbance	Weight (grams)
- 6	0.026	0.0321
-5	0.023	0.0303
- 4	0.021	0.0292
- 3	0.019	0.0252
- 2	0.018	0.0243
- 1	0.017	0.0238
+ 5	0.018	0.0266
+ 1	0.020	0.0269
+ 1.75	0.021	0.0273
+ 3.25	0.023	0.0291
+ 3.75	0.024	0.0301
+ 5.0	0.026	0.0308
+ 6.0	0.028	0.0322

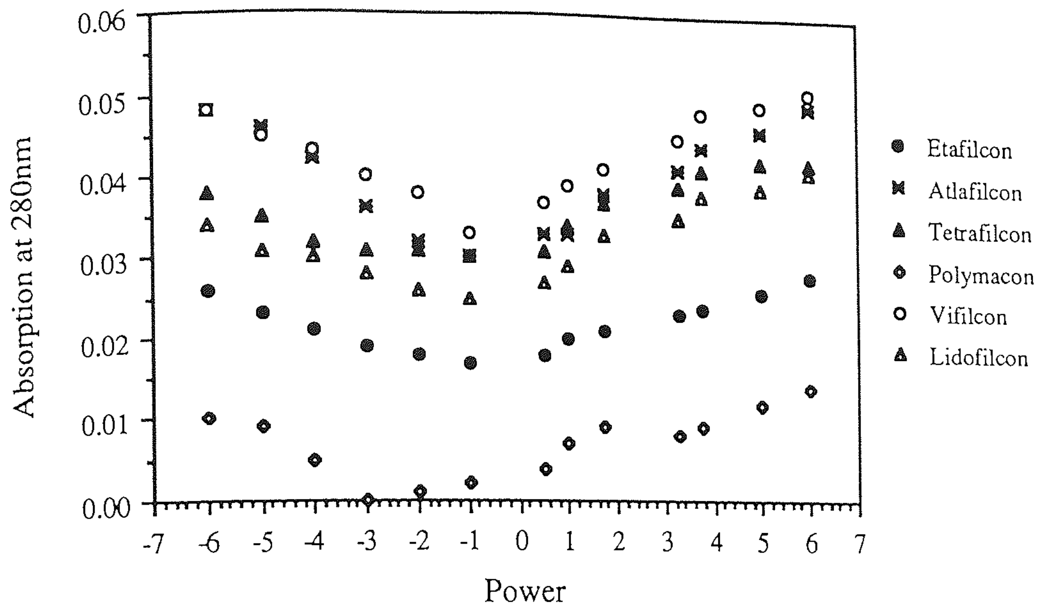


Figure 8.2 The absorption of different blank lenses with different powers.

Table 8.3 The absorption values of different blank lenses.

Power	Absorption at 280 nm for					
	Etafilcon	Atlafilcon	Tetrafilcon	Polymacon	Vifilcon	Lidofilcon
- 6.0	0.026	0.048	0.038	0.010	0.048	0.034
- 5.0	0.023	0.046	0.035	0.009	0.045	0.031
- 4.0	0.021	0.042	0.032	0.005	0.043	0.030
- 3.0	0.019	0.036	0.031	0.000	0.040	0.028
- 2.0	0.018	0.032	0.031	0.001	0.038	0.026
- 1.0	0.017	0.030	0.030	0.002	0.033	0.025
+ 0.5	0.018	0.033	0.031	0.004	0.037	0.027
+ 1.0	0.020	0.035	0.034	0.007	0.039	0.029
+ 1.75	0.021	0.038	0.037	0.009	0.041	0.033
+ 3.25	0.023	0.041	0.039	0.008	0.045	0.035
+ 3.75	0.024	0.044	0.041	0.009	0.048	0.038
+ 5.0	0.026	0.046	0.042	0.012	0.049	0.039
+ 6.0	0.028	0.049	0.042	0.014	0.051	0.041

8.2 The Effect of Group and Water Content

8.2.1 Introduction

In mid-1986 the FDA adopted a classification system for hydrogel lenses that groups them according to the ionic nature of the lens polymer matrix and their water content, as follows:

Group I:	Low water content, non-ionic matrix
Group II	High water, non-ionic
Group III	Low water content, ionic
Group IV	High water, ionic

As both Group II and IV materials are high water content, the comparison of protein deposition between these two groups, will give an indication of the effect of lens material itself. This *in-vivo* study was carried out to outline the effect of the lens material. A series of *in-vitro* spoilation studies were also carried out using the same contact lenses spoiled using individual protein solutions.

8.2.2 Methodology

Six patients wore Focus™ (Ciba Vision Group IV) in one eye and Medalist 66™ (Bausch & Lomb, alphafilcon A, visibility tinted, Group II) in the other eye. Miraflow™ was used for cleaning according to the manufacturer's recommended procedure and 10:10 peroxide for disinfection.

The *in-vitro* spoilation was carried out using 0.3 mg/ml solutions of lysozyme, lactoferrin and albumin. Individual lenses from Group II (Medalist66™) and Group IV (Focus™) were spoiled in 3.0 ml of the protein solutions at room temperature for 72 hours.

The amount of protein deposited on each lens (*in-vivo* and *in-vitro* spoiled) were measured quantitatively at 280 nm by U.V. spectrophotometer. Each lens was rinsed with distilled water once before the measurement and a blank of the same lens material was used as reference.

8.2.3 Results:

The results are presented in Table 8.4 and Figure 8.3 . These *in-vivo* results confirm the values obtained from *in-vitro* experiments with the individual and mixtures of proteins.

The comparison also shows that in all cases, it is the lysozyme which is absorbed most to both groups contact lenses and is the most likely to be blamed for the problems of protein spoilation. This is very obvious from the results of individual lysozyme spoilation *in-vitro* and the *in-vivo* total spoilation which show very similar values. This study also confirms that the spoilation is very much patient dependent.

Table 8.4 Comparison of the protein up-take by different patients.

Subjects	Left eye		Right eye	
	Lens type	Protein (mg/ml)	Lens type	Protein (mg/ml)
1	Focus™	0.496	Medalist™	0.042
2	Medalist™	0.033	Focus™	0.439
3	Medalist™	0.039	Focus™	0.482
4	Focus™	0.562	Medalist™	0.052
5	Focus™	0.483	Medalist™	0.052
6	Medalist™	0.035	Focus™	0.490

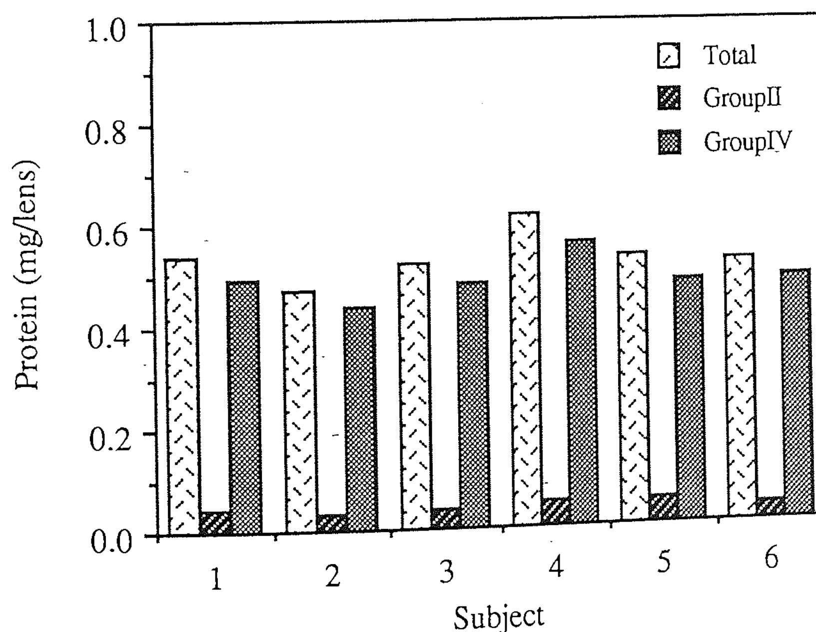


Figure 8.3 The effect of water content and patient variation on protein up-take.

8.2.4 Discussion

Protein deposition onto hydrophilic lenses continues to be a major problem for contact lens wearers, limiting the lifetime of the lens and subjecting the cornea, lid and accessory structures to potential injury. It is, however, the water content of the hydrogel which has

been shown to affect most of its potential contamination by protein. Group II lenses have shown an affinity for protein which is greater than Group I, but significantly less than Group IV. However, the non-ionic polymer matrix prevents additional favourable interactions between the protein and the lens.

8.3 Reproducibility of the Spoilation Results in Group II and Group IV

8.3.1 Introduction

The previous set of *in-vivo* and *in-vitro* experiments show that the degree of spoilation is highly dependent on the water content and lens grouping, and also there are great patient differences. It would be interesting and useful to know how these results can be reproduced during a longer period of study. The interpretations would then be more reliable and accurate.

Groups II and IV contact lenses are both high water content hydrogel and they differ in their ionic characteristics. In this *in-vivo* study the spoilation of these two groups was studied during three months wear.

8.3.2 Methodology

Twenty volunteers wore one Focus™ (from Ciba Vision, Group IV) in one eye and one Precision™ (from Pilkington, Vasurfilcon A, Group II) in the other eye for three months. The lenses were replaced at the end of each month using the same type of lens in each eye every time. Miraflow™ was used as a surfactant cleaner (Ciba Vision, preservative and enzyme free). The quantity of the proteins absorbed was measured using the Lowry procedure on the extraction solutions as the Precision™ lenses contain U.V. blockers, and the direct U.V. absorbance on the contact lens was not possible. Only 60% of the total protein deposited was extracted into the ReNu™ solution, this factor was taken into consideration when calculating the quantity of proteins per lens.

8.3.3 Results

The amount of protein deposited on each type of lens are shown in Table 8.5. As shown in this table, the higher values belong to the Group IV lens and the Group II lenses deposited less protein. Comparison of the quantity of deposition during each month of the study is shown in Figure 8.4.

The extraction solutions of the three pairs of lenses for each patient were then run on the electrophoresis for comparison of the type of the protein extracted from each lens type for the different patients. In the case of Group II lenses the concentration of the extracted proteins were not high enough to be detectable in a polyacrylamide gel. Whereas the extracted solutions from Group IV lenses were detectable by electrophoresis, although the differences in patients and wearing time could not be seen sharply on a stained gel. Figure 8.4 shows the electrophoresis gel stained with Brilliant Blue G contaning. The wells from top to bottom belong to proteins extracted from contact lenses worn for the first month on the right eyes of patients number 1, 2, 15, 11 and 12 in Table 8.5.

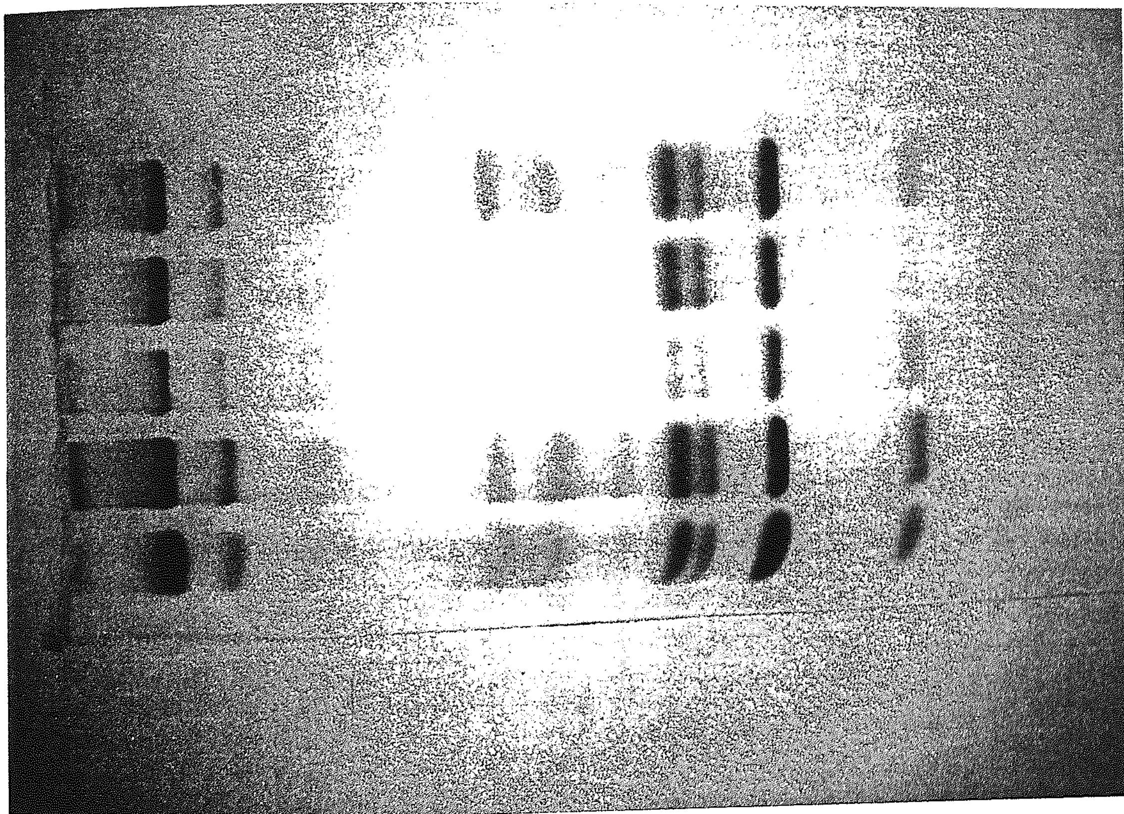


Figure 8.4 Electrophoresis gel containing lens extractions from Group IV lenses.

8.3.4 Discussions and Conclusions

The U.V. results show that the protein spoilation is much more patient dependent than time or period dependent. The sharp differences in the protein uptake between Group II and IV lenses are also confirmed by this study. The important point of this study is that the protein spoilations are fairly similar and reproducible during each different month of wear and in most cases the protein uptake increases slightly as the time of wearing contact lenses increases (slightly higher for the third month than for the first month). Looking at the electrophoresis results proves that there has been a high quantity of the proteins on the Group IV lenses and the middle line (from patient 15) in Figure 8.4

contains slightly less protein than the other lines. Table 8.5 shows that patient number 15 has deposited comparatively the lowest protein on the Group IV lens worn on the right eye.

It can be concluded that Group IV lenses deposit high quantity of protein and this is patient dependent and less related to time or period. On the other hand, Group II lenses which have high water content are more resistant to protein deposition and this is due to their non-ionic character.

Table 8.5 The spoilation of Groups II and IV over three months.

Subject	Protein deposited (mg/lens)					
	Right eye			Left eye		
	Month 1	Month 2	Month 3	Month 1	Month 2	Month 3
1	0.462	0.471	0.477	0.015	0.015	0.017
2	0.575	0.578	0.586	0.040	0.042	0.045
3	0.038	0.033	0.035	0.491	0.498	0.505
4	0.028	0.024	0.025	0.464	0.472	0.476
5	0.582	0.575	0.588	0.044	0.046	0.048
6	0.446	0.457	0.562	0.024	0.021	0.023
7	0.456	0.462	0.468	0.032	0.026	0.025
8	0.031	0.026	0.031	0.462	0.468	0.475
9	0.041	0.037	0.037	0.520	0.529	0.534
10	0.016	0.019	0.021	0.450	0.458	0.467
11	0.466	0.460	0.472	0.024	0.029	0.032
12	0.039	0.038	0.042	0.485	0.492	0.498
13	0.012	0.012	0.015	0.442	0.448	0.454
14	0.484	0.492	0.498	0.021	0.030	0.033
15	0.416	0.419	0.423	0.012	0.011	0.014
16	0.032	0.036	0.039	0.484	0.492	0.498
17	0.461	0.467	0.469	0.024	0.025	0.025
18	0.498	0.511	0.517	0.033	0.036	0.038
19	0.041	0.042	0.049	0.552	0.560	0.567
20	0.035	0.038	0.041	0.528	0.531	0.528

8.4 Repeatability study over a Year for Group II Contact Lenses

8.4.1 Introduction

Having established the differences between the protein uptake by Groups II and IV contact lenses, it was possible to study the effect lens material ionicity. The reliability and repeatability of the results obtained in Section 6.3 was studied in this part of the experimentation.

This part of *in-vivo* experiment the spoilation of one type of Group II lenses was studied over a long wear period.

8.4.2 Procedure

Ten patients wore Medalist 66™ (Bausch & Lomb, Alphafilcon A™ polymer, visibility tinted, Group II) for one year. The lenses were changed at the end of each month for a fresh pair. Their collected lenses were stored in ReNu™ which was also used for the cleaning. The quantity of the protein absorbed on their lenses were measured by the use of direct U.V. on the lenses.

At the end of six months, all the collected lenses from each patient were extracted by vigorously shaking the lenses in 0.5 ml of ReNu™ for 24 hours and the extraction solutions were added together.

The activity was measured in the extraction solution of one patient (No. 9) who had deposited the highest amount of the protein during the first 6 months. In the case of this patient a more severe extraction technique was used which made it possible to extract the maximum protein quantity of the lens.

The extraction solutions were then used for electrophoresis and the same set of experiments were carried out for the second set of lenses collected during the next 6 months to compare the repeatability of the results in each case.

8.4.3 Results

The results of first 6 months are shown in Table 8.6. The quantity of the proteins deposited by the same patient are very similar each month and the patient variations occur in all cases. This suggests that the long term protein build up on high water content hydrophilic lenses depends upon factors such as hygiene, compliance with instruction for

cleaning, environmental factors and the patient tear film quality and quantity. These factors make the protein deposition even more patient dependent.

Figure 8.6 shows the average protein deposited by both eyes for three patients at the end of each month during 6 months of the study. Figure 8.5 compares the patient variation by showing the average protein for 6 month in the case of all ten patients. As it can be seen from both these figures, there are both patient and time variations. The patient variation, however, is more pronounced than other factors as most of the care and hygiene factors also depend on the patient. The average of total protein during 6 month for patient No. 9 was 0.045 mg/lens. The extraction solutions from both lenses during six months (12 lenses in total) were added together and the total protein in the extraction solution for this patient was 0.314 mg/ml the activity of which was 0.284 mg/ml. This shows that 89.6% of the total extracted protein was still active.

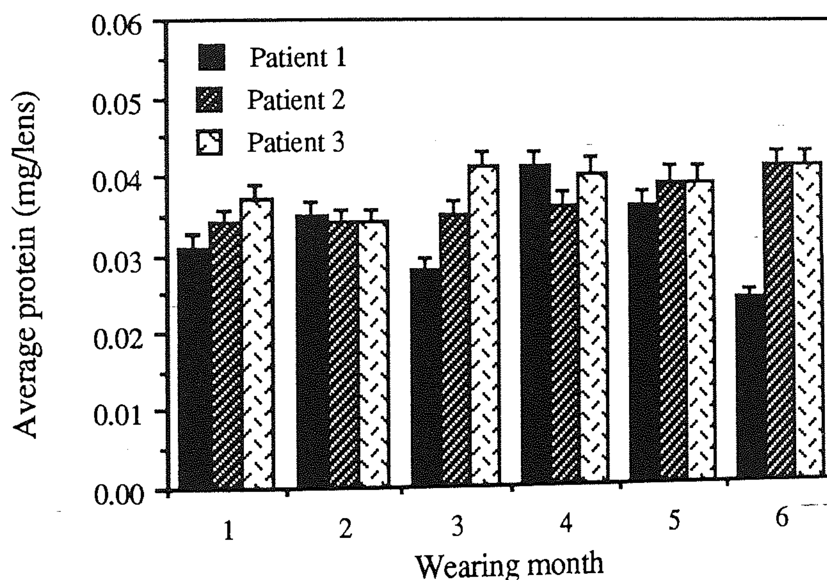


Figure 8.5 Mean protein values deposited in each month by three patients showing the effect of time and wearer variation.

8.4.4 Discussion

The protein deposition on Group II contact lenses depends on many factors of which the patient and care system are the most important ones. The quantities of the protein attracted to Group II lenses are higher than Group I lenses which is due to their considerably higher water content. This study also shows that most of the lysozyme deposited to Group II lenses is active and, within the experimental errors, the quantity of lysozyme deposited and remaining active are identical.

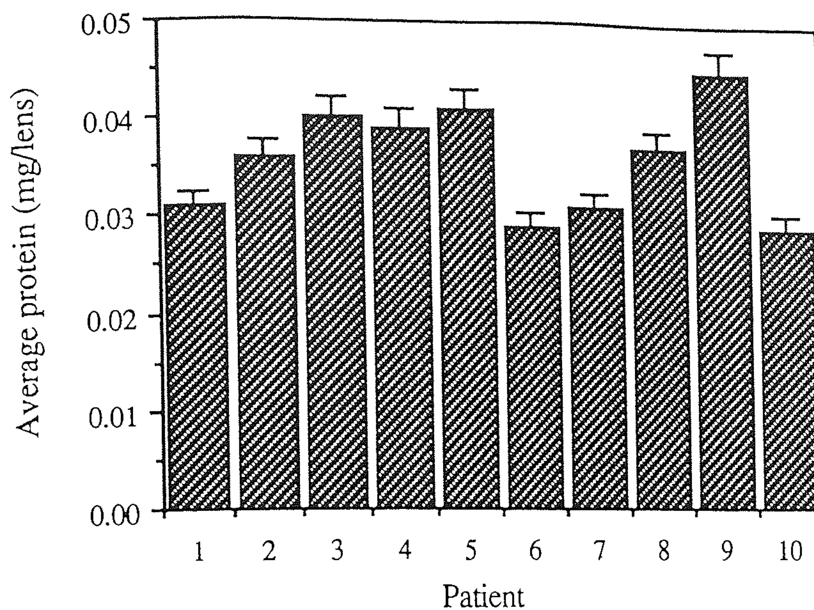


Figure 8.6 The average quantities of protein deposited by different patients in six months.

8.4.5 Conclusions

This study was designed for one year and it is still ongoing. The results of first 6 months study showed that the activity of the protein did not change due to absorption to Group II lenses. It also was proven that the protein absorption is very much patient dependent and the results are reproducible during the first 6 months of the study. The lenses for the next three month have also been analysed and the results confirm the findings for the first 6 months. It can be predicted that the results are reproducible during one year and they will show similar pattern in the second 6 months period of the study.

Table 8.6 Comparison between the protein deposited during 6 month study by different patients.

Subject	Protein deposited (mg/lens) (± 0.002)							
	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6	Average	Total extracted
1	R 0.032 L 0.030	R 0.038 L 0.034	R 0.029 L 0.025	R 0.040 L 0.042	R 0.035 L 0.037	R 0.025 L 0.023	0.031	0.229
2	R 0.035 L 0.034	R 0.033 L 0.036	R 0.036 L 0.032	R 0.038 L 0.034	R 0.039 L 0.039	R 0.042 L 0.043	0.036	0.267
3	R 0.039 L 0.035	R 0.043 L 0.048	R 0.036 L 0.037	R 0.034 L 0.032	R 0.041 L 0.043	R 0.044 L 0.040	0.040	0.283
4	R 0.036 L 0.037	R 0.035 L 0.032	R 0.043 L 0.044	R 0.041 L 0.043	R 0.039 L 0.041	R 0.040 L 0.037	0.039	0.291
5	R 0.038 L 0.035	R 0.039 L 0.034	R 0.042 L 0.041	R 0.045 L 0.046	R 0.047 L 0.043	R 0.044 L 0.041	0.041	0.302
6	R 0.025 L 0.027	R 0.023 L 0.024	R 0.028 L 0.026	R 0.032 0.031	R 0.035 L 0.038	R 0.030 L 0.028	0.029	0.208
7	R 0.042 L 0.041	R 0.029 L 0.031	R 0.023 L 0.025	R 0.036 L 0.037	R 0.034 L 0.032	R 0.039 L 0.038	0.031	0.222
8	R 0.034 L 0.033	R 0.033 L 0.034	R 0.037 L 0.039	0.039 0.033	0.032 0.032	0.039 0.038	0.037	0.214
9	R 0.039 L 0.036	R 0.039 L 0.041	R 0.043 L 0.042	R 0.045 L 0.048	R 0.048 L 0.050	R 0.049 L 0.051	0.045	0.314
10	R 0.025 L 0.023	R 0.029 L 0.030	R 0.024 L 0.023	R 0.033 L 0.034	R 0.035 L 0.030	R 0.035 L 0.033	0.029	0.205

8.5 Early *in-vivo* Protein Deposition on Group IV Lenses (Acuvue™)

8.5.1 *Introduction*

The amount of protein deposited *in-vivo* has been shown to be dependent at least on two major factors: the ionic character and water content of the lens material. Therefore, the protein deposition follows the order of FDA lens material grouping system. Group IV lenses (high water, ionic) absorb the highest quantity of the protein. The absorption process takes place instantly as soon as the lens is inserted into the eye. The small tear protein, lysozyme, is absorbed to a higher degree and is then easily desorbed after the removal of the lens from the eye, leaving it into storage solution. This *in-vivo* clinical study demonstrates the early deposition of tear proteins on two different Group IV lens material.

8.5.2 *Methodology*

A group of volunteers wore a new synthesized Group IV (Ultra-15) and Acuvue™ (Group IV) lenses for short periods (10 and 30 minutes). The lenses were then stored in saline solutions individually and their absorbed proteins were measured with the normal U.V. detection directly on the lenses and the quantity of the protein in their storage solutions was also estimated. Both of these lens types absorbed only small amount of protein in this short time and about half of the deposited protein had leached out into the storage solutions. The interesting point about Ultra-15 lenses was that the blank had a very high U.V. absorbance at 280nm.

8.5.3 *Results*

The results of 10 and 30 minutes deposition on both lens materials are shown in Table 8.7. The quantity of the protein leached into the storage solutions was also measured and can be seen in this table. Figure 8.7 compares the two Group IV lenses for their protein uptake and the amount remaining on the lens after leaching into the storage solution.

8.5.4 *Discussions*

As it can be seen from Figure 8.7, the protein deposited on both Group IV lenses is comparatively low during short wear time. Although the lenses are high water content ionic hydrogel lenses, the short wear time and considerable period left in the storage solution before measurement, has reduced the amount of protein remained on the lenses. This suggests that the frequently disposable lenses have the advantage of not

being highly deposited by multilayers of protein and that a monolayer is not very difficult to remove during the storage time.

Table 8.7 The *in-vivo* spoilations of Group IV lenses in short wear times.

Subject	The quantity of protein (mg/lens or ml)							
	Ultra-15™				Acuvue™			
	10 Min.		30 Min.		10 Min.		30 Min.	
	Lens	Solution	Lens	Solution	Lens	Solution	Lens	Solution
1	0.009	0.006	0.011	0.010	0.017	0.016	0.022	0.020
2	0.008	0.006	0.010	0.009	0.018	0.016	0.022	0.022
3	0.009	0.008	0.010	0.010	0.016	0.017	0.023	0.024
4	0.008	0.005	0.012	0.010	0.019	0.021	0.025	0.023
5	0.009	0.006	0.014	0.011	0.017	0.015	0.018	0.020
6	0.006	0.005	0.010	0.010	0.016	0.017	0.022	0.024
7	0.006	0.005	0.008	0.007	0.015	0.016	0.023	0.024
8	0.009	0.006	0.009	0.006	0.016	0.016	0.024	0.022
9	0.010	0.007	0.013	0.012	0.019	0.020	0.026	0.025
10	0.011	0.006	0.013	0.010	0.020	0.021	0.028	0.026
11	0.008	0.005	0.010	0.010	0.017	0.018	0.021	0.022
12	0.009	0.009	0.012	0.008	0.018	0.019	0.024	0.023
13	0.008	0.008	0.013	0.010	0.016	0.016	0.022	0.024
14	0.006	0.005	0.009	0.006	0.013	0.013	0.028	0.026
15	0.009	0.006	0.010	0.010	0.014	0.012	0.021	0.020
16	0.008	0.006	0.011	0.007	0.016	0.017	0.021	0.022
17	0.009	0.005	0.012	0.008	0.018	0.019	0.024	0.023
18	0.007	0.006	0.013	0.010	0.017	0.020	0.026	0.027
19	0.007	0.006	0.011	0.009	---	---	---	---

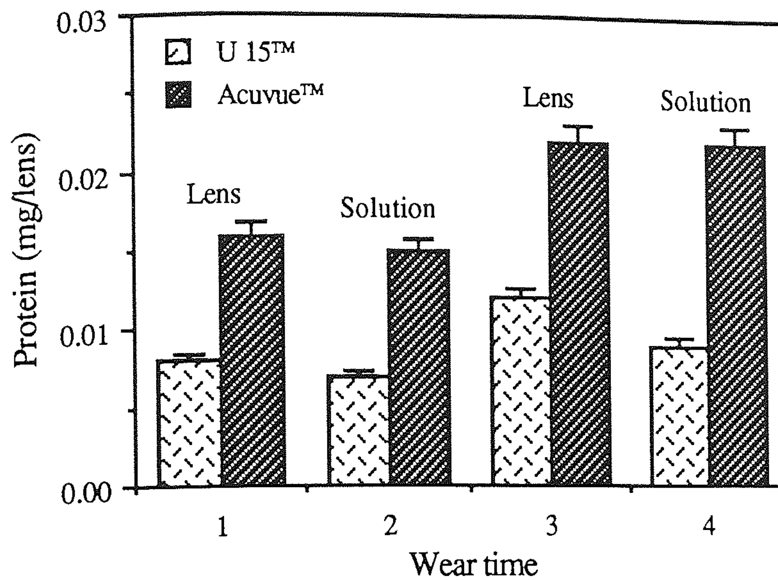


Figure 8.7 The protein remained on the lens and leached into solution from Acuvue™ and U 15™ lenses. 1 and 2 are 10 minutes wear while 3 and 4 refer to 30 minutes wear time

8.6 *In-vivo* spoilation Behaviour of Disposable Contact Lenses

8.6.1 Introduction

It has been suggested that some of the spoilation problems may be solved by the use of frequent replacement lenses. However the degree of spoilation on these lenses may be high and it is important to study their spoilation.

This study involved the spoilation and clinical performances of Group II disposable contact lenses when used on a monthly versus three monthly replacement.

8.6.2 Methodology

Twelve patients used a Group II lens (Pilkington "Precision™ UV") and cleaned their lenses with Bausch & Lomb's "Multi-Purpose Solution" solution (ReNu™). The lenses were then replaced after one month with a new lens of the same type. The experiment continued for three months after which they replaced their lenses with another clean lens, used ReNu™ for cleaning and changed them after three months.

The direct U. V. measurement on the lenses was not possible as they contained UV blockers. Therefore the lenses were cut into quarters and shaken in 1 ml ReNu™ solution overnight and the quantity of the deposited protein in each case was measured

by the use of U. V. on their extraction solutions. The amount of the protein in their storage solutions were also measured by U. V. spectrophotometry at 280 nm. The Lowry micro assay method for protein measurement was also used in some cases to compare the results. The activity of the extracted protein (lysozyme) was measured by the *Micrococcus lysodeiktikus* method (Section 2.4.2). The fact that the proteins were not totally extracted from the lenses was taken into account when calculating the total protein spoilation.

8.6.3 Results

The quantity of protein deposited for some of the subjects are shown in Figure 8.8. The values for one month study shown in this figure are the mean values of the one month spoilation. The full data obtained for this experiment is given in Table 8.8. Figure 8.8 also resembles the mean (± sd) protein values for the whole group.

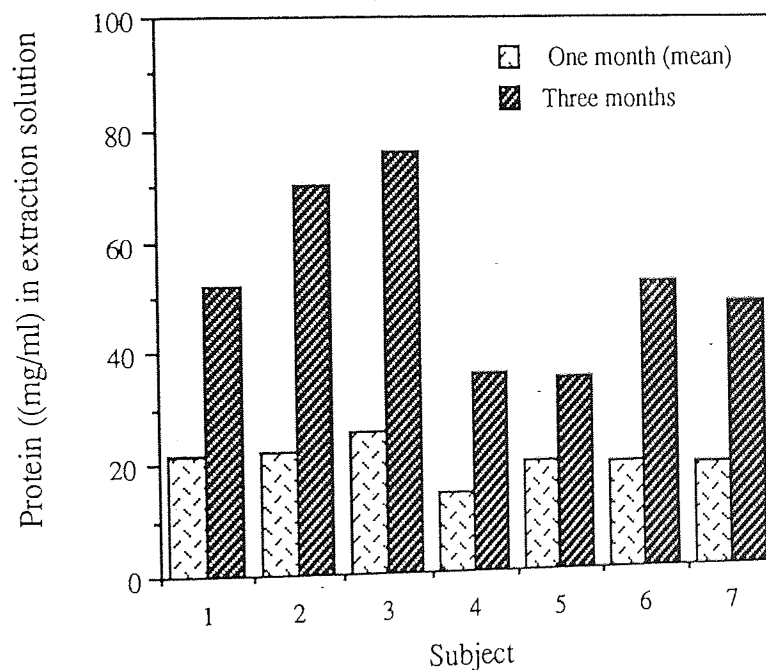


Figure 8.8 Quantities of proteins deposited *in-vivo* on Precision™ lenses during one and three month wear.

Table 8.8 The quantity and activity of the proteins extracted from *in-vivo* spoiled frequently disposable lenses (month 4 is the three month wear).

Subject	Month	Total protein (mg/ml) in				Activity (mg/ml) in Extraction solution from	
		Storage solution		Extraction solution			
		Left eye	Right eye	Left eye	Right eye	Left	Right
1	1	0.015	0.030	0.011	0.012	0.010	0.011
	2	0.017	0.027	0.010	0.013	0.009	0.012
	3	0.033	0.032	0.012	0.012	0.011	0.011
	4	0.048	0.051	0.071	0.062	0.045	0.048
2	1	0.021	0.026	0.024	0.030	0.020	0.026
	2	0.028	0.030	0.032	0.031	0.028	0.027
	3	0.018	0.022	0.018	0.022	0.014	0.019
	4	0.070	0.068	0.046	0.052	0.041	0.048
3	1	0.030	0.026	0.032	0.028	0.029	0.023
	2	0.019	0.022	0.022	0.024	0.018	0.021
	3	0.028	0.030	0.029	0.033	0.025	0.028
	4	0.076	0.071	0.062	0.056	0.058	0.051
4	1	0.012	0.015	0.016	0.021	0.014	0.017
	2	0.018	0.016	0.023	0.019	0.021	0.015
	3	0.013	0.015	0.016	0.019	0.012	0.015
	4	0.036	0.039	0.046	0.051	0.042	0.048
5	1	0.016	0.012	0.018	0.016	0.014	0.012
	2	0.015	0.014	0.016	0.018	0.013	0.012
	3	---	---	---	---	---	---
	4	---	---	---	---	---	---
6	1	0.022	0.020	0.025	0.026	0.021	0.022
	2	0.019	0.016	0.023	0.019	0.019	0.014
	3	0.018	0.015	0.023	0.020	0.019	0.016
	4	0.035	0.038	0.042	0.048	0.039	0.045
7	1	0.017	0.016	0.019	0.022	0.015	0.018
	2	0.019	0.030	0.023	0.029	0.018	0.023
	3	0.021	0.031	0.019	0.028	0.016	0.024
	4	0.052	0.055	0.056	0.061	0.051	0.057
8	1	0.019	0.023	0.022	0.026	0.018	0.022
	2	0.016	0.012	0.021	0.026	0.017	0.022
	3	0.021	0.026	0.022	0.030	0.018	0.025
	4	0.048	0.051	0.053	0.056	0.049	0.051
9	1	0.011	0.016	0.015	0.019	0.011	0.015
	2	0.013	0.016	0.016	0.017	0.012	0.014
	3	0.014	0.017	0.018	0.021	0.013	0.018
	4	---	---	---	---	---	---

8.6.4 Discussion

It can be seen from the results shown in Figures 8.8 and 8.9 that the protein deposition is highly subject dependent, even in the case of one month replacement lenses. The

protein deposition is higher in the case of three month wear, which suggests that the protein deposition builds up during the wear period and that the initially deposited protein is a base for further protein attachment to the lens. However, the sum of the deposited protein is nearly constant for 3 x one month and three month wear period. It can be concluded that the frequent replacement lenses are a useful way for the deposit control, and to avoid the protein accumulation on the lens. Considering the activity results, it can be seen that in all cases about 80% of the total protein is still active after absorption and extraction from the lenses. In this case the factor for the extractable protein has been taken into account.

Table 8.9 The mean values of total and active proteins.

Wearing period	Protein (mg/lens) (total) (± 0.002)	Activity remained	
		mg/lens	%
Month 1	0.040	0.036	79
Month 2	0.042	0.034	81
Month 3	0.040	0.033	83
Mean for 3 months	0.041	31.9	78
Three months	0.112	0.096	86

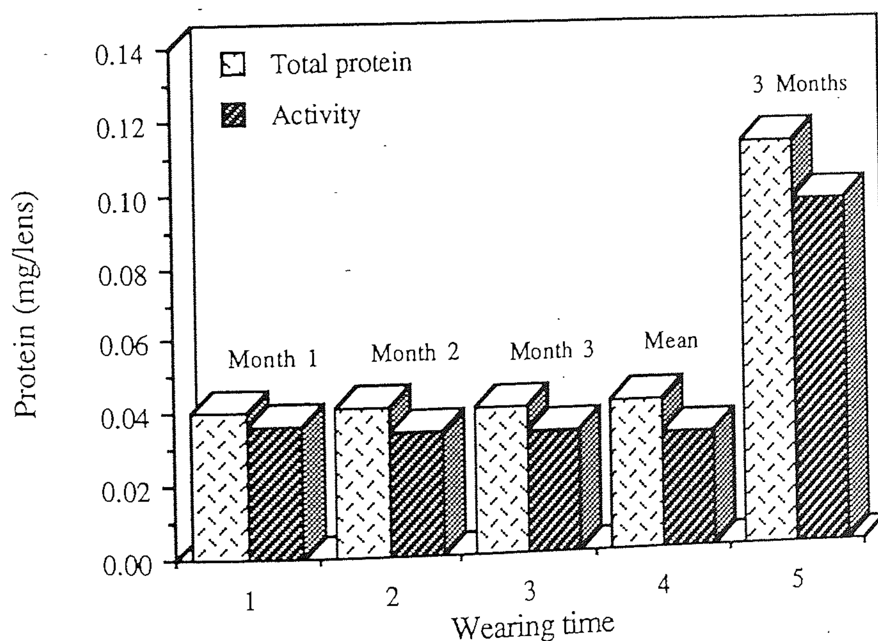


Figure 8.9 The mean values of total and active proteins

8.7 The Effect of Lens Material on *in-vivo* Protein Deposition

8.7.1 Introduction

In continuing the research on the effect of the lens material while keeping the water content constant, two lens materials with similar water content (Group I) together with a Group II lens material were used to study the protein spoilation *in-vivo*. The specific properties of the lens materials used in this study together with some other available lens materials are shown in Table 8.10. The *in-vitro* studies had shown that all these materials were deposit resistance materials. The quantity of spoilation caused by lipids were also measured by other members of the research group. The Vistagel plus™ (DR 40 material) was synthesised in our group at Aston University. This polymer is a low water content material based on water structuring groups and controlled sequence distribution (Figure 8.10). The controlled sequence backbone prevents the "blockiness" problem that is found in NVP based polymers [120] and causes enhanced spoilation. Water structuring groups shield the polymer surface from the deposits, preventing them from firmly adhering.

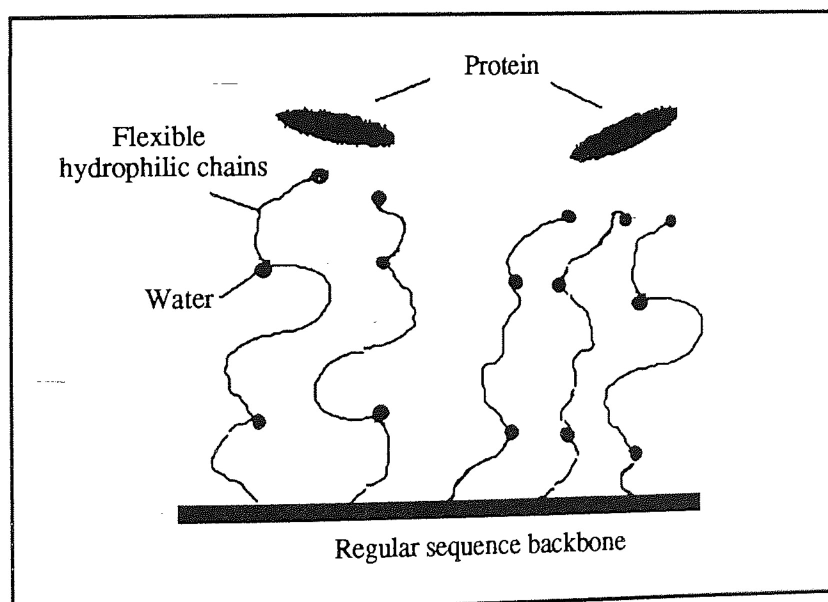


Figure 8.10 Diagrammatic representation of DR40 concept.

8.7.2 Procedure

Twelve patients randomly used three lens types daily disposable lenses for one month. Their lenses were changed for another lens type at the end of the month, but the same lens type was used in both eyes. LC65™ was used for surfactant cleaning and Oxysept™ II-Step for disinfection and enzyme cleaners were not used during the study.

After one month their lenses were collected and the quantity of the protein was measured by direct U. V. on the lenses at 280 nm.

Table 8.10 The properties of the lenses used in different clinical and many *in-vitro* spoilation studies.

Name	Company	EWC %	FDA group	USAN name	Manufacture	Monomer
Classic™	Pilkington	42.5	I	Tetrafilcon	Lathed	MMA, VP, HEMA
DR 40™	Vista	40	I	N/A	Lathed	Complex
Excelens™	CIBA	64	II	Atlafilcon	Moulded	PVA-based
ES 70™	Lunelle	70	II	N/A	Lathed	AMA/VP
Z6™	Hydron	38	I	Polymacon	Lathed	HEMA
Precision™	Pilkington Barnes-Hind	74	II	Vasurfilcon	Moulded	MMA/VP
Surevue™	Vistakon	58	IV	Etafilcon	Moulded	HEMA/MA
Rythmic™	Lunelle	73	II	N/A	Moulded	MMA/VP

8.7.3 Results

The quantities of proteins for a number of patients on each type are shown in Figure 8.11 and Table 8.11.

The protein deposited on the lenses in all cases was so low that the extraction by ReNu™ followed by electrophoresis on the extraction solutions showed no detectable protein bands.

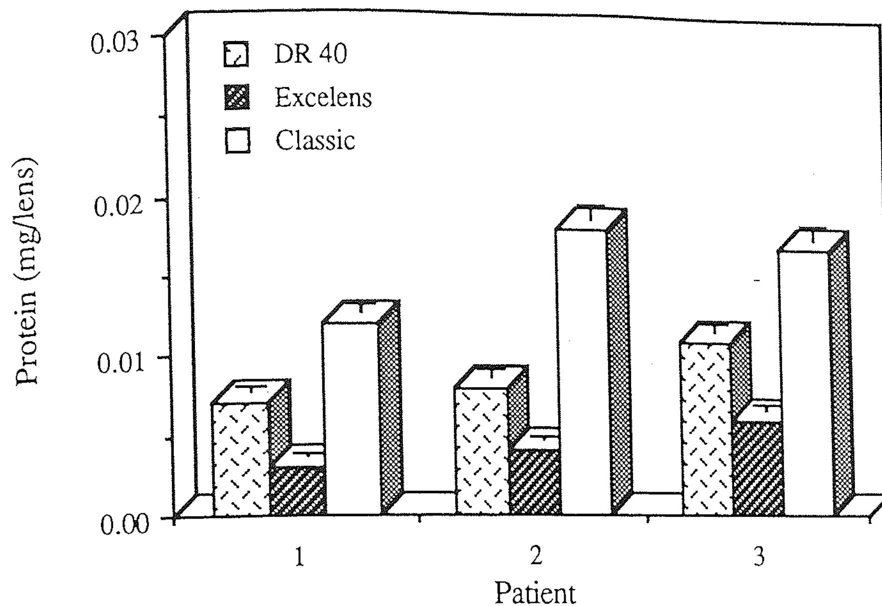


Figure 8.11 Typical protein deposition on different spoilation resistant materials.

Table 8.11 The proteins deposited *in-vivo* on Group I lenses.

Patient	Protein deposited (mg/lens) on		
	DR 40™	Excelens™	Classic™
1	0.007	0.003	0.012
2	0.008	0.004	0.018
3	0.011	0.006	0.017
4	0.009	0.005	0.015
5	0.008	0.006	0.013

8.8 The Contralateral Eye Study

8.8.1 *Introduction*

Although both Group II and IV lenses are high water content materials, but they have different chemical structure especially in terms of ionicity. The chemical structure of the lens material has a remarkable effect on the uptake proteins from the tear fluid. Several commercial lens cleaners have been used to clean the lenses. However, the cleaning efficacy of these solutions have only been investigated in commercially scales. In this study the differences between Group II and IV lenses in terms of protein deposition together with the effect of the care regimes in removing the deposition have been studied.

8.8.2 *Methodology*

Ten male patients wore a Group II lens in one eye and a Group IV lens in the other eye (Table 8.10 shows the materials). The lenses were worn on a daily basis and changed them for a new one at the end of the month. The study was carried out for five consecutive months during which the patients used one of the following care solutions for each month. The care solutions were used according the manufacturers recommended procedure. The procedures to use all the care solutions and surfactant cleaners are included in Appendix V.

1. Oxysept™ I-step (Allergan).
2. Oxysept™ II-step (Allergan).
3. ReNu™ (Bausch & Lomb)
4. Optifree™ (Alcon) with prior cleaning with Opticlean (Alcon).
5. Softab™ (Alcon) with prior surfactant cleaning with Alcon Pliagel™.

The protein was extracted from the lenses after their total protein was measured using U. V., and the activity of lysozyme was measured in the extraction solutions by the *Micrococcus* method.

8.8.3 *Results and Discussions*

Table 8.12 shows the total quantity for the first two month study, while Table 8.13 gives the amount extracted by ReNu™ together with the quantity of the active lysozyme in the extraction solutions.

Table 8.12 The results of protein deposition on Group II and Group IV contact lenses.

Patient	Lens type	Month 1		Month 2	
		Protein (mg/lens)	Care system	Protein (mg/lens)	Care system
1L 1R	Surevue™ Rythmic™	0.375 0.066	Softab™	0.352 0.043	Oxysept I™
2L 2R	Surevue™ Rythmic™	0.362 0.056	Oxysept II™	0.458 0.067	Oxysept I™
3L 3R	Surevue™ Rythmic™	0.330 0.032	Optifree™	0.446 0.058	ReNu™
4L 4R	Rythmic™ Surevue™	0.033 0.259	ReNu™	0.044 0.260	Oxysept II™
5L 5R	Surevue™ Rythmic™	0.301 0.059	ReNu™	0.259 0.043	Oxysept I™
6L 6R	Rythmic™ Surevue	0.041 0.481	Softab™	0.043 0.491	Oxysept II™
7L 7R	Rythmic™ Surevue™	0.045 0.396	Oxysept II™	0.067 0.412	Softab™
8L 8R	Rythmic™ Surevue™	0.055 0.345	ReNu™	0.076 0.473	Oxysept I™
9L 9R	Rythmic™ Surevue™	0.053 0.364	Oxysept II	0.063 0.341	Optifree™
10L 10R	Surevue™ Rythmic™	0.431 0.093	Oxysept I	0.452 0.101	Optifree™

Figure 8.12 shows the quantity of the protein deposited by various patients on each lens material. Figure 8.13 compares the effect of the cleaning regime on the activity of lysozyme remaining after the extraction. The values used to obtain the figures are the mean values for the patients and the error bars show the subject variations. The differences between the lens group is obvious from Figure 8.12 and it was shown that there are some patient variations as well as lens material. The quantity of the protein remained on the lenses are higher in the case of softab™than other care solutions and ReNu™ has been able to remove the proteins to a higher degree.

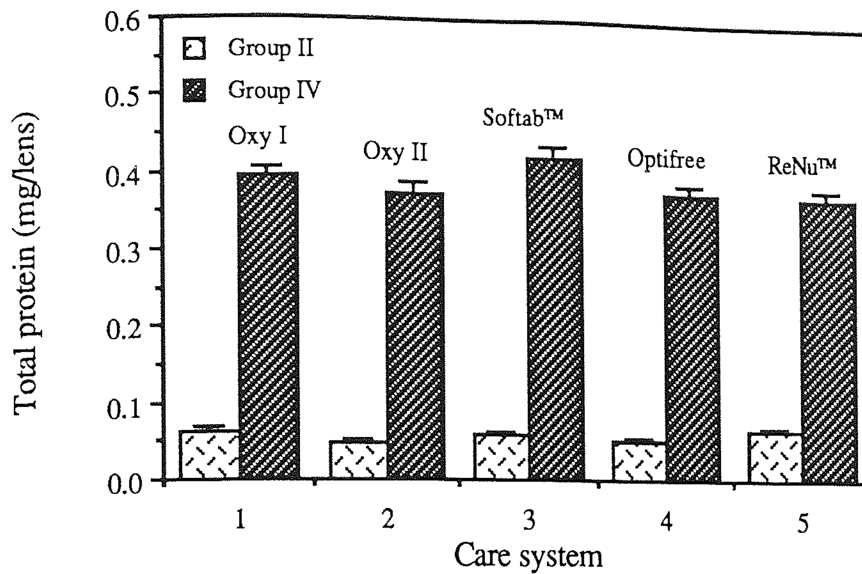


Figure 8.12 The quantity of the protein deposited on lenses cleaned with different care solutions.

It can be seen from Figure 8.13 that the activities have slightly been affected depending on the care system used. ReNu™ has the least effect on the activity, while Oxysept™ systems have more destroyed the activity. Softab™ has the highest effect on the lysozyme activity and this can be due to the oxidant effect of the two later care systems. The loss of activity is more significant in the case of Group II lenses and it is shown in Figure 8.13 that the activity of lysozyme extracted from Group IV lenses is retained between 92-96% depending on the care solution used , while in the case of Group II up to 30% of the activity is lost. This can be due to the lower quantities of the proteins in the case of Group II lenses. The ionic nature of Group IV lenses gives the protein more mobility and can cause the retain of the activity.

Table 8.13 The effect of care system on the loss of activity.

Subject	Month 1			Month 2		
	Protein (mg/ml)		Care system	Protein (mg/ml)		Care system
	Total	Active		Total	Active	
1L	0.221	0.218	Softab™	0.231	0.208	Oxysept I
1R	0.021	0.018		0.108	0.008	
2L	0.231	0.226	Oxysept II	0.250	0.232	Oxysept I
2R	0.040	0.032		0.042	0.031	
3L	0.018	0.013	Optifree™	0.260	0.253	ReNu™
3R	0.160	0.156		0.042	0.039	
4L	0.168	0.162	ReNu™	0.151	0.143	Oxysept II
4R	0.035	0.028		0.021	0.012	
5L	0.208	0.202	ReNu™	0.160	0.142	Oxysept I
5R	0.030	0.028		0.028	0.020	
6L	0.023	0.019	Softab™	0.022	0.013	Oxysept II
6R	0.321	0.313		0.381	0.361	
7L	0.023	0.019	Oxysept II	0.035	0.031	Softab™
7R	0.286	0.262		0.296	0.276	
8L	0.246	0.241	ReNu™	0.042	0.032	Oxysept I
8R	0.042	0.038		0.316	0.239	
9L	0.041	0.037	Oxysept II	0.036	0.031	Optifree™
9R	0.259	0.239		0.231	0.223	
10L	0.328	0.316	Oxysept I	0.308	0.269	Optifree™
10R	0.053	0.046		0.062	0.058	

Table 8.14 The mean protein values on lenses cleaned by different solutions.

Care system	Protein (mg/lens) on	
	Surevue™	Rythmic™
Oxysept I™	0.394	0.064
Oxysept II™	0.375	0.048
Softab™	0.422	0.058
Optifree™	0.374	0.065
ReNu™	0.378	0.051

Table 8.15 The mean value of active lysozyme in extraction solution of lenses from both eyes cleaned with different care systems.

Care system	% active Protein	
	Surevue™	Rythmic™
Oxysept I™	91.4	74.8
Oxysept II™	92.5	73.8
Optifree™	92.1	80.8
Softab™	96.1	82.5
ReNu™	96.8	89.8

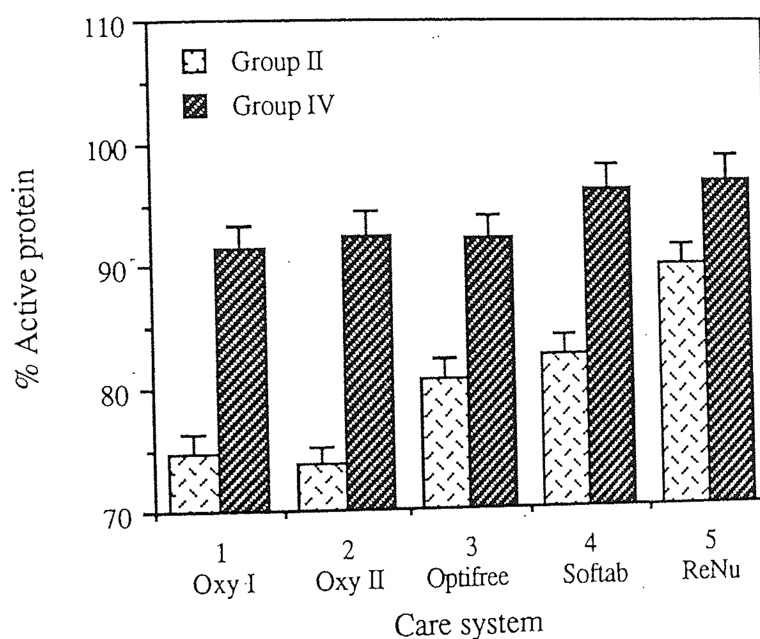


Figure 8.13 The effect of care system on the loss of activity of protein deposited on Groups II and IV contact lenses.

8.9 The Cross-Over study between Group II Lenses (ES70™ and Excelens™)

8.9.1 Introduction

The method of manufacture of the lens may have some effect on its properties especially in terms of spoilation behaviour. In fact different groups of lens material have been shown to be spoiled in different ways, but the lenses from the same group are expected to show similar pattern of spoilation and protein uptake. In this set of clinical experiments two Group II lenses from different manufacturers were compared and were used in a cross-over study to determine the patient response to changing the lenses to another type within the same FDA group and the effect of long term use of these lenses on protein absorption.

8.9.2 Methodology

In this six month, 2-way cross-over study, 6 contact lens wearers randomly used either ES70™ or Excelens™ lenses on a daily wear basis. Miraflow™ was used for surfactant cleaning and 10:10 peroxide solution for disinfection during this time. After 3 months the lenses were changed for the other lens type and at the end of next 3 months the lenses were collected for analysis. No enzyme cleaner was used during this 6 months study. The quantities of protein deposited was measured by direct U. V. at 280 nm on the lenses after a rinse with distilled water.

6.9.3 Results and Discussion

The quantities of protein are shown in Table 8.16. These results indicate that the VP based lens material (ES70™) deposit more protein than newer poly vinyl alcohol (PVA) based materials (Excelens™). Despite the similarity of the water content and ionicity (both are Group II lenses) these two lens types showed variation in protein spoilation behaviour. The lipid and calcium analysis were also performed by other members of the research group and it was shown that VP based lenses deposited greater amounts of these species. The FDA categorisation is not always a complete factor in specifying the spoilation resistance of the lens material and the polymer composition has a significant effect on the quantity of the protein uptake by the lens. The results also show a wide range of patient variation which seems to be significant in all types of lenses and their spoilation behaviour.

Table 8.16 The quantity of protein on Group II lenses in three months wear.

Patient No. (Eye)	Quantity of protein (mg/lens) on ES70™	Quantity of protein (mg/lens) on Excelens™
1 (Left)	0.057	0.012
1 (Right)	0.052	0.008
2 (Left)	0.069	0.011
2 (Right)	0.063	0.013
3 (Left)	0.068	0.004
3 (Right)	0.069	0.005
4 (Left)	0.082	0.000
4 (Right)	0.086	0.003
5 (Left)	0.064	0.021
5 (Right)	0.069	0.018
6 (Left)	0.051	0.010
6 (Right)	0.048	0.008

8.9.4 Conclusion

Group II lenses can vary widely in their behaviour in contact with tear proteins. Although they are classified as high water content non- ionic lenses, but the nature of the polymer used for their manufacture affects their spoilation by proteins and other tear components. This study showed that poly vinyl alcohol based materials have a higher resistance to the protein deposition than VP lens materials.

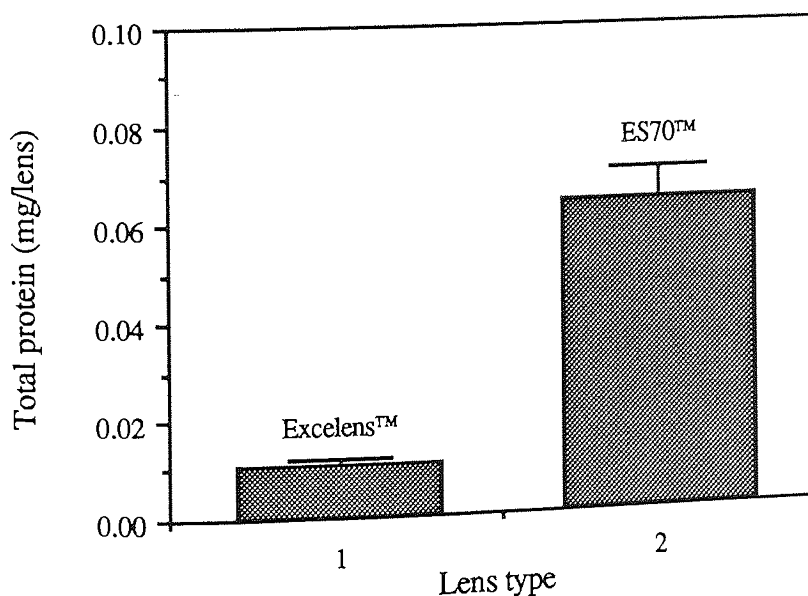


Figure 8.14 The average protein spoilation on Group II lenses.

8.10 The Protein Absorption on Group I Lenses

8.10.1 Introduction

This study aimed to show the effect of long term use of different Group I lenses on their protein uptake. The lenses were selected from those made with polymers based on biomimetic principle.

8.10.2 Methodology

Twelve patients enrolled on a three month, 2-way cross-over study in which two lens types on a daily wear basis were used. LC65™ was used as surfactant cleaner and Oxysept™ II-Step as a disinfectant according to the manufacturers recommended procedures. As in the previous study no enzyme tablets were used during this study. As the DR40 is a new biomimetic material, this study showed its difference with poly(HEMA) (Z6™) which is an industry standard material prescribed for daily wear.

8.10.3 Results and Discussion

The material details are indicated in Table 8.10. The quantity of the total protein deposited on each lens are shown in Table 8.17. The mean protein values are presented in Figure 8.15. These results shown in Table 8.17 demonstrate that the protein deposition on these lenses is highly patient dependent. The higher quantity of protein on DR40 lens material than expected for a Group I lens suggests that these lenses may have been contaminated with residual methacrylic acid (MAA) during the polymerisation process. It has been shown that methacrylic acid can enhance the uptake of lysozyme [121]. It is normally very difficult to remove all residual MAA from the final product of HEMA contact lens production. The *in-vitro* lysozyme assay experiment which was carried out (Chapter 4) indicated the differences between the poly(HEMA) lenses in terms of MAA contamination. It can be concluded that nearly all poly(HEMA) lenses contain some trace of methacrylic acid which causes the protein deposition in most cases. The degree of the MAA impurities depends on the polymerisation process, the distillation and purification of the final product and the care taken when using the polymer to manufacture the contact lens.

Table 8.17 The *in-vivo* protein deposition on Group I lenses.

Subject	Total protein (mg/lens) on	
	Vista DR40™	Hydron Z6™
1	0.044	0.023
2	0.040	0.020
3	0.051	0.030
4	0.057	0.033
5	0.038	0.025
6	0.032	0.020
7	0.030	0.018
8	0.047	0.032
9	0.053	0.034
10	0.040	0.026
11	0.047	0.027
12	0.050	0.020

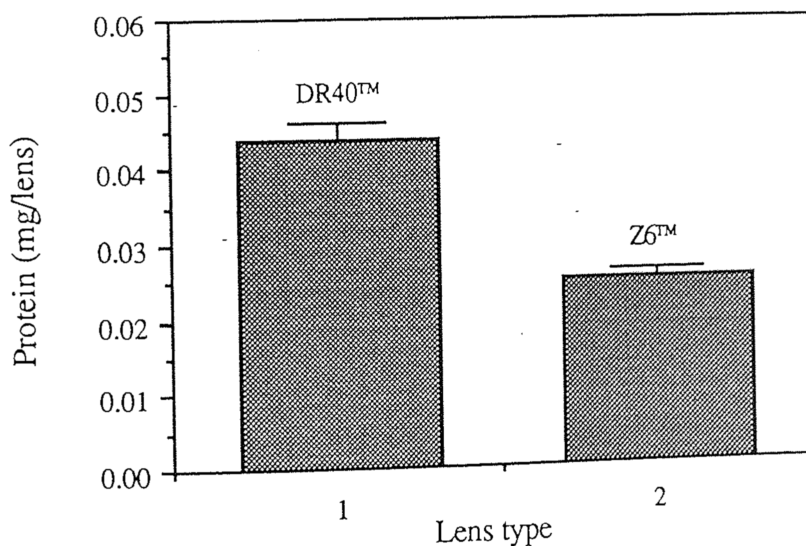


Figure 8.15 The differences between DR40™ and Z6™ (Group I) lenses.

8.11 Tear Analysis

Human tears contains a complex mixture of different proteins as well as lipids and a wide range of other constituents. Some of the proteins identified in tears are shown in Table 1.4. The composition of tear proteins may vary in different people and it depends various factors such as age and different infections which have been resulted from some

eye related problems. In this part of work a series of tear samples collected from some patients in Section 8.3.2 were used to measure their lactoferrin (by Lactoplate® Section 2.4.6) and lysozyme (by radial immunodiffussion assay on diluted samples, Section 2.4.3). Some tear samples were also used for the identification of their proteins using electrophoresis. The tears were collected every month and the total collected samples were added together to make enough volume for the experiments.

The results of electrophoresis is shown in Figure 8.15. In this figure, the lines are the tear samples from patients 1, 2, 5, 17, 18, 6 and 7. Looking back to the level of the protein these patients deposited and comparing them with the electrophoresis results show that the quantity of the protein in the tear affects the spoilation. This is more pronounced in the case of Group IV lenses. The proteins were extracted from Group IV lenses of some of theses patients and their electrophoresis gel stained with Brilliant Blue G is shown in Figure 8.4.

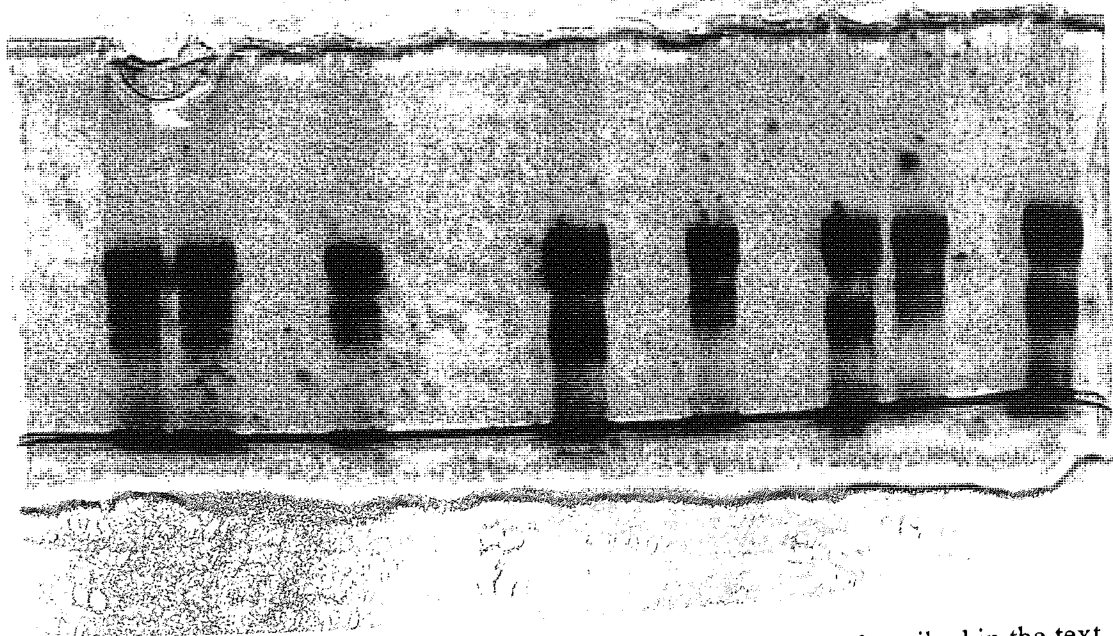


Figure 8.16 The electrophoresis gel containing tear samples as described in the text.

The quantities of lysozyme and lactoferrin in the tear samples were calculated for patient number 1 and 15 which deposited comparably highest and lowest protein respectively on thier lenses. It was shown that the quatities of these proteins are higher for patient 1 than patient 15. It can be concluded that the composition of the tear is another important factor which affect the contact lens spoilations.

Chapter 9

Removal of Proteins from Contact Lenses

Chapter 9

Removal of Proteins from Contact Lenses

9.1 General Introduction

Within 30 minutes of inserting never-worn soft contact lenses, the anterior surface is approximately 50 % covered by a scattered coating of mucus and proteins. After eight hours of wear, lenses are approximately 90 % covered by a complex, layered coating [122]. Manufacturing methods and water content have been shown to affect the degree of deposition (Chapter 5).

There are many commercially available cleaning regimes for removing the proteins from hydrophilic contact lenses. Fowler [123] have studied the deposited contact lenses by scanning electron microscopy and concluded that cleaning solutions have not been very effective in removing the lens deposits. The failure of patients to strictly adhere to proper care systems increases many of the problems associated with the lens spoilation. Multi-purpose contact lens care products have been developed to provide wearers with simpler lens care regimes.

9.2 The Effect of Various Multi-Purpose Cleaning Solutions on the Quantity and Activity of Proteins

9.2.1 Introduction

It has been shown that the small, positively charged proteins such as lysozyme penetrate into the matrix of anionic hydrogels and, therefore, their complete removal is almost impossible. In the present study similar *in-vitro* procedures were used to study the efficacy of seven different commonly used all-in-one solutions in removing the proteins from various group hydrogel lenses.

9.2.2 The Spoilation Procedure

A number of lenses from different FDA Groups (Table 9.1) were individually spoiled *in-vitro* in 2 millilitres of 0.5 mg/ml protein solutions (lysozyme, albumin and lactoferrin) for 5 days with constant stirring. They were cleaned using different care systems and the protein remained on each after cleaning procedure was then measured by measuring the absorption at 280 nm after a quick rinse with distilled water. The cleaning solutions were

used according to the manufacturers recommended procedures, which in most cases were a simple one step cleaning followed by leaving the lens in the solution for a short period. The U. V. absorptions were again measured after cleaning. The specific procedures to use each care system are presented in Appendix V.

Table 9.1 The properties of contact lenses used for cleaning study.

Name	Company	EWC %	FDA group	Power	Material
Z6™	Hydron	38	I	- 0.5	HEMA
Rythmic™	Lunelle	73	II	- 0.5	VP, MMA
B. H Thin™	Pilkington Barnes Hind	41	III	- 0.5	HEMA, MAA

The individual proteins were then extracted from the lenses by the use of small volume of ReNu™ and shaking overnight. The activities of extracted lysozyme from all three group lenses were measured by *micrococcus lysodeikticus* method (Section 2.4.2). This is a method based on the decrease in the turbidity, i.e.. the U.V. absorption of a *micrococcus lysodeikticus* solution due to the reaction of lysozyme on this substrate. A standard lysozyme solution was also used under the same conditions to measure the activity of the unknown samples.

The lenses were stored in the care solutions for one and three weeks without changing the solutions and disturbing the removal process. Their U.V. absorptions were then measured in order to compare the results obtained before long term storage.

9.2.3 Results and Discussions

The quantities of each individual protein deposited on three different Group lenses are shown in Tables 9.2 - 9.5 and Figures 9.1 - 9.4 together with type of cleaning solutions. The effectiveness of these four commercially available multi-purpose solutions can be compared. It is apparent from the results that all of these solutions can remove only about 60-65 % of the deposited proteins. The higher the amount of the spoilation the more effective the care solution, and lysozyme is harder to be removed from the lenses than lactoferrin and albumin. This is because lysozyme is a small protein and can penetrate into the matrices of lenses and, therefore, is more difficult to be washed off. It can also be concluded that ReNu™ is the most effective multi-purpose solution among those which were studied.

The electrophoresis of the extracted proteins showed no detectable (visible) protein extracted from any of the three lens groupings, because the quantities of the extracted proteins were very low and the techniques are not suitable for the detection of protein samples less than 0.2 mg/ml.

Lysozyme was extracted from all of the lenses and its activity was measured by micrococcus method, which is based on the decrease in turbidity (A_{580}) following lysis of a suspension of *Micrococcus lysodeikticus* (Section 2.4.2). The total protein remained after cleaning with different care systems was measured by direct U.V. measurement on the lenses. The results of total and active lysozyme are shown in Tables 9.6 and 9.7.

It can be seen that the activity has not been affected due to the absorption by the different lens groups . The cleaning procedure and the type of cleaner do not have a marked effect on the quantity and activity of the proteins absorbed to these three group lenses. It was shown before that the activity of the enzyme remained almost unaffected after absorption to Groups I and IV lenses (section 5.1). It can also be concluded from this study that lysozyme is an active enzyme which retains its activity against treating with various cleaning solutions.

The quantities of proteins after one and three weeks storage in the appropriate care solutions did not show significant difference with those obtained just after the cleaning procedures. The storage of the spoiled lens in a ReNu™ solution for long term with changing the care solution every day, however, was one of the preferred processes to extract the proteins from ionic lens materials (Section 9.3).

Table 9.2 The effect of ReNu™ (Bausch & Lomb) multi-purpose solution in removing the proteins from contact lenses.

Protein type	Quantities of proteins on lenses (mg/lens) (± 0.002)					
	Before cleaning			Remained after cleaning		
	Group I	Group II	Group III	Group I	Group II	Group III
Lysozyme	0.028	0.062	0.221	0.016	0.040	0.116
Lactoferrin	0.021	0.046	0.181	0.012	0.028	0.123
Albumin	0.022	0.032	0.058	0.018	0.026	0.042

Table 9.3. The effect of Complete™ (Allergon) all-in-one solution in removing the proteins from contact lenses.

Protein type	Quantities of proteins on lenses (mg/lens) (± 0.002)					
	Before cleaning			Remained after cleaning		
	Group I	Group II	Group III	Group I	Group II	Group III
Lysozyme	0.026	0.064	0.220	0.014	0.044	0.102
Lactoferrin	0.022	0.045	0.181	0.016	0.030	0.128
Albumin	0.021	0.030	0.056	0.018	0.015	0.044

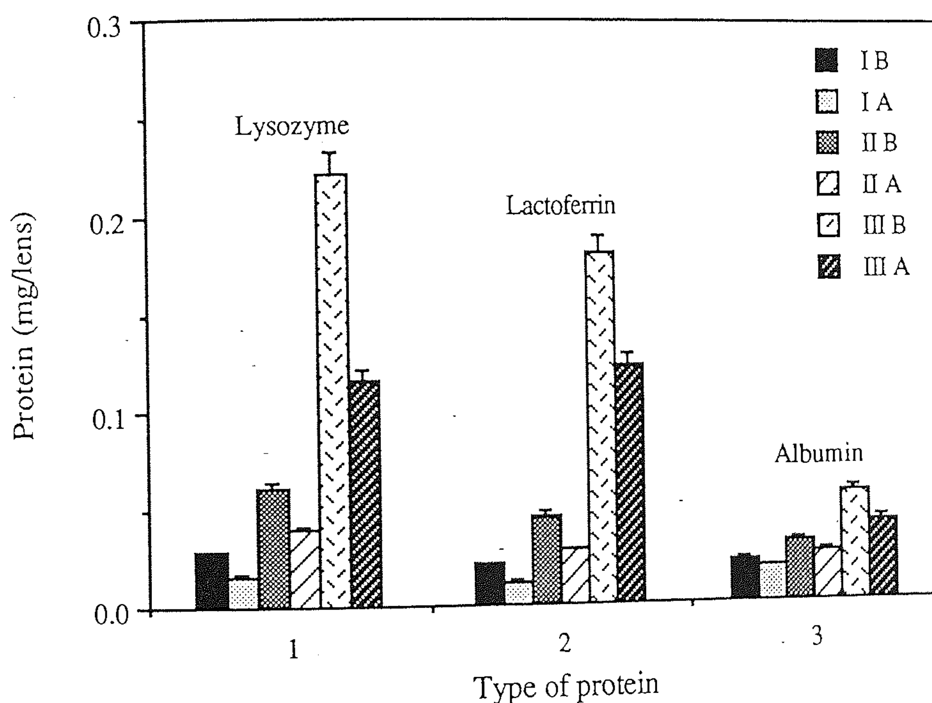


Figure 9.1 The effect of ReNu™ multi-purpose solution in removing the proteins from different lenses (B = Before and A = After cleaning, I, II and III refer to the Groups of the lens material).

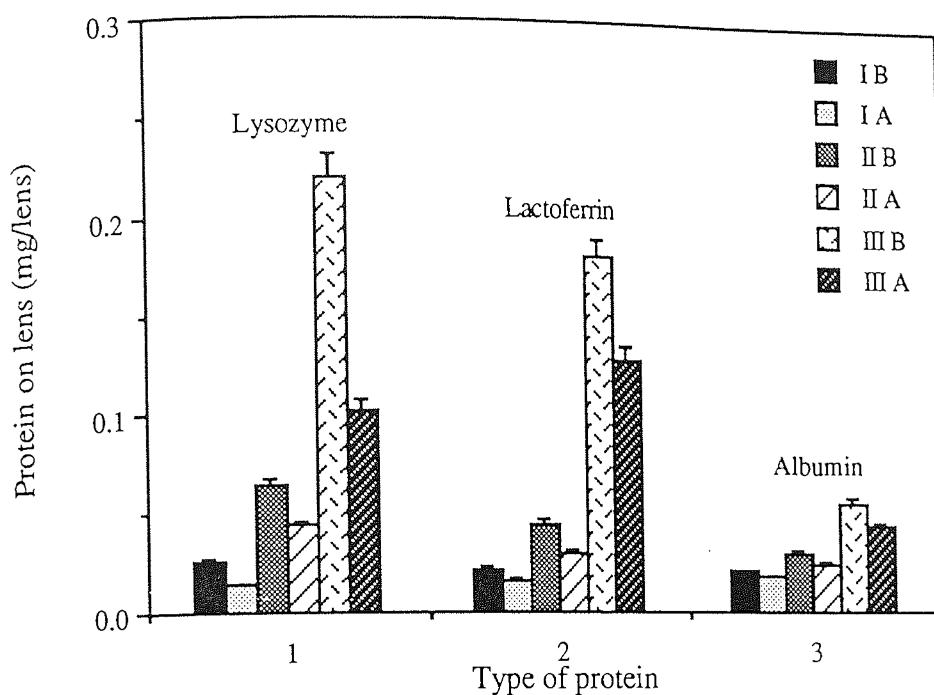


Figure 9.2 The effect of Complete™ all-in-one solution in removing the proteins from different lenses (B = Before and A = After cleaning, I, II and III refer to the Groups of the lens material).

Table 9.4 The effect of Concerto™ (Essilor) all-in-one solution in removing the proteins from contact lenses.

Protein type	Quantities of proteins on lenses (mg/lens) (± 0.002)					
	Before cleaning			Remained after cleaning		
	Group I	Group II	Group III	Group I	Group II	Group III
Lysozyme	0.028	0.061	0.223	0.014	0.040	0.122
Lactoferrin	0.022	0.043	0.180	0.014	0.028	0.132
Albumin	0.024	0.034	0.055	0.019	0.028	0.048

Table 9.5 The effect of Sauflon™ all-in-one solution in cleaning the contact lenses from deposited proteins.

Protein type	Quantities of proteins on lenses (mg/lens) (± 0.002)					
	Before cleaning			Remained after cleaning		
	Group I	Group II	Group III	Group I	Group II	Group III
Lysozyme	0.030	0.060	0.226	0.014	0.042	0.132
Lactoferrin	0.023	0.044	0.182	0.016	0.030	0.128
Albumin	0.020	0.031	0.056	0.016	0.026	0.046

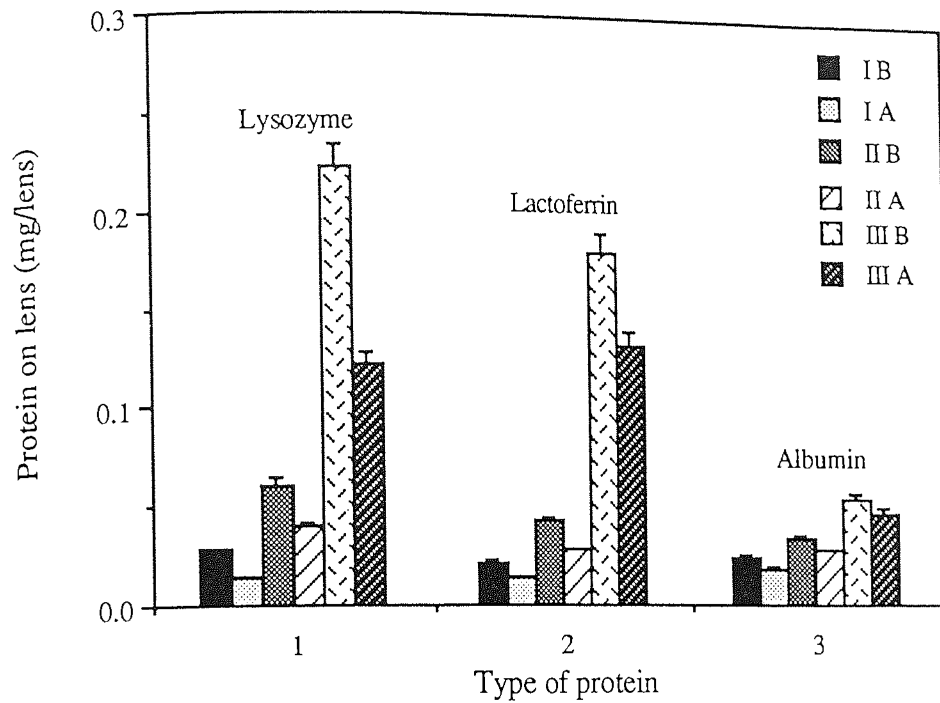


Figure 9.3 The effect of Concerto all-in-one solution in removing various proteins from different lenses (B = Before and A = After cleaning, I, II and III refer to the Groups of the lens material).

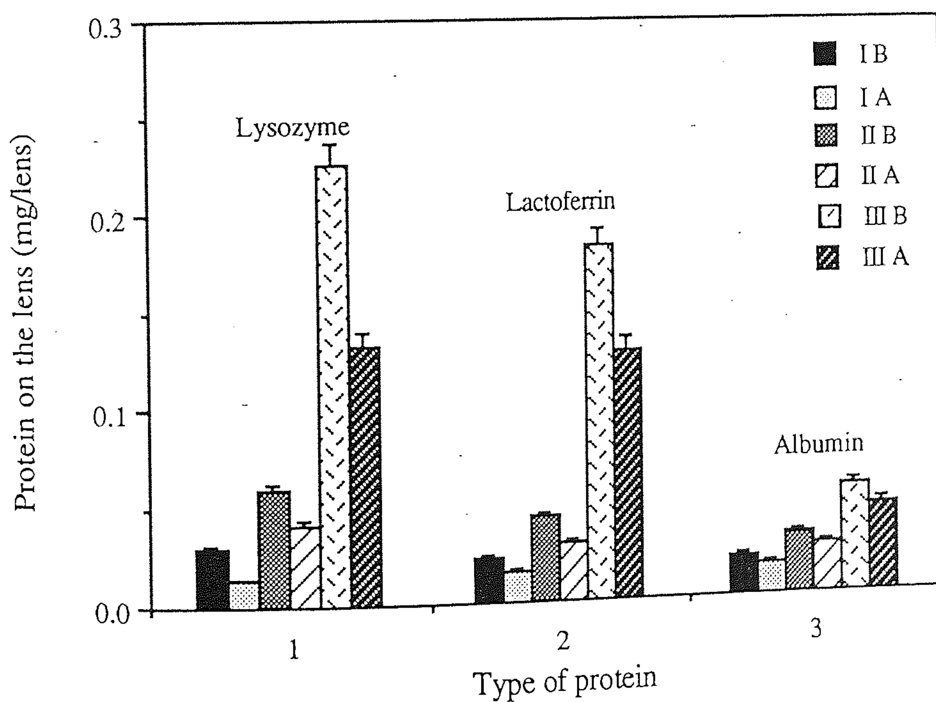


Figure 9.4 The effect of Sauflon™ all-in-one solution in removing the various proteins from different lenses (B = Before and A = After cleaning, I, II and III refer to the Groups of the lens material).

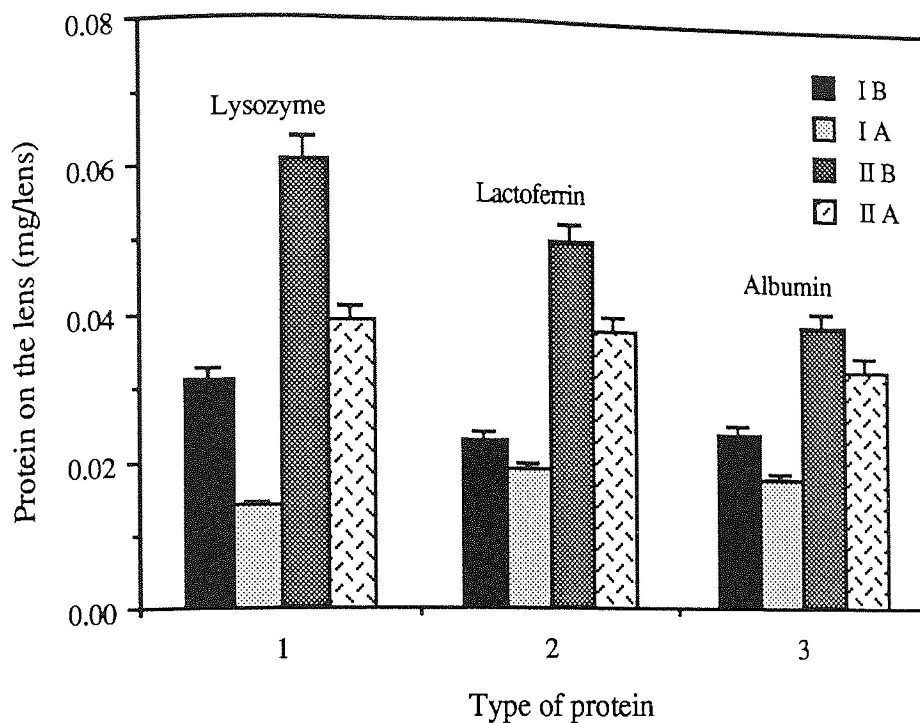


Figure 9.5 The effect of Quick Care™ in removing various proteins from different lenses (B = Before and A = After cleaning, I, II and III refer to the Groups of the lens material).

Table 9.6 The effect of Quick Care™ (Ciba Vision) multi-purpose solution in removing the deposited proteins from contact lenses.

Protein type	Quantities of proteins on the lenses (mg/lens) (± 0.002)					
	Before cleaning			Remained after cleaning		
	Group I	Group II	Group III	Group I	Group II	Group III
Lysozyme	0.031	0.061	0.200	0.014	0.039	0.124
Lactoferrin	0.023	0.050	--	0.019	0.038	--
Albumin	0.024	0.039	--	0.018	0.033	--

Table 9.7 The effect of Opti-Free™ (Alcon) solution in removing the proteins from contact lenses.

Protein type	Quantities of proteins on the lenses (mg/lens) (± 0.002)					
	Before cleaning			Remained after cleaning		
	Group I	Group II	Group III	Group I	Group II	Group III
Lysozyme	0.026	0.065	0.221	0.014	0.042	0.122
Lactoferrin	0.021	0.041	--	0.018	0.036	--
Albumin	0.019	0.033	--	0.016	0.028	--

Table 9.8 The effect of Opti-One™ multi-purpose (Alcon) solution in removing the proteins from contact lenses.

Protein type	Quantities of proteins on the lenses (mg/lens) (± 0.002)					
	Before cleaning			Remained after cleaning		
	Group I	Group II	Group III	Group I	Group II	Group III
Lysozyme	0.026	0.062	0.220	0.012	0.034	0.118
Lactoferrin	0.021	0.044	--	0.018	0.036	--
Albumin	0.022	0.041	--	0.016	0.033	--

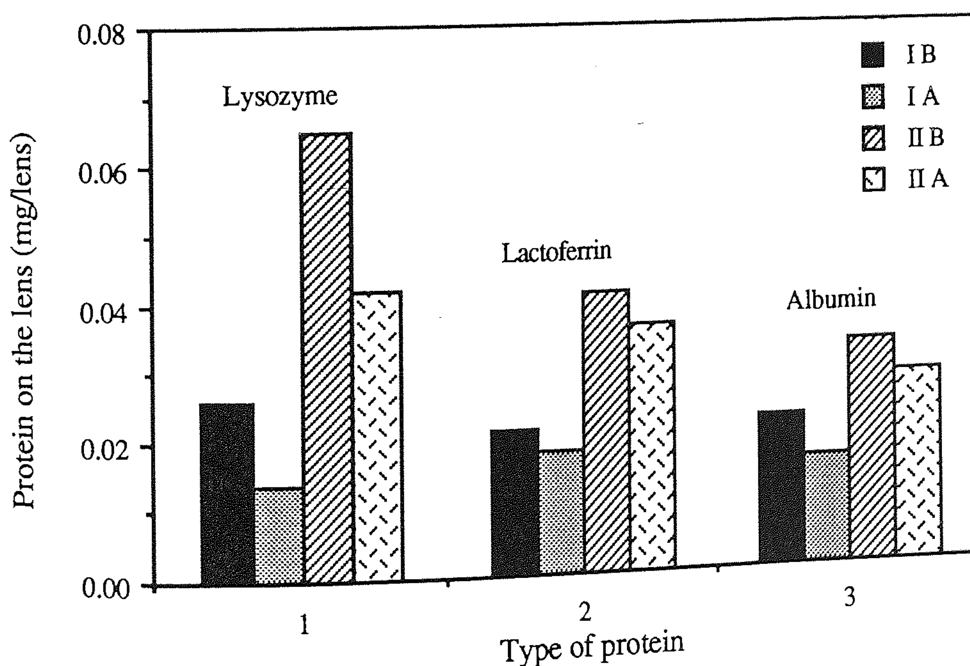


Figure 9.6 The effect of Opti-Free™ solution in removing various proteins from different lenses (B = Before and A = After cleaning, I, II and III refer to the Groups of the lens material).

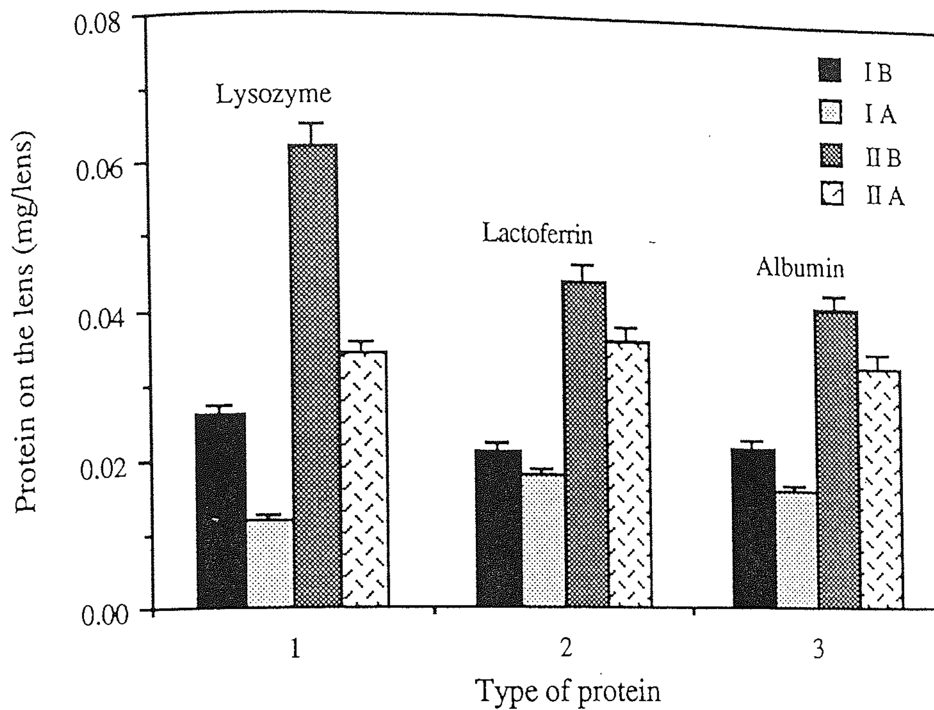


Figure 9.7 The effect of Opti-One™ multi-purpose solution in removing various proteins from different lenses (B = Before and A = After cleaning, I, II and III refer to the Groups of the lens material).

Table 9.9 The total and active lysozyme removed from different groups of contact lenses by different cleaning solutions.

Cleaning solution	Quantities of lysozyme extracted (mg/ml) (± 0.002)					
	Total			Active		
	Group I	Group II	Group III	Group I	Group II	Group III
ReNu™ multi purpose	0.013	0.026	0.126	0.012	0.024	0.120
Complete™ all-in-one	0.015	0.024	0.132	0.013	0.022	0.124
Concerto™ all-in-one	0.016	0.022	0.134	0.014	0.020	0.128
Sauflon™ all-in-one	0.020	0.024	0.136	0.018	0.022	0.130
Quick Care™ solution	0.012	0.022	0.124	0.010	0.020	0.118
Opti-Free™ solution	0.010	0.024	0.124	0.008	0.020	0.118
Opti-One™multi-purpose	0.012	0.022	0.120	0.010	0.020	0.114

Table 9.10 The percentage of active lysozyme extracted from various contact lenses into different care solutions.

System No.	Care solution	Active lysozyme %		
		Group I (±5%)	Group II (±3%)	Group III (±2%)
1	ReNu™ multi-purpose	92	92	95
2	Complete™ all-in-one	87	92	94
3	Concerto™ all-in-one	88	91	95
4	Sauflon™ all-in-one	90	92	95
5	Quick Care™ solution	83	91	95
6	Opti-Free™ solution	80	83	95
7	Opti-One™ multi-purpose	83	91	95

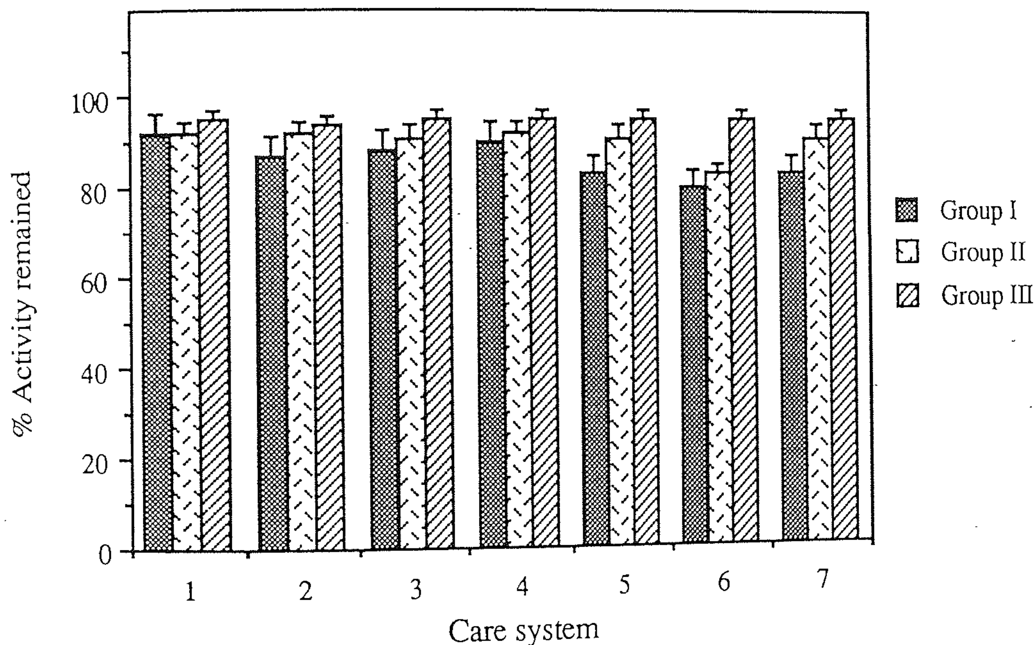


Figure 9.8 The comparison between the % activity of lysozyme extracted from different lenses cleaned with various care solutions (the numbers refer to the systems given in Table 9.10).

9.2.4 Conclusions

The results presented in Tables 9.2-9.8 show that all of the cleaning systems used in this study have similar effect on the removal of the protein deposits from hydrogel contact lenses. ReNu™, however, have a comparatively higher efficiency with respect to the other all-in-one solutions. Although about 30 to 50 % of the proteins have been removed by the use of cleaning solutions, considerably high quantities of protein still

remained on the lenses. This protein layer is mostly the first layer which, especially in the case of ionic lenses (Group III), have penetrated into the matrix. The first monolayer of protein is very hard and practically impossible to be removed from the lens.

The quantity of the remaining protein depends on the quantity of the absorbed protein. The activity measurements on the extracted lysozyme from different lenses shows that, within the experimental errors, most of lysozyme remained active after cleaning with different cleaning systems. The slight decrease in the activity depends more on the type of the lens than on the care regime used for cleaning the lenses. The standard deviation for the activity measurement varied between 2-5 %.

9.3 The Effect of Extraction Techniques on the Removal of Proteins

9.3.1 Introduction

Although the care solutions produced by different manufactures have slightly different efficiency on the removal of proteins from contact lenses, however it can be seen from Section 9.2 that most of the protein remains in the lens even after cleaning with the most effective solutions.

In most of the analytical studies about the proteins deposited on contact lenses, however, it is necessary to extract the protein from the lens matrix. Measuring the activity by the method or the identification of the nature of protein by electrophoresis and isotachophoresis techniques are not possible while the protein is absorbed in the lens.

As discussed in Section 2.4.1 attempt was made to extract the most possible extractable protein from different contact lenses. This was a procedure consisting of different extraction solvents and mixture of solvents together with a number of different extraction times and techniques.

In a preliminary experiment in Section 2.4.1 it was found that long term extraction in ReNu™ was more effective than other chemical extraction solutions. In this study, ReNu™ was compared with some other care solutions and surfactant cleaners.

9.3.2 Methodology

The lenses used in this study were selected from different lens groups and they are shown in Table 9.11. The lenses were spoiled *in-vitro* in 2 ml of lysozyme and lactoferrin solutions at room temperature for 5 days. They were vibrated frequently on a vibrator at low speed during the spoilation. They were then rinsed once with distilled water and the total protein deposited on them was measured by U.V. at 280 nm. Using the following methods (1 - 8) the proteins were extracted from the lenses and the protein remained on each lens was measured again by U. V. at 280 nm. The percentage of each type of protein removed from the different lenses was calculated.

1. Rub the lens with LC 65™ surfactant cleaner using fingers and rinse with saline.
2. Clean with surfactant cleaner (LC 65™) followed by shaking in ReNu™ for at least one hour.

3. Clean the lens with ReNu™ and leave in the fresh solution overnight while shaking vigorously on a shaker.
4. Clean the lens with saline and leave in the fresh solution overnight while shaking vigorously on a shaker.
5. Wash with ReNu™ and shake for one week in fresh ReNu™ solution.
6. Clean and rinse the lens with saline and leave saline solution for 10 minutes.
7. Clean and rinse the lens with saline and leave in Oxysept™ solution for 10 minutes.
8. Clean and rinse the lens with saline and leave in Oxysept™ solution for 20 minutes.

The quantity of each protein absorbed on different lens types was measured before the extraction and after the attempt to extract the protein. The direct U.V. method was used to measure the quantity of the remained protein against a blank of the same lens material.

Table 9.11 The characteristics of the contact lenses used for extraction study.

Lens	Company	EWC	Monomers	USAN	Manufacture	FDA
Z6™	Allergon	38	HEMA	Polymacon	Lathed	I
Rythmic™	Lunelle	73	VP-MMA	---	Moulded	II
Acuvue™	Vistakon	58	HEMA+MAA	Etafilcon	Moulded	IV

9.3.3 *Results and Discussions*

The percentages of lysozyme and lactoferrin removed from different group lenses are compared in Figure 9.9 and 9.10, respectively. It can be seen that lysozyme has been removed more easily than lactoferrin and albumin from all the lenses. The use of ReNu™ as a cleaner and storage solution has the highest efficiency to extract the protein. Saline has a similar, but slightly lower effect than ReNu™ in the removal of the proteins. It was shown in previous studies [106] that the mobility of the proteins is higher in solutions with a slightly alkaline pH and this is the reason for the higher efficiency of long term storage of the lens in ReNu™ (pH 7.2).

The quantities of the remained proteins on each lens type after extraction are shown in Table 9.12. As it can be revealed from this figures, although a high percentage of the

deposited protein have been extracted the actual quantities which remained after the extraction, however, is still considerable. The quantity depends on the lens type as well as the extraction technique.

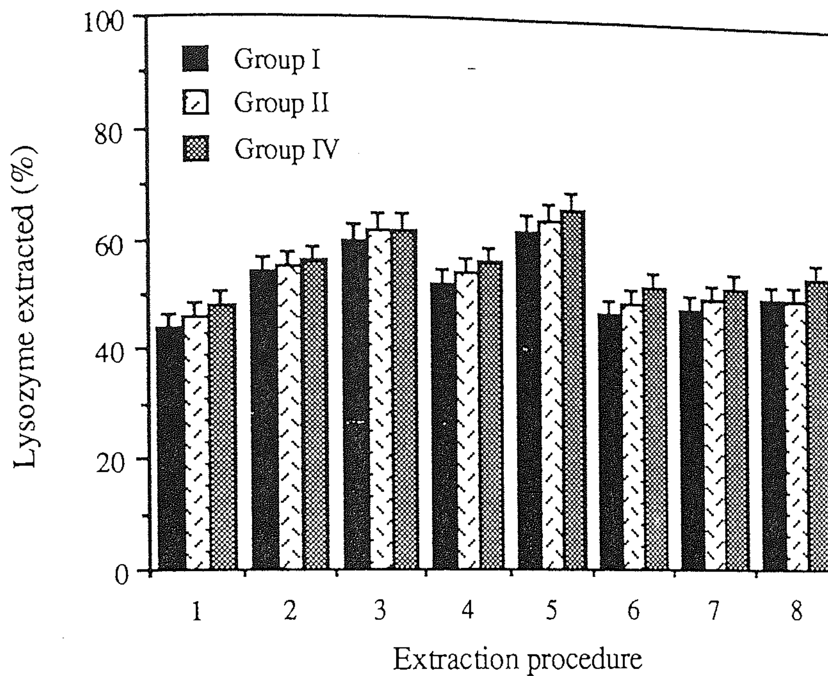


Figure 9.9 The percentage of lysozyme extracted from different lens types by various extraction techniques (the numbers refer to the techniques discussed in 9.3.2).

However, the extraction has been more effective in the case of more heavily deposited lenses i. e. lysozyme extracted from Group IV lenses has a higher percent than the quantity of lysozyme extracted from Group I lenses in each case. In the case of lactoferrin the amount of extracted protein depends on the type of the lens, but to a lower degree than lysozyme. The reason for this difference between lysozyme and lactoferrin may be due to the small size of lysozyme compared to lactoferrin which makes it more mobile and less attached to the lens.

It must be emphasised here that the *in-vitro* nature of these experiments has one major advantage over the *in-vivo* studies. The efficiency of different cleaning systems can be compared without patient-related experimental complications such as variation in wearing time, tear compositions and using the care regimes. It appears that the care systems available are more effective in removing lysozyme and have more difficulties in cleaning the lens from other proteins such as lactoferrin and albumin. Albumin tends to be very soluble in water and negatively charged at physiological pH and, therefore it may remain soluble and difficult to remove. Lactoferrin displays a strong affinity for

binding to other proteins [124] and might readily interact with an immobile glycoprotein embedded in the lens matrix and/or with solubilized albumin.

Table 9.12 Milligrams of lysozyme (a) and lactoferrin (b) (± 0.002) remaining on and percentage of a and b removed (± 7.0) from lenses after extraction.

Extraction Technique	Group I		Group II		Group IV		Protein type
	mg Protein remained	% Protein removed	mg Protein remained	% Protein removed	mg Protein remained	% Protein removed	
1	0.016	44	0.034	46	0.164	48	a
	0.019	35	0.040	36	0.192	38	b
2	0.013	54	0.030	55	0.142	56	a
	0.016	45	0.034	47	0.160	50	b
3	0.012	60	0.028	62	0.122	62	a
	0.014	50	0.032	52	0.146	54	b
4	0.014	52	0.036	54	0.140	56	a
	0.017	42	0.038	42	0.172	45	b
5	0.012	62	0.024	64	0.110	66	a
	0.014	54	0.034	46	0.130	59	b
6	0.016	47	0.034	49	0.152	52	a
	0.018	38	0.038	40	0.178	44	b
7	0.016	48	0.032	50	0.152	52	a
	0.018	38	0.040	38	0.190	40	b
8	0.014	50	0.030	50	0.144	54	a
	0.016	40	0.040	42	0.178	44	b

9.3.4 General Conclusions on Protein Removal

None of the commercially available cleaning systems tested was completely effective at removing proteinaceous material from hydrophilic lenses under our experimental conditions. The effort to extract protein to study the effect of different care systems on the activity of lysozyme was successful only up to about 65 % extraction of lysozyme.

It is suggested that the removal of charged proteins is easier from the ionic lenses than non-ionic contact lens materials. Figure 9.9 shows that the percentage of lysozyme extracted from a Group IV (high water content, ionic) lens is higher than from both Groups I and II which are non-ionic.

However, it can be concluded that the long term extraction with ReNu™ is the most effective way to remove the proteins from the contact lenses especially if it is followed by rubbing the lens with a surfactant cleaner. Lysozyme is a very mobile protein and, although it is absorbed strongly to high water content ionic lenses, it is most easily removed. Once protein is absorbed to the lens, it is almost impossible to be extracted totally and the most effective solution and extraction process can only remove up to 65% of the protein. In any case there is still high quantity of the protein remained on all lens Groups after cleaning with various care regimes and despite the hardest method of extraction.

It would be explained from the results obtained in this part of study that the cleaning systems and extraction techniques studied have been designed to remove lysozyme from spoiled lenses with less reference to the other proteins that can deposit. In any case, it is strongly suggested that the manufacturers of cleaning solutions should develop more efficacious cleaning procedures for the removal of a broader array of proteins from hydrophilic contact lenses.

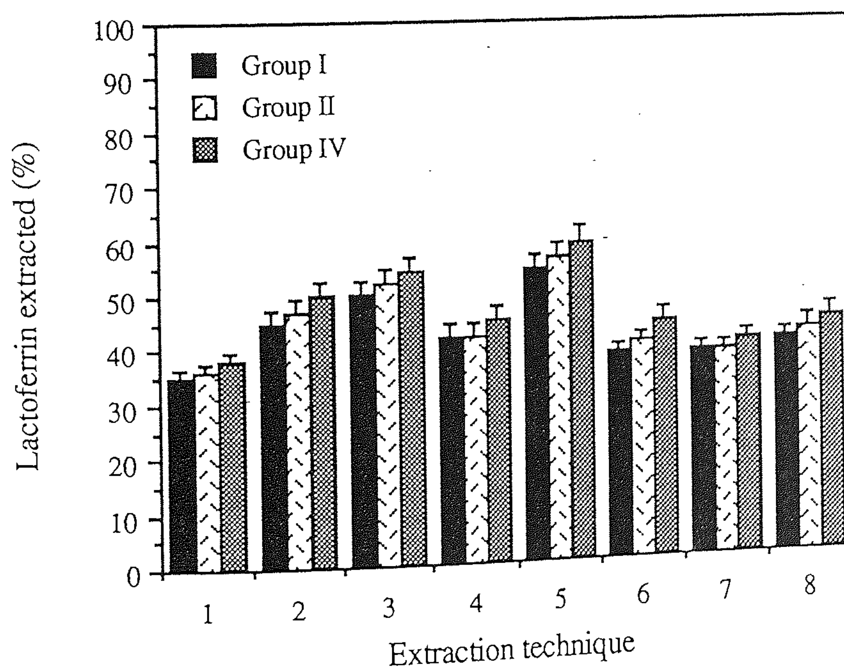


Figure 9.10 The percentage of lactoferrin extracted from different lens types by various extraction techniques (the numbers refer to the techniques discussed in 9.3.2).

Chapter 10

General Conclusions

Chapter 10

General Conclusions

10.1 General Conclusions

The absorption of proteins on charged surfaces is largely determined by electrostatic interaction. This can be concluded from the following observations:

1. The amount absorbed from single protein solutions increases with increasing charge contrast between the protein and the surface.

2. Sequential absorption occurs only if the second protein has a more electrostatic interaction with the surface.

3. It is also suggested that the protein having the most favourable electrostatic interaction absorbs preferentially from a mixture, so that the final composition of the absorbed layer essentially consists of this protein.

The plateau-values for the absorption of all tear proteins on different hydrogel surfaces and lens materials is reached at around 0.4 mg/ml. The time required to reach this value is about 5 days. Therefore, most of the *in-vitro* spoilation experiments were carried out under these conditions.

Proteins have the highest absorption at around their isoelectric point. Albumin is known for its high conformational adaptability towards changing environmental conditions. The reduction in the absorption at either side of the isoelectric point is due to the structural rearrangements in the adsorbing molecule (albumin).

Although the absorption is strongly dependent upon structural stability of most proteins, the influence of the interaction between the charge of the protein and the charge on the surface can also be observed. The density of negative charge on the protein increases with the increase in pH, as a result the absorption isotherms on negative surface are at lower levels at higher pH values.

The absorption of lysozyme on HEMA increases with both increasing pH and the amount of negative charge on the sorbent surface. This again proves the previous

The effect of temperature on the absorption of the proteins is different for different system studied. Different authors have reported different absorption behaviour of various proteins due to the change of temperature.

The results of our studies showed that the lysozyme and lactoferrin have different behaviour due to the increase in the temperature of the spoiling solution. The pH and other conditions such as concentrations and physical factors are kept constant. Both lysozyme and lactoferrin are absorbed more at 37°C to the hydrophilic soft contact lenses. Their absorption is lower at very high and very low temperatures.

Therefore, it can be suggested that the temperature has not a straight forward effect on the protein absorption into contact lenses. At low and very high temperatures the absorption decreases while the highest absorption takes place around physiological temperature. This phenomena makes the tear protein absorption on contact lenses even more considerable. The prediction of contact lens spoilation by proteins at different temperature is almost impossible and it may depend on different factors such as the pH of the solution, protein type and protein concentration.

The absorption of tear proteins to hydrogel contact lenses is a complex phenomena which includes the diffusion of the protein particle through the aqueous solution and the collision and interaction of the protein at the interface. In the case of small proteins with charges opposite to the high water content hydrogels the primary absorption is followed by the penetration of the protein into the hydrogel matrix. The protein absorption depends on many factors such as the protein concentration, protein type, size and charge, the chemical structure of the sorbent surface. Temperature and the pH of the spoilation solution also influence the absorption, as the pH affects the charge of the protein and the hydrogel surface.

Our work suggests that it is primarily the ionic nature of a hydrogel and secondarily its water content that determine the extent of its potential contamination by proteins. After the protein has been permeated the water of the lens, favourable polar interactions with an ionic polymer matrix further enhance the deposition process. Thus, the groups I and II lenses show relatively little protein deposition due their no-ionic nature. Group II lens material shows higher protein deposition compared to group I due to its higher water content, but shows lower deposition compared to a group IV which is also ionic. Group IV lenses are able to absorb relatively large amounts of protein because of their ionic character and high water content. In the case of group IV lenses, once the protein has permitted the lens, the chances of its remaining are enhanced by binding to the ionic polymer matrix.

ionic character and high water content. In the case of group IV lenses, once the protein has permitted the lens, the chances of its remaining are enhanced by binding to the ionic polymer matrix.

Although, there are many factors which may cause error in these types of experiments. The results obtained are, however, very reliable and reproducible, as the sum errors caused from different sources is less than 5% in all cases.

The following conclusions are made on the absorption of the proteins to various hydrogel materials:

1. Both size and charge of proteins are influential in their deposition onto contact lenses.

2. The uptake of different proteins into the matrices of Group IV lenses is more influenced by the charge of proteins than their size.

3. Lysozyme and lactoferrin adsorb onto the surface of Group IV lenses to a high degree, whereas albumin and tear specific pre-albumin are less adsorbed.

4. Lactoferrin, as well as lysozyme, penetrates into the matrix of Group IV lenses despite its greater size.

5. Lysozyme is mobile and can be leached out of the matrices of Group IV lenses stored at pH > 7 in, for example, ReNu™.

6. The biological activity of lysozyme is not detectably changed by the absorption and desorption processes.

7. The inclusion of MPEGs increases the resistance of hydrogels to protein absorption.

8. Increasing the concentration of a particular MPEG in the terpolymer causes a decrease in the protein absorption.

9. The higher the molecular weight of the methoxy polyether derivative, the greater the decrease in the protein absorption, i.e. MPEG-2000 is more effective than MPEG-550 in reducing the protein deposition by the hydrogel.

10. In the case of hydroxy terminated polyether derivatives the protein absorption increases with the incorporation of higher concentrations with similar molecular weights.

11. The longer the hydroxy terminated PEG used in terpolymer synthesis, the higher is the protein absorbed into it at a similar concentration.

12. The effect of the polyether derivatives is, however, more pronounced in the case of methoxy terminated PEGs.

13. The cell adhesion studies also confirm these results, but in the case of PEGOHs the protein deposition does not follow a pattern. However, in the case of PEGOH-1000 the protein absorption is greater or similar to the obtained for a pure poly(HEMA:MMA).

14. The spoilation studies on the synthesised positively charged hydrogels showed that they possess a higher resistance to the proteins especially the positively charged proteins such as lysozyme. The protein absorption on these copolymers, however, depends on the degree of cross-linking and their equilibrium water contents (EWC).

15. The presence of impurities in HEMA polymers can cause many problems the most important of which is that they promote the protein absorption to HEMA. The care taken in the synthesis and distillation processes can increase the purity in the hydrogel.

From the studies on several commercially available care solutions it was concluded that:

1. Although the care solutions produced by various manufactures have slightly different efficiency on the removal of proteins from contact lenses, but most of the protein remain in the lens even after cleaning with the most effective solutions.

2. The most important tear protein, lysozyme, can be removed more easily than lactoferrin and albumin from the surface and bulk of ionic soft contact lenses. The quantity of the protein remained on the lens depends on the quantity of the primarily absorbed protein.

In most of the analytical studies on proteins deposited on contact lenses, however, it is necessary to extract the protein from the lens matrix. Measuring the activity by the method or the identification of the nature of protein by electrophoresis and isotachophoresis techniques are not possible while the protein is absorbed in the lens. The extraction techniques used in this work proved to be more efficient than the care regimes in extraction of the protein. However, long term soaking in slightly alkaline solution such as ReNu™ is the best way to extract higher amount of protein.

Attempt to extract the most extractable protein from different contact lenses led us to perform the most effective way of cleaning the hydrogel lens materials and extract the absorbed protein.

It was found that long term extraction in ReNu™ is more effective than other extraction solutions (Section 2.4.1). In this study, ReNu™ was compared with some other care solutions and surfactant cleaners.

The mobility of the protein between the bulk, surface and the storage solution depends on the pH of the solution and it can be suggested that soaking the lens into slightly acidic solution before the spoilation process can prevent the lens from protein absorption. It may, however, have a negative effect on the lens material and its tonicity and reduce the tensile strength. More studies and *in-vitro* experiments should be done before using this technique for the real *in-vivo* spoilations.

10.2 Suggestions for Further Work

Arising from the experimental studies carried out in this work and the results obtained, some suggestions can be made for future work:

1. Isotachophoresis is a very useful method which gives accurate results for the analysis of tear proteins. However, it is necessary to apply this method to the lens extract as it needs very small sample volume and it would be possible to reduce the volume of the extraction solution and increase its protein concentration.

2. The biotin-avidin staining technique on the nitro-cellulose membrane did not show very sharp and detectable bands. It is suggested that if a cell culture experiment was carried out on the membrane the presence of the proteins can be shown more precisely.

3. The extraction methods used here only extracted up to 65 % of the proteins while it may be possible to extract most of the proteins using a mixture of the extraction solvents and care methods.

4. Some other copolymers with positive charged surfaces should be synthesised and their protein up-take monitored. These can be consisted of different combinations of the comonomers which have been used so far (Chapter 6) or some other novel comonomers.

5. The negative charges on the Group IV lenses can be neutralised by pre-soaking the lens in acidic solution before it is worn. Some *in-vitro* experiments should be carried out to examine the lens characteristics after it is soaked in acidic solution.

6. It has been shown that the activity of lysozyme is unaffected due to different experimental conditions and concluded that lysozyme is a stable enzyme. The biological activity of other tear proteins such as lactoferrin should be measured. Lactoferrin can also penetrate into the matrices of high water content anionic contact lenses and, therefore, its stability can be important.

7. Most of the clinical studies were carried out for short periods, therefore, their repeatability was not recognised. Some clinical studies should be done in long periods such as one year to show the accuracy and repeatability of the results.

8. Tear analysis using isotachophoresis technique should be continued and the composition of tear proteins examined in order to relate the spoilation of soft contact lenses to patient factors.

9. The effect of soaking the ionic lenses in acidic solution prior to the spoilation experiment need to be studied *in-vivo* and *iv-vitro*.

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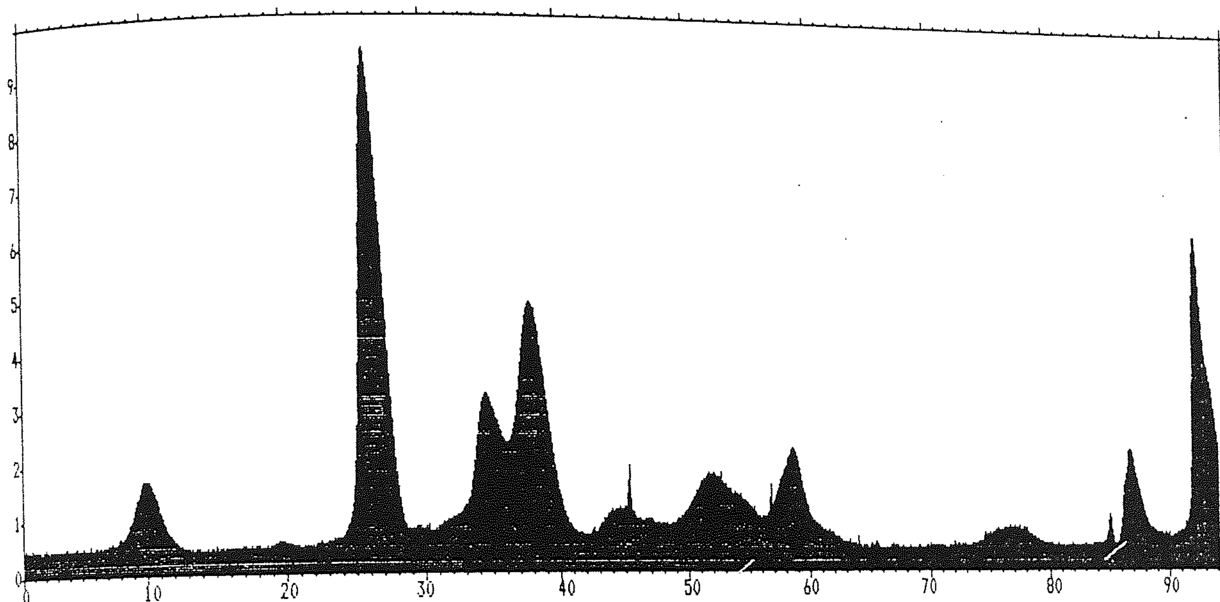
Appendix I

Appendix I

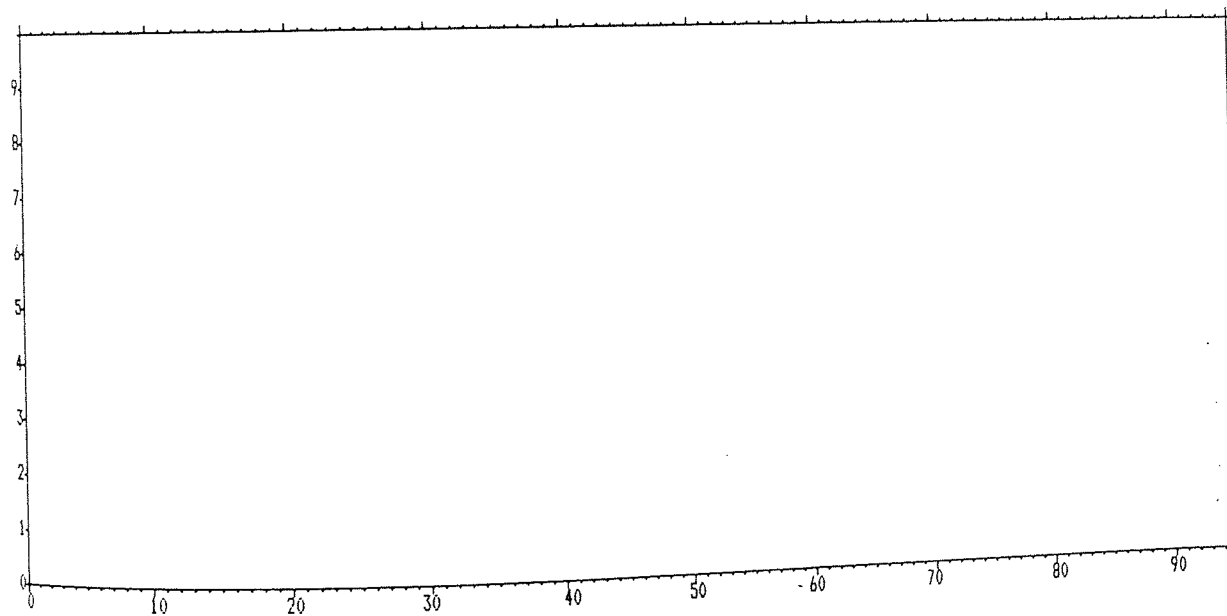
Densitograms obtained using Ultra-Scan Laser Densitometer



INTENSITY PROFILE of

Date : 24-Feb-84

(peaks, Scale = 10, AbsR.= 4.0)



INTENSITY DATA of

(Scale = 10, AbsR.= 4.0)

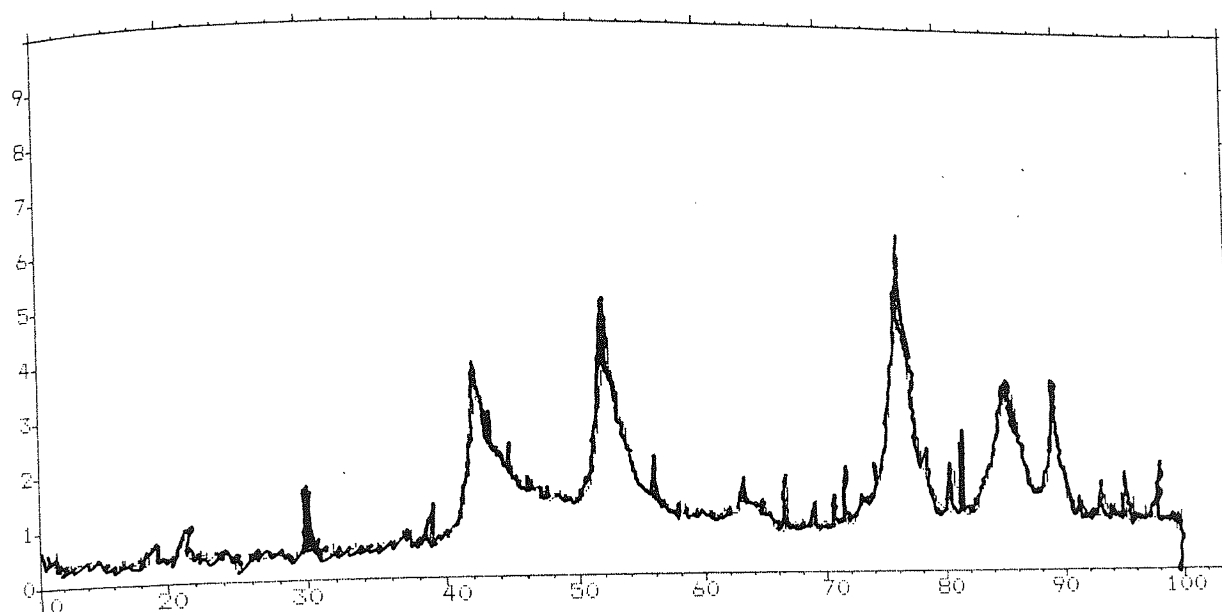
Figure A.1.1. The densitogram of well number 2 containing 10μl of Sigma high molecular weight standards from the Brilliant Blue G stained electrophoresis gel shown in page 83.

INTENSITY PROFILE of

GEL 1

Date : 24-Feb-94

(meas., Scale = 13, AbsR.= 0.5)

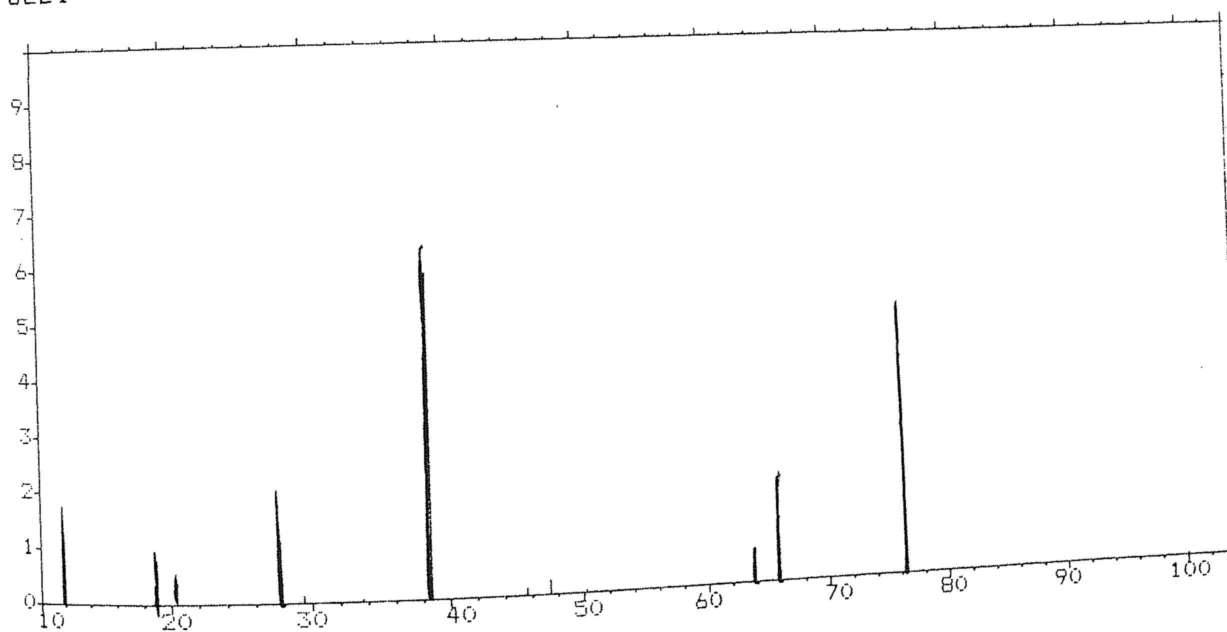


INTENSITY PROFILE of

GEL 1

Date : 24-Feb-84

(peaks, Scale = 13, AbsR.= 0.5)

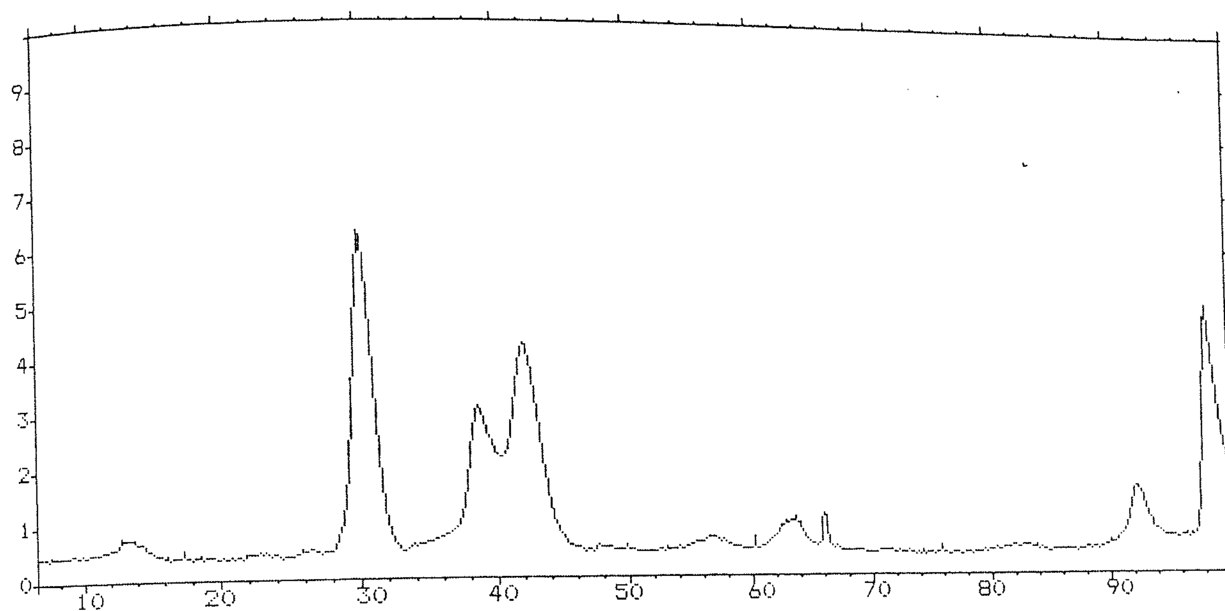


INTENSITY DATA of

GEL 1

(Scale = 13, AbsR.= 0.5)

Figure A.1.2. The densitogram of well number 6 containing a mixture of albumin, lysozyme, lactoferrin and γ-globulins (total protein concentration 0.6 mg/ml) from the Brilliant Blue G stained electrophoresis gel shown in page 83.



INTENSITY PROFILE of

TEAR02

Date : 24-Feb-84

(peaks, Scale = 10, AbsR.= 1.0)

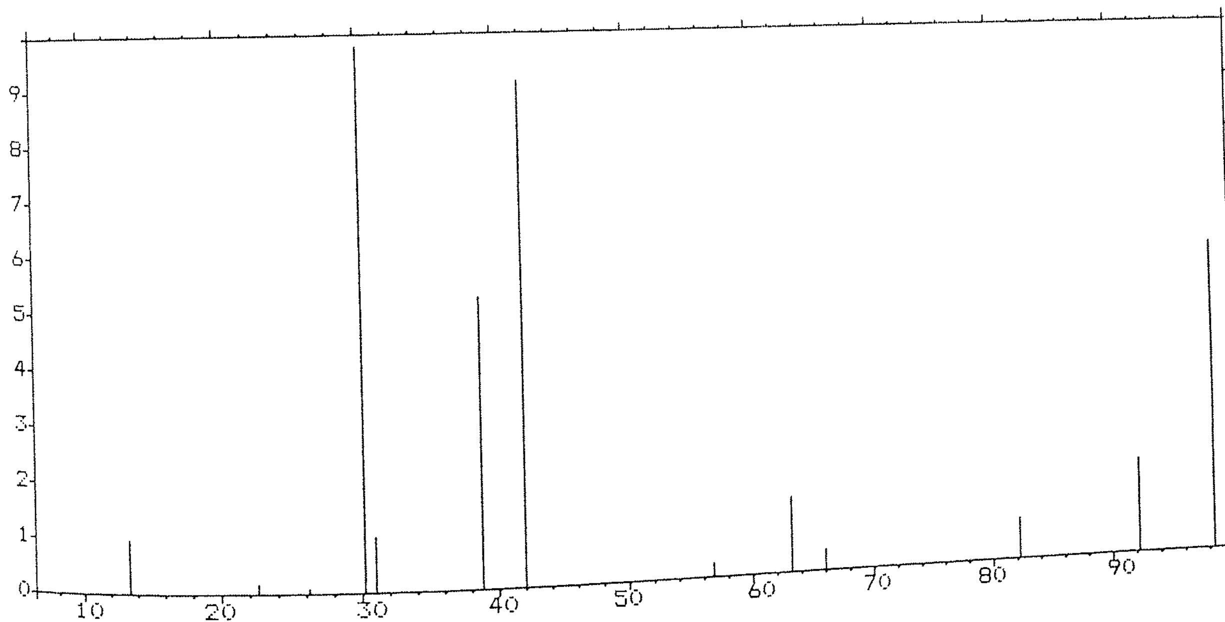


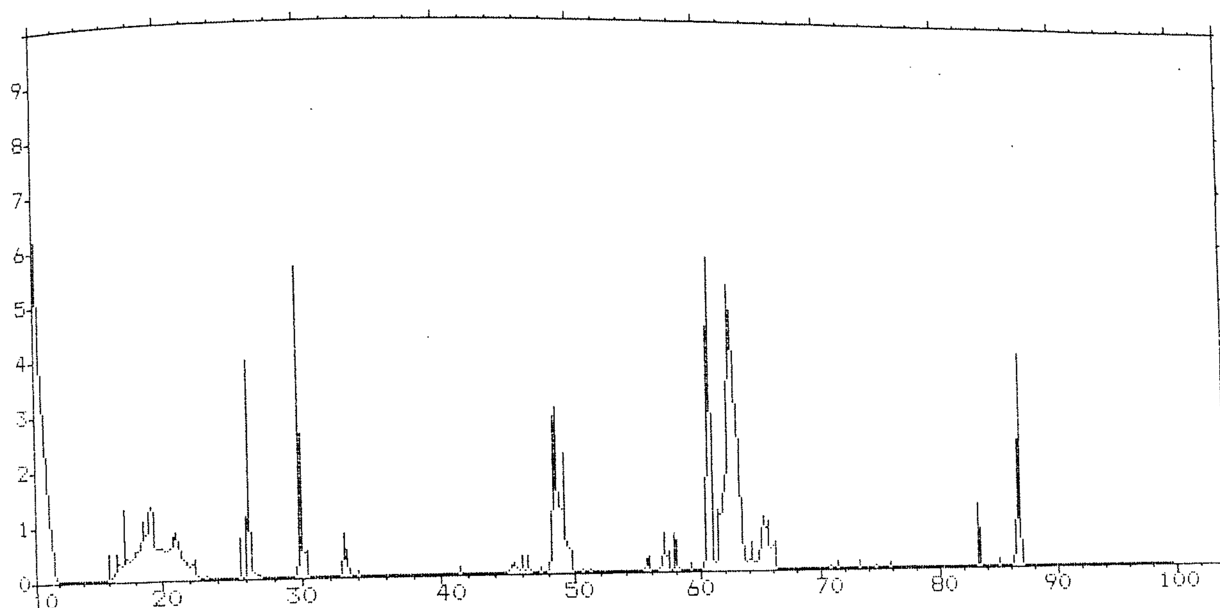
Figure A.1.3. The densitogram of well number 2 containing 5μl of Sigma high molecular weight standards from the Brilliant Blue G stained electrophoresis gel shown in page 88.

INTENSITY PROFILE of

GEL21

Date : 24-Feb-94

(meas., Scale = 13, AbsR.= 0.5)



INTENSITY PROFILE of

GEL21

Date : 24-Feb-84

(peaks, Scale = 13, AbsR.= 0.5)

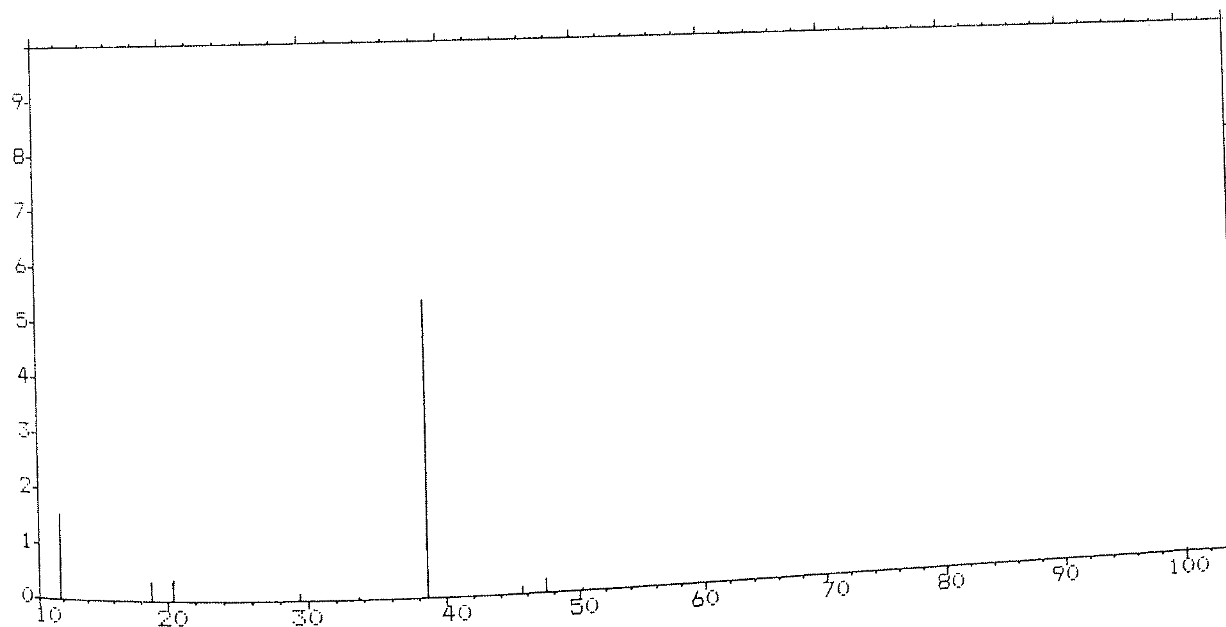


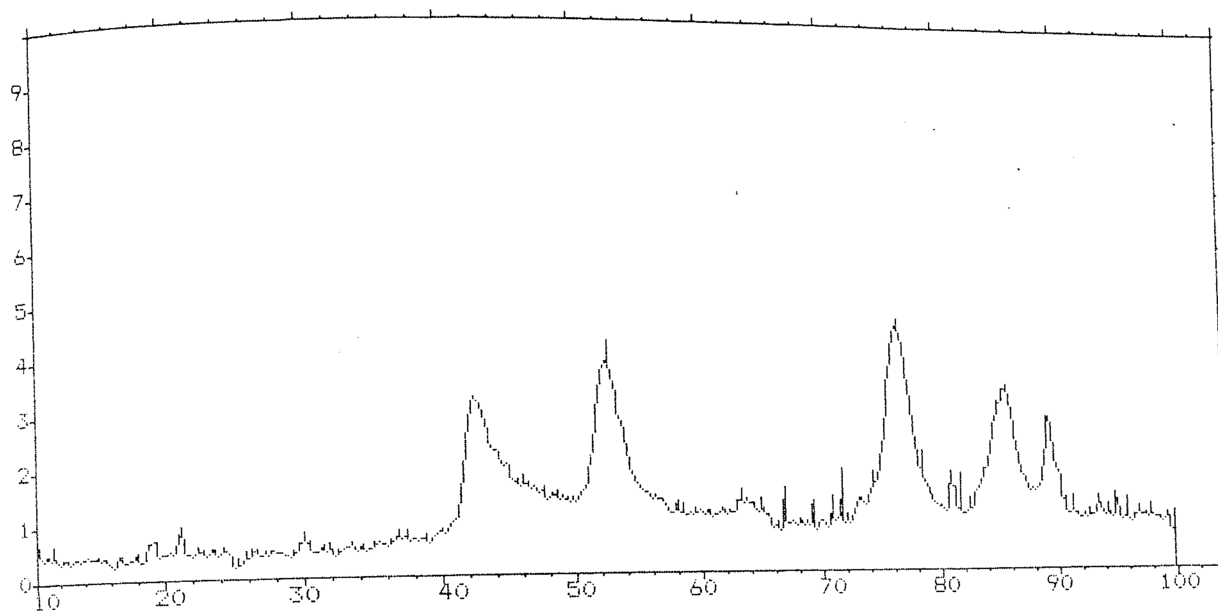
Figure A.1.4. The densitogram of well number 2 containing a tear sample from the Brilliant Blue G stained electrophoresis gel shown in page 216.

INTENSITY PROFILE of

GEL19

Date : 24-Feb-94

(meas., Scale = 13, AbsR.= 0.5)

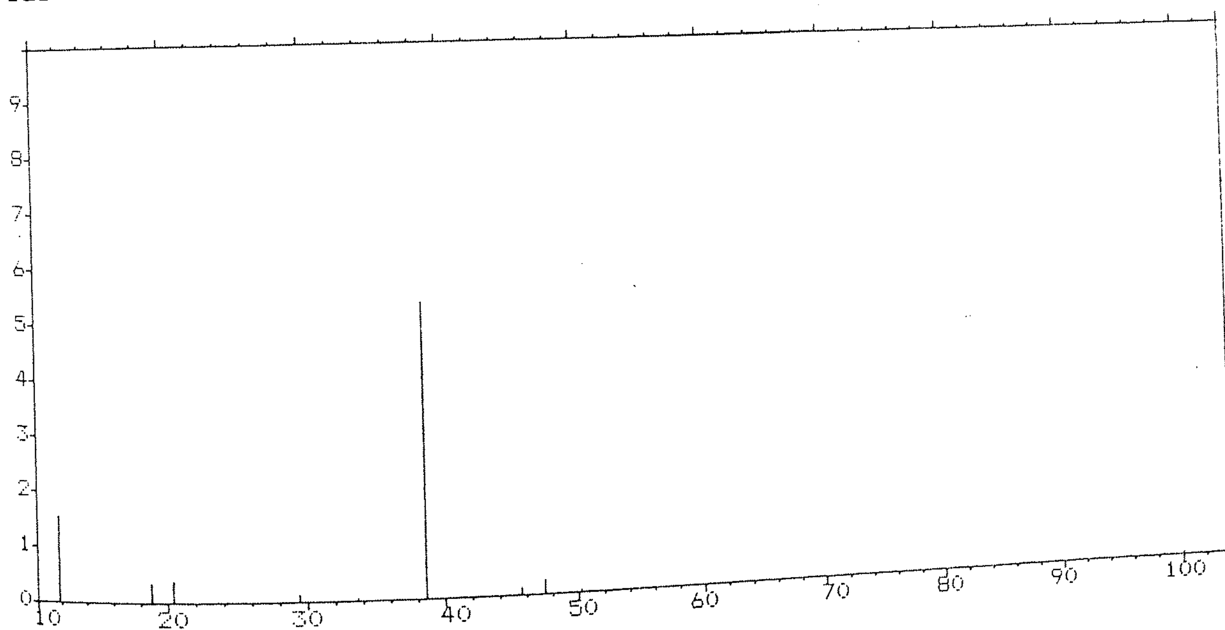


INTENSITY PROFILE of

GEL19

Date : 24-Feb-84

(peaks, Scale = 13, AbsR.= 0.5)



INTENSITY DATA of

GEL19

(Scale = 13, AbsR.= 0.5)

Figure A.1.5. The densitogram of a mixture of albumin, lysozyme, lactoferrin and γ-globulins (total protein concentration 0.6 mg/ml).

Appendix II

Appendix II

Fluorescence Spectra obtained using Hitachi F-4500 Spectrofluorimeter

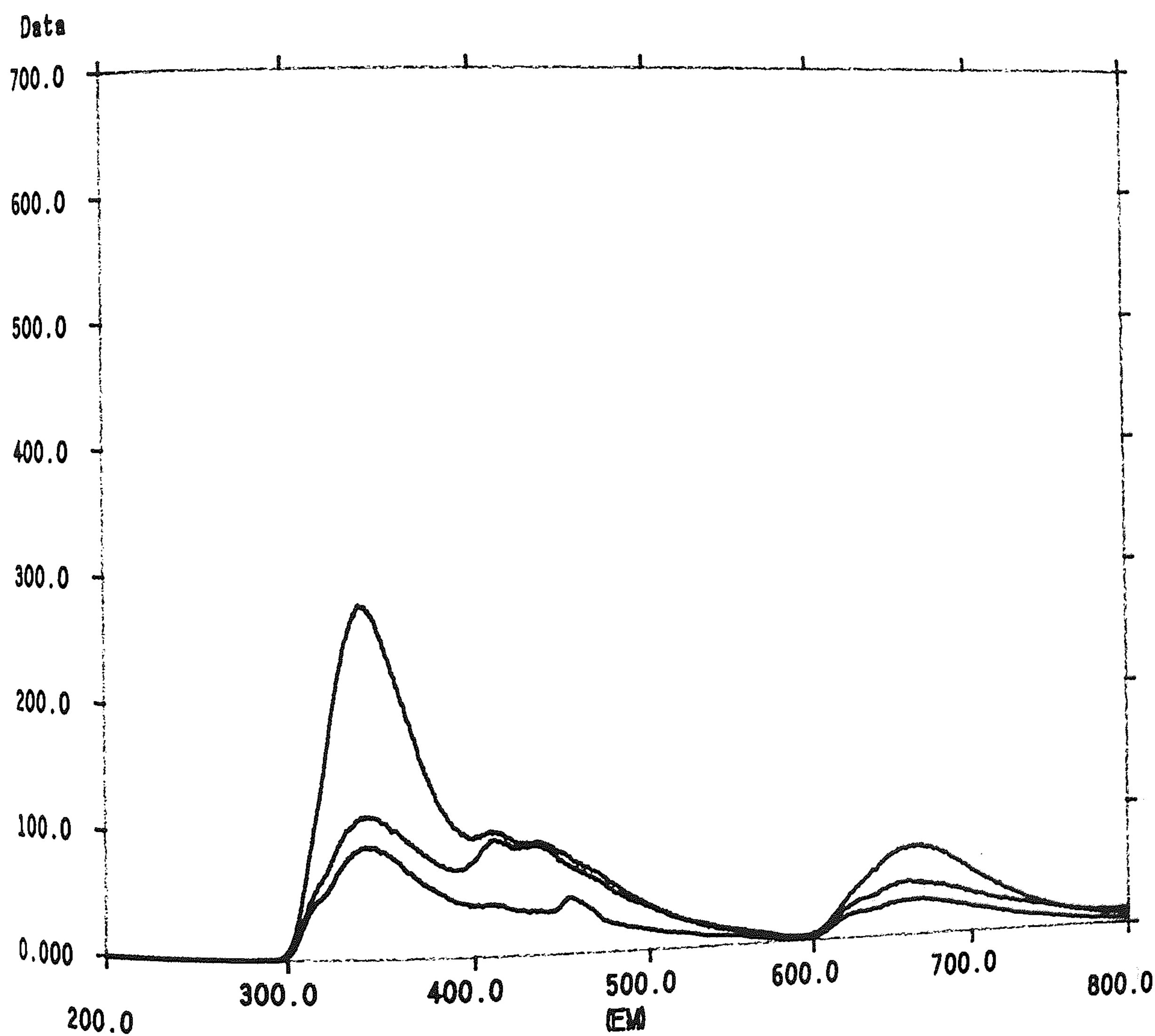


Figure A.2.1. The fluorescence spectra of an Acuvue™ lens soaked in 0.1, 0.2 and 0.4 mg/ml lysozyme solutions for one hour.

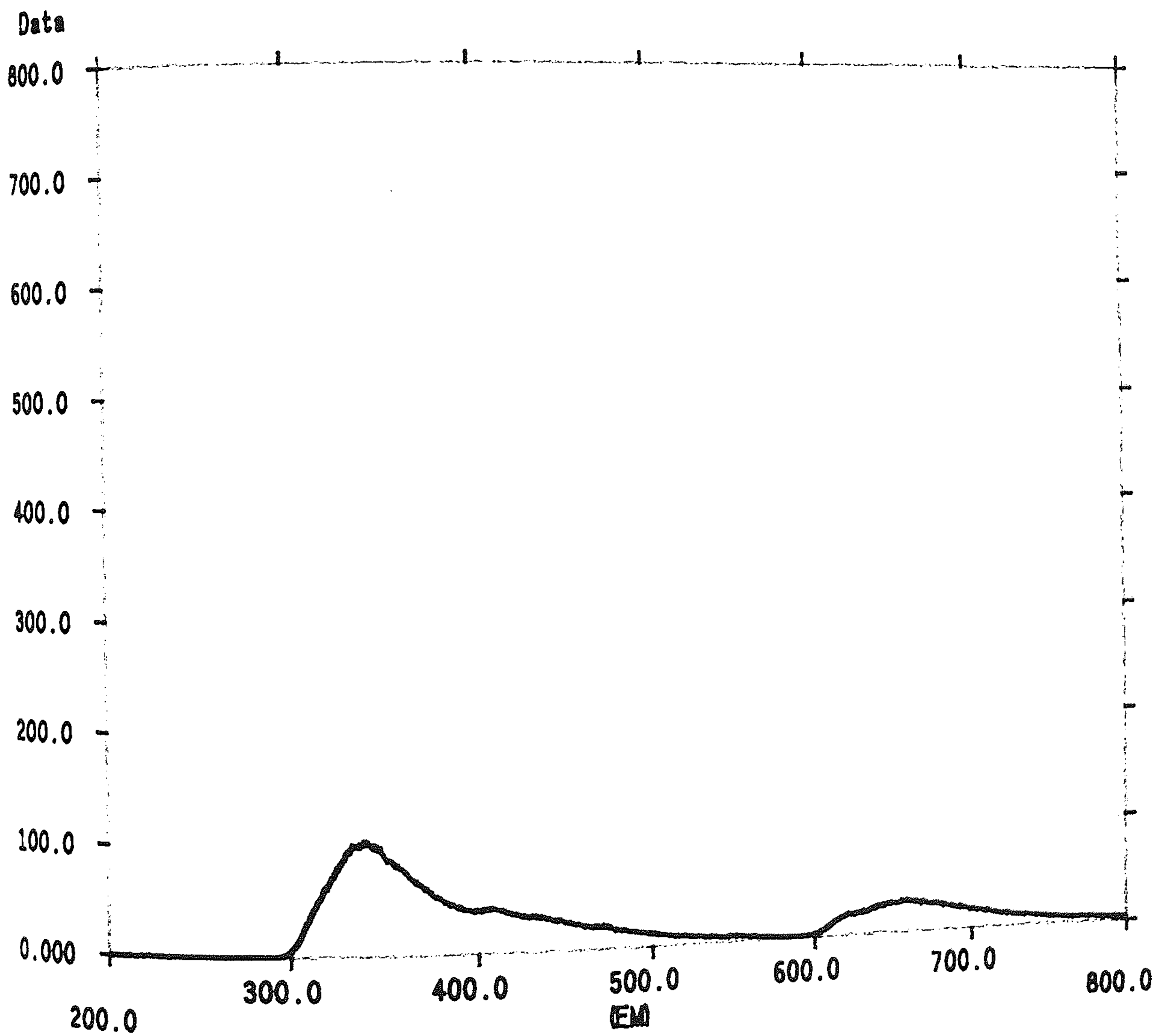


Figure A.2.2. The fluorescence spectra of the ReNu™ solution extracted from an Acuvue™ *in-vivo* spoiled lens.

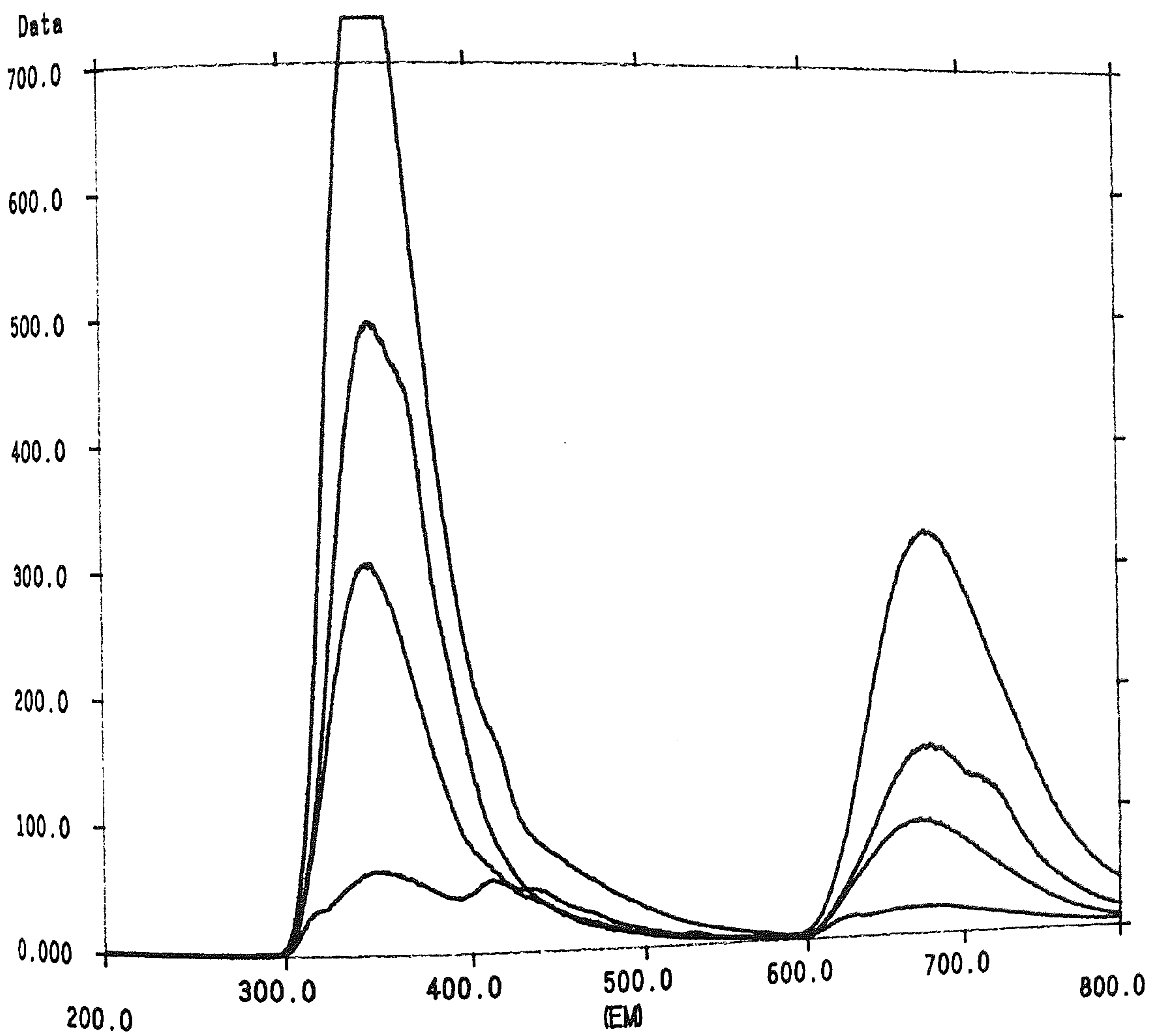


Figure A.2.3. The fluorescence spectra of an Acuvue™ lens soaked *in-vitro* for 5 days in 0.6 mg/ml lysozyme solution.

Appendix III

Appendix III

Isotachophoresis

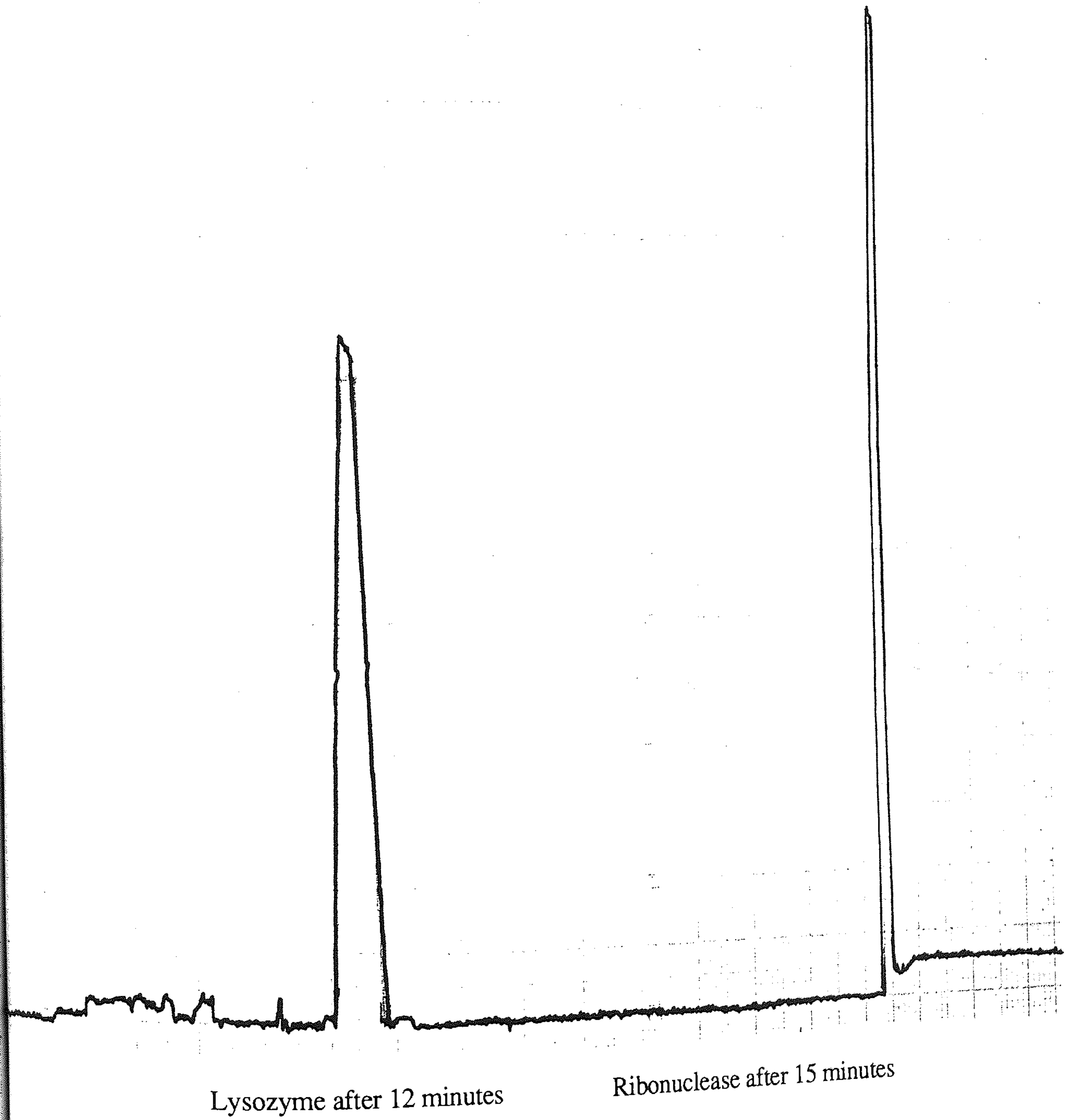
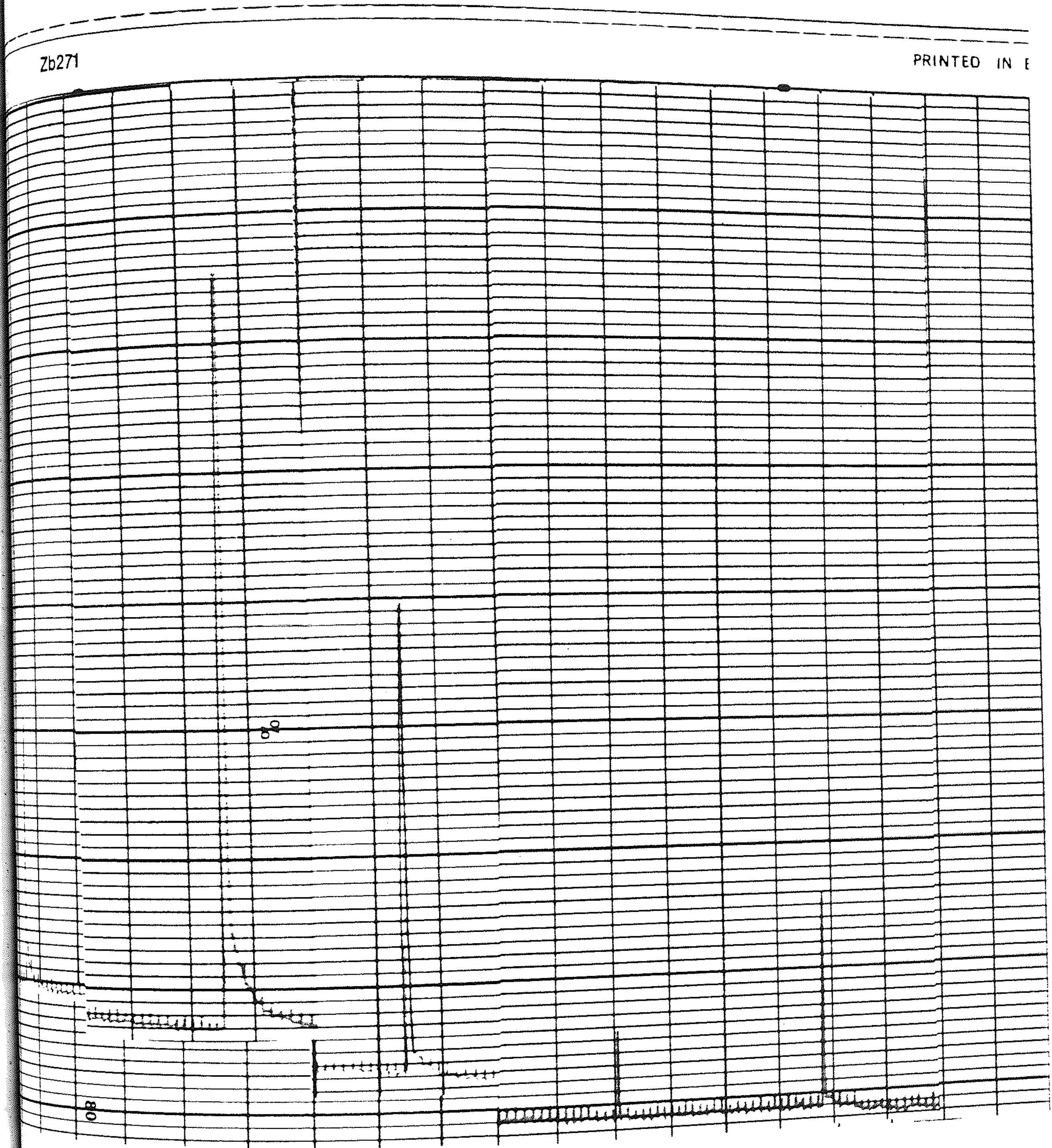


Figure A.3.1. Isotachogram of 10 ml of lysozyme and 15 ml of ribonuclease recorded on Servoscribe 1S chart recorder.



Insulin (5 minutes), Lysozyme (12 min.), ribonuclease (14 min.), Albumin (25 min.), Lactoferrin (26 min.)

Figure A.3.2. Isotachogram of 10 ml of insulin, lysozyme, ribonuclease albumin and lactoferrin, recorded on Howe YT 1000 chart recorder.

Start of the day screen

* * * TRIO * * *

09:38 05/04/95
Channel A on line
Channel B on line

Time 09:38
Date 05/04/95

Channel	A	B
Default method	External	Test
On-line	Y	Y

Methods
Run sequence
Parameters
Automatic parameters
Program select
Stored results
Disk functions

The procedure set to run the Trio attached to the LKB isotachophoresis instrument.

METHODS

		09:30	05/04/95
		Channel A	ready
Method Name	EXAMPLE	Channel B	ready
Method text	from Default		

Methods
TEST
EXAMPLE

Data collection	Run time-Minutes	30.00
	Select Peak width	10.0
	Select input voltage	10000

Peak detection	Start time End time	0.00	60.00
	Sensitivity	1	
	Minimum baseline time	0.02	
	Peak and slope	1	
	Skim Ratio	0	

Report calculation
Preset values

Delete method

Save method on disk
Load method from disk

Configuration
Version 3.2

09:47 05/04/95

Channel A	ready
Channel B	ready

Start/Stop type	Channel A	0
	Channel B	0
Data source	Channel A	External
	Channel B	External
Ram configuration	Sequence table	33
	Peak results	12.50
	Raw data	133.25
	Program swapping	N
	Start on line	N
	Host port for BCD	N

Parameters

Channel A	09:43	05/04/95
Channel A	ready	
Channel B	ready	

Plotter	On-line plot	Y
	Expansion	1.000
Printer	Orientation	H
	Print Format	0
	Device	Aux 1
Sequence	Initial sample number	1
	Configuration	

Appendix IV

Appendix IV

The accuracy and sensitivity of the U.V. measurements

Sources of Errors

In most of the experiments for the quantitative measurement of proteins by U.V. there are a number of possible errors as follows:

1. The error in preparing individual and mixture of the protein solutions with known concentrations for the in-vitro spoilations. To reduce this type of error higher concentrations of required proteins were made as stock solutions and appropriate dilutions and mixture of proteins were prepared just before use. The stock protein solutions were prepared in saline and stored in refrigerator (- 4 °C) for maximum of two weeks (in the case of albumin four days).

2. The volume of the proteins used for all spoilation experiments was kept constant to reduce the effect of the concentration of the protein in the up-take process.

3. Each spoilation experiment was repeated on at least three samples under similar conditions and the quantities of protein measured by U.V. were taken as a mean value (± SD).

4. The absorption of each blank material was measured before it was spoiled with the protein and the value was deducted from the result obtained for the spoiled sample. In this way, the error due to the hydrogel material was reduced to near zero.

5. The U.V. absorptions of all the blank hydrogel materials and the spoiled samples were measured while the sample was immersed in distilled water and the lenses/disks were placed at the same position in the cuvette in all experiments.

6. The error in the change of temperature was reduced by performing most of the experiments at room temperature as (20 ± 2°C). A slight variation in the temperature, howevere, had no significant effect on the U.V. spoilation processes.

7. A standard curve was obtained for each protein and the mixtures of proteins and it was used to calculate the quantity of the protein from its U.V. absorption.

8. A standard plot of error band against frequency of the results was obtained for each experiment from which the standard deviations were calculated.

9. The standard deviations for each set of experiment was calculated as below:

$$SD = \sqrt{\frac{\Sigma(x_i - \bar{x})^2}{n - 1}}$$

Where n is the number of results obtained for each similar experiment, x_i is the value of each test and $\bar{x}$ is the mean value.

A typical cumulated distribution curve (error band) obtained for deposition of lysozyme from a 0.5 mg/ml solution onto a Group I contact lens is presented in Figures A.4.1 and A.4.2. This type of distribution curve was obtained for each set of U.v. experiment.

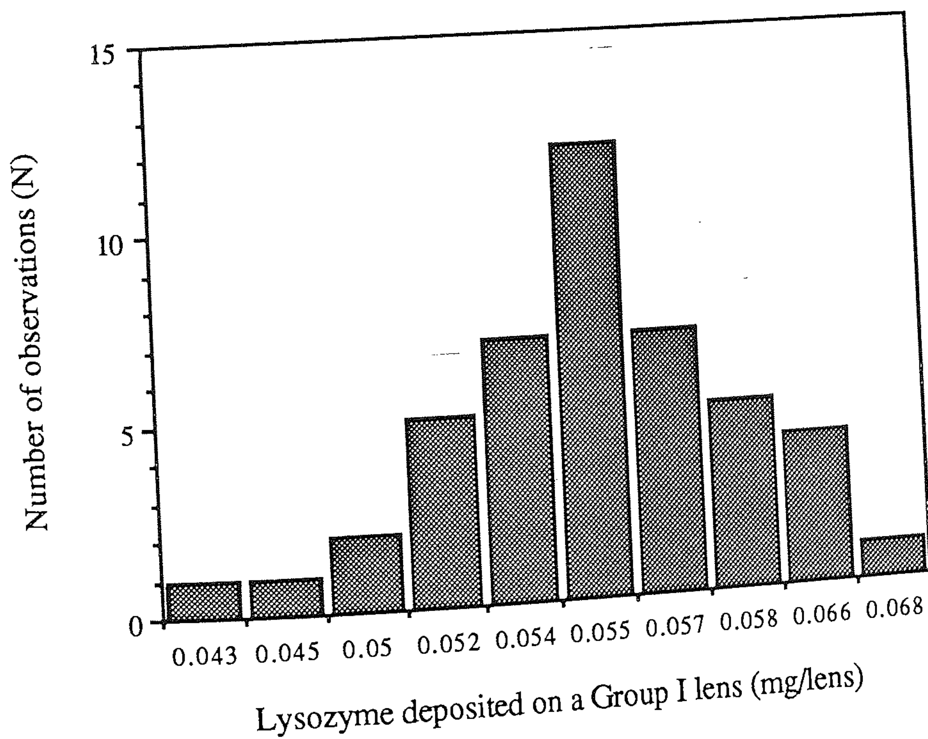


Figure A.4.1 The variation in lysozyme concentration on a Group I contact lens measured by direct U.V. on the lens.

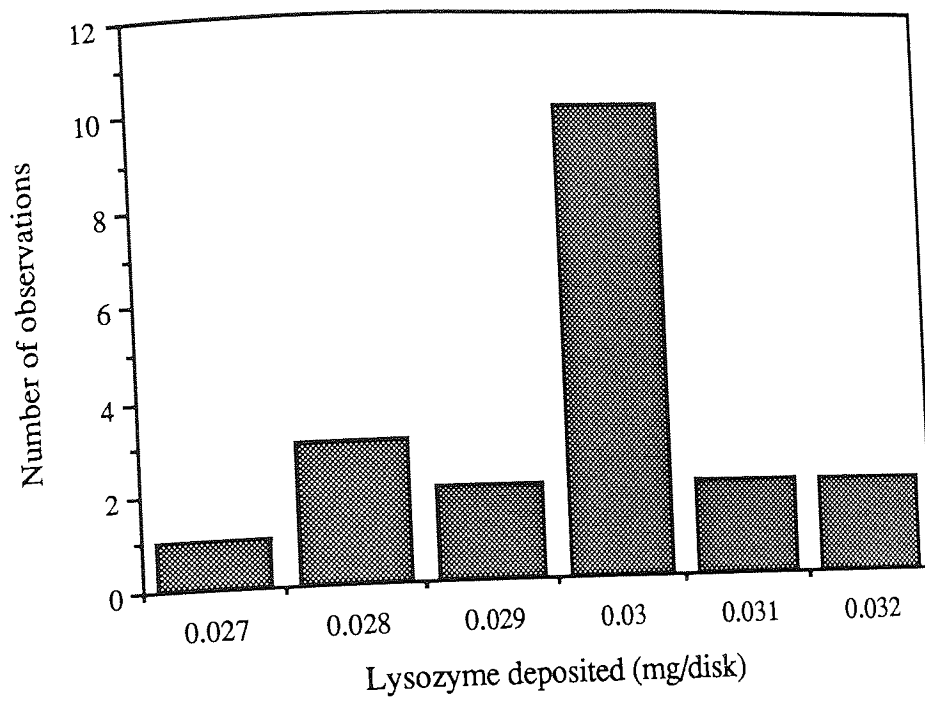


Figure A.4.2 The variation in lysozyme concentration on a HEMA/NVP copolymer measured by direct U.V on a disk of $1cm^2$.

Appendix V

Appendix V

Care Solutions

The following contact lens care solutions were used in different parts of the study, the manufacturers recommended procedures for each is discussed below:

ReNu® Multi-Purpose Solution (Bausch & Lomb)

This is a sterile, isotonic solution that contain boric acid, sodium borate and sodium chloride preserved with DYMED™ (polyaminopropyl biguanide) 0.00005% and edetate disodium 0.1%. It disinfects, daily cleans and rinses. It is used in two steps:

1. Cleaning and rinsing

   Place three drops of ReNu™ multi-purpose Solution onto each side of the lens. Thoroughly clean the lens by gently rubbing each surface for 20 seconds between the palm and forefinger of the other hand. Rinse the lens completely with a steady stream of the same solution.

2. Disinfecting and storing

   Fill the lens storage case with fresh ReNu™ and place the newly cleaned lenses in the case and tightly close the cap. Leave the lens to soak at least four hours in ReNu™ Multi-Purpose Solution.

Sauflon™ All-in-One Solution (Essilor)

This is a disinfectant and cleaning solution which contains disodium edetate 0.3% weight per volume and polyhexanide 0.0005%. It was used as follows:

1. Wash hands and rinse thoroughly with water. Apply two drops of Sauflon™ All-in-One solution to each lens surface. Rub each side of the lens with forefinger of the other hand gently.

2. Thoroughly rinse both surface of the lens with the same solution for at least 15-20 seconds to remove all the traces of the cleaner.

3. Fill the storage case and soak the lens in Opti-Free™ solution for at least 4 hours or leave to soak overnight.

Quick Care™ Solution (Ciba Vision®)

The Quick Care system consists of a starting and fishing solution. The starting solution is a strile aqueous solution that contains purified water, isopropanol, sodium chloridepolyoxypolyethylene-polyoxyethylene block copolymer and disodium lauroamphodiacetate. The finishing solution is a strile isotonic solution consisting of sodium borate, boric acid and sodium perborate (generating up to 0.006% hydrogen peroxide, stabilised with phosphonic acid). The two solutions were used as follows:

1. Apply 10 drops of Quick Care 2 starting solution onto the lens surface and rub the lens for 30 seconds.

2. Thoroughly rinse the lens with the finishing solution and soak it in the fresh finishing solution for at least one minute to ensure the removal of the starting solution.

Complete™ Solution (Allergan)

This is also a two step cleaning system which cleans, rinses and disinfects. The procedure is as follows:

1. Wash and dry the hands and place three drops of Complete™ solution on the lens and rub both sides gently for 20-30 seconds. Rinse thoroughly with the same solution to remove the traces of dirt.

2. Place the lens in the lens case and fill the case with enough Complete™ solution to cover the lens. Allow to soak for a minimum of four hours.

Opti-Free™ Solution (Alcon vision care)

This is a rinsing, disinfection and storage solution which contains citrate buffer and sodium chloride, with edetate disodium 0.05% and POLUQUAD® (poluquaternium-1) 0.001% as preservative. The cleaning system consists of the three steps as follows:

1. Wash hands and rinse thoroughly with water. Apply two drops of Opti-Free™ solution to each lens surface. Rub each side of the lens with forefinger of the other hand gently for 20 seconds.

2. Thoroughly rinse both surface of the lens with the same solution for at least 2-3 seconds to remove all the traces of the cleaner.

3. Fill the storage case and soak the lens in Opti-Free™ solution for at least 4 hours.

Opti-One™ Multi-purpose Solution (Alcon vision care)

This multi-purpose solution is a strile, buffered, isotonic, aqueous solution containing sodium citrate and sodium chloride, with edetate disodium 0.05% and POLYQUAD® (polyquaternium-1) 0.001% as preservatives. The following three steps were used:

1. Wash hands and rinse thoroughly with water. Apply two drops of Opti-One™ multi-purpose solution to each lens surface. Rub each side of the lens with forefinger of the other hand gently for 10 seconds.

2. Thoroughly rinse both surface of the lens with the same solution for at least 20 seconds.

3. Fill the storage case and soak the lens in the multi-purpose solution for 4 hours or leave the lens to soak overnight.

LC-65™ Surfactant Cleaner

This is a non-abrasive, preservative free daily contact lens cleaner which can be used for all lens types. The following procedure was carried out:

1. Wash the hands thoroughly before touching the lens. Place the lens in the palm of the hand and add two to three drops of LC-65™ solution onto the lens surface. Rub the lens for 15-20 minutes.

2. Rinse the lens thoroughly with strile saline solution to remove the surfactant cleaner and store in saline if required.

Oxysept® Solution

This care system consists of two solution, Oxysept® 1 disinfecting solution and Oxysept® 2 rinsing, neutralising and storing solution.

1. Clean and rinse
   Wash hands and clean the lens with a surfactant cleaner such as LC-65™ rinse lens thoroughly with Oxysept® 1 or strile saline solution.

2. Disinfection-Oxysept 1 Solution
   Place the lens in the storage case and fill the case with Oxysept 1 solution for at least 10 minutes or store overnight.

3. Neutralisation-Oxysept 2 Solution
   After disinfection, discard the Oxysept 1 solution from the lens case and shake the excess solution. Fill the case with Oxysept 2 solution and leave to soak for at least 10 minutes. Lenses can be stored in oxysept 2 solution overnight if required.