ANALYTICAL TECHNIQUES FOR THE STUDY OF SOFT CONTACT LENS SPOILATION

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Doctor of Philosophy

UNIVERSITY OF ASTON

IN BIRMINGHAM

OCTOBER 1991

THE UNIVERSITY OF ASTON IN BIRMINGHAM

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Submitted for the Degree
of Doctor of Philosophy

October 1991

SUMMARY

Soft contact lens wear has become a common phenomenon in recent times. The contact lens when placed in the eye rapidly undergoes change. A film of biological material builds up on and in the lens matrix. The long term wear characteristics of the lens ultimately depend on this process. With time, distinct structures made up of biological material have been found to build up on the lens. A fuller understanding of this process and how it relates to lens chemistry could lead to contact lenses that are better tolerated by the eye.

The tear film is a complex biological fluid, it is this fluid that bathes the lens during wear. It is reasonable to suppose that it is material derived from this source that accumulates on the lens.

To understand this phenomenon it was decided to investigate the make up and conformation of the protein species that are found on and in the lens. As inter individual variations in tear fluid composition have been found, it is important to be able to study the proteins on a single lens. Many of the analytical techniques used in bio-research are not suitable for this study because of a lack of sensitivity.

Work with poly acrylamide electrophoresis showed the possibility of analysing the proteins extracted from a single lens. The development of a biotin avidin electro-blot and an enzyme linked antibody electro-blot, lead to the high sensitivity detection and identification of the proteins present.

The extraction of proteins from a lens is always an incomplete process. A method that analyses the proteins *in situ* would be a great advancement. Fourier transform infrared microscopy was developed to a point where a thin section of a contact lens could yield information about the protein present and their conformation.

The three dimensional structure of the gross macroscopic structures termed white spots was investigated using confocal laser microscopy.

Keywords: contact lenses, tear proteins, fourier transform infrared spectroscopy, confocal microscopy, poly-acrylamide gel electrophoresis.

Dedicated to my family

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

ATR	attenuated total reflection
AZBN	azo bis isobutyronitrile
°C	degrees centigrade
CSLM	confocal scanning laser microscopy
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EDXA	energy dispersive X-ray analysis
EGDMA	ethyleneglycol dimethacrylate
ELISA	enzyme linked immunosorbent assay
EWC	equilibrium water content
FCS	foetal calf serum
FDA	Food and Drug Administration (USA)
FSD	Fourier self deconvolution
FTIR	Fourier transform infrared
g	gram
HEMA	hydroxyethyl methacrylate
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
IgA	immunoglobulin A
IgE	immunoglobulin E
IgG	immunoglobulin G
ITP	isotachophoresis

K_m	Michaelis Menten constant (in enzyme kinetics)
M	mole per litre
MA	methacrylic acid
MCT	mercury-cadmium telluride
mg	milligram
ml	millilitre
μl	microlitre
MMA	methyl methacrylate
MIR	multiple internal reflection
NMR	nuclear magnetic resonance (spectroscopy)
PAGE	poly-acrylamide gel electrophoresis
pH	1/log the hydrogen ion concentration
pHEMA	poly hydroxyethyl methacrylate
pVP	poly N-vinyl-2-pyrrolidone
RI	refractive index
SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
SLR	single lens reflex (camera)
TEM	transmission electron microscopy
TIRF	total internal reflection fluorescence
TLC	thin layer chromatography
TSPA	tear specific prealbumin
UV	ultra violet
VP	N-vinyl-2-pyrrolidone

Footfalls echo in the memory
Down the passage which we did not take
Towards the door we never opened
Into the rose garden.

from Four Quartets, 'Burnt Norton'.
by T.S. Eliot

CHAPTER 1

Introduction:- Biocompatibility and the eye

1.1 Biocompatibility: The problem

The widespread use of man made materials to replace and augment structures in the body has made the study of the interactions that take place between the implant and the host tissue an important area of study. The implanted material, termed a biomaterial, when placed in contact with a biological environment undergoes various interactions, in that the material affects the environment and is itself affected by the surrounding tissues [1]. The degree of success of a biomaterial at this interface is described as its biocompatibility.

It is not surprising that the implant encounters problems when interfaced with a living system; the living system has developed without external influence for millions of years, into a complex physical and chemical equilibrium that is highly dynamic by its very nature. In placing a man made material into this situation we ask the near impossible, to deceive a highly discriminative multi-component environment that a relatively simple synthetic material is not foreign. This fact does not make the study of biomaterials and biocompatibility any less worthwhile, as long as we accept that full integration of host tissue and man made implant, where required, is a very far distant goal in many fields. At best we will make only small improvements in the short term in the majority of biomaterial applications. The work carried out has lead to improved materials over those that were initially used, these early materials were chose on purely empirical grounds based on availability, ease of sterilization and speculation as to their suitability.

The biocompatibility of a material depends on complex, and as yet not fully

understood, interactions both physical and chemical between the implanted biomaterial and its host environment. Some workers have speculated as to what properties lead to desirable interactions, these have included rates of differential protein adsorption,[2] and also the relationship between surface energy and biocompatibility has been studied [3]. A complicating factor in the study of biocompatibility is that different body sites offer very different environments; vascular and arterial, urinary tract and the ocular milieu, for example are all very diverse, and each site thus requires different things from a biomaterial.

1.2 Contact lens wear and implications for biocompatibility

As a primary area of study, contact lenses may seem a frivolous subject, whilst other body sites involve disorders that affect mobility or are life threatening. This greatly over simplifies the case, the eye because of its ease of access, complexity of tears, and the large commercial importance of contact lenses is a field worthy of study.

The implications of biocompatibility in contact lens wear are many fold, running from the submicroscopic protein layers that build up after a very short period of wear, to gross macro structures termed white spots that are visible to the naked eye. The various types of contact lens deposits and their analysis reported in the literature will be discussed in Chapter 3.

The process of deposit formation is termed spoilation. It reduces wear comfort, increases the risk of microbial contamination, diminishes optical clarity and necessitates the use of a variety of expensive and not totally effective, cleaning agents. The cleaning agents used range from proteolytic enzyme cleaners to detergent-based solutions. The

use of these agents is a complicating factor when considering the interaction of a polymer placed in contact with the eye, it becomes another variable in an already complex system. The role of enzyme cleaners will be looked at in detail in chapter 4.

1.3 Project aims

The aim of the project at the outset was to understand fully the role of protein-containing species in the mechanism of spoilation on soft contact lenses. Of particular interest were the initial events in deposit formation, as it was believed that these initial events could be common to a number of biocompatibility problems. The ocular environment is a good site to study as mentioned earlier, as it is easily accessible, and tear film, although a complex biological fluid, is simpler than blood. It contains none of the cellular components and lacks other species, e.g. complement and platelets which complicate the study of blood contact devices.

The project aims to study the biological material that builds up on a single contact lens, rather than on pooled samples. Many analytical techniques used in bio-research were developed to analyse much larger sample sizes, thus modifications are necessary if they are to be used. The study of contact lens deposits has a parallel in forensic sciences, in that often the sample size is very small with no option of increasing it. It is interesting to note that FTIR microscopy has proved useful in both disciplines (see chapter 4).

Analytical techniques that study the material *in situ* are advantageous as no removal regime is involved. When removal is required it should be remembered that it is likely that the material of most interest will be the most tightly bound.

1.4 Achieving biocompatibility

To achieve a fully biocompatible surface it is envisaged that the material would be fully accepted by the host tissue without any adverse effects on the organism or the implant. This can be brought about in several ways; the material can mimic the biological milieu[4] thus the host would see the implant as 'self ', or the implant could be 'invisible', i.e. not detected by the organism. A third and more complex route would be to encourage the adsorption of certain biological species from the host onto the prosthesis rapidly after implantation, it would be this overlying layer that is seen by the recipient tissue rather than the man made material it conceals.

In the search for the 'Holy Grail', of a highly bio-tolerant contact lens material, we can use the method of testing *in vitro* and *in vivo* many potential materials, and by guess work and deduction attempt to predict suitable materials from the results. Another method that can be complimentary to the above is to study contact lenses that have been worn by patients. If the chemical make-up, conformation of the adsorbed protein and its spatial arrangement was fully understood we would be in a position to look at the pathway to contact lens spoilation and suggest a point along the pathway to inhibit the deposition mechanism. These two methods, along with anecdotal evidence from clinicians are the route envisaged to lead to a solution of this convoluted problem.

1.5 Materials used in contact lens manufacture

Three major types of material are presently used for contact lenses; hard contact lenses manufactured from poly methyl methacrylate, gas permeable contact lenses made principally from co-polymers of siloxymethacrylates and methyl methacrylate and finally soft contact lenses made normally from polyhydroxyethylmethacrylate (HEMA)

and co-polymers of HEMA or methylmethacrylate with vinylpyrrolidone. It is the biocompatiblity of this last group that the project sets out to study.

It was the work of Wichterle and Lim [5] that lead to the discovery of acrylic hydrogels, it was suggested that even at this early stage that these materials would be suitable as contact lens materials. Through the use of a range of monomers, hydrogels have indeed become important in this field.

Hydrophilic soft contact lenses can be classified into three general types based on composition as shown in table 1.1

Table 1.1 General classification of contact lenses by monomer composition.

1   Those consisting of polyhydroxyethyl methacrylate.

2   Those consisting of polyhydroxyethyl methacrylate co-polymerised with one or more co-monomers such as N-vinyl-2-pyrrolidone or methyl methacrylate.

3   Those principally poly N-vinyl-2-pyrrolidone normally co-polymerized with at least one other monomer.

All contain a small amount of cross-linking agent, the most common being ethyleneglycol dimethacrylate (see fig 1.1 for structure), and they have equilibrium water contents between 30 and 80%.

Figure 1.1 Structure of ethyleneglycol dimethacrylate (EGDMA).

$$
\begin{array}{cccc}
& CH_3 & & CH_3 \\
& | & & | \\
H_2C\!=\!\!C & & C\!=\!\!CH_2 \\
& | & & | \\
& C\!=\!\!O & O\!=\!\!C \\
& | & & | \\
& O\!-\!C\!-\!C\!-\!O \\
& \quad H_2 \;\; H_2 \\
\end{array}
$$
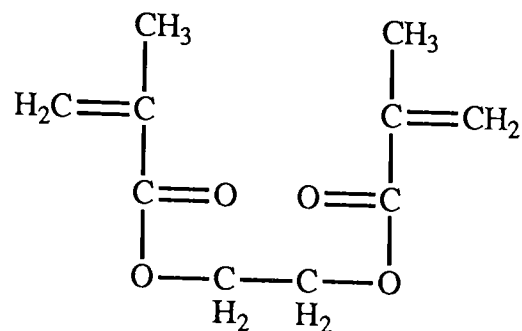
The structure of a number of the monomers mentioned in table 1.1 are shown in figures 1.2 to 1.4. Included are several other monomers found in soft contact lenses in smaller amounts and not mentioned in the general discussion on soft contact lens types, these monomers are nonetheless important in altering the physical properties of hydrogels by their inclusion.

Figure 1.2 Structure of hydroxyethyl methacrylate (HEMA).

$$
\begin{array}{c}
CH_3 \\
| \\
H_2C\!=\!\!C \\
| \\
C\!=\!\!O \\
| \\
O \\
| \\
CH_2 \\
| \\
CH_2 \\
| \\
OH \\
\end{array}
$$
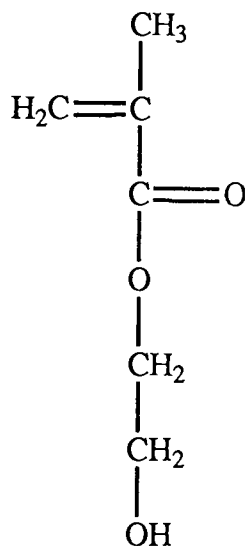
Figure 1.3 Structure of N-vinyl-2-pyrrolidone (VP).

$$H_2C\!=\!CH$$

$$H_2C \quad N \quad C\!=\!O$$

$$H_2C\!-\!CH_2$$
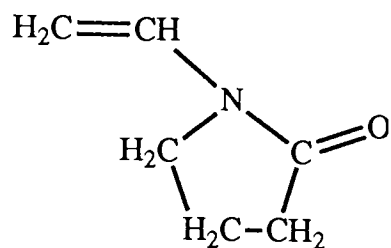
Figure 1.4 Structure of methacrylic acid (MA).

$$CH_3$$
$$H_2C\!=\!C$$
$$C\!=\!O$$
$$OH$$
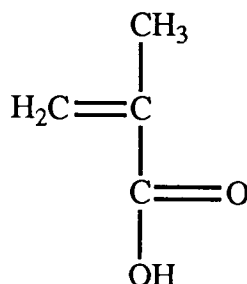
Figure 1.5 Structure of methyl methacrylate (MMA).

$$CH_3$$
$$H_2C\!=\!C$$
$$C\!=\!O$$
$$O$$
$$CH_3$$
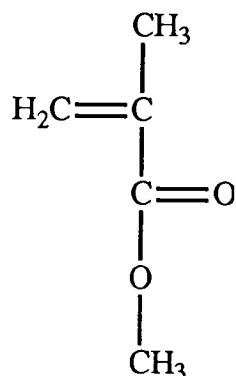
The monomers are reacted as in the general scheme shown in figure 1.6, the monomer solution is thoroughly degassed with dry nitrogen and azo-bis-isobutyronitrile (AZBN), is added as an initiator. The polymers formed from these monomers, when hydrated, are water-swollen gels with a net-like molecular structure as in the schematic diagram fig 1.7.

Figure 1.6 Schematic conversion of hydrogel monomers to polymer

$$H_2C = CH + H_2C = CH$$

$$\mid X \qquad \mid Y$$

$$\downarrow$$

$$\underset{C}{\overset{H_2}{\sim\!\!\sim\!\!\sim C}} - CH - \underset{C}{\overset{H_2}{C}} - CH \sim\!\!\sim\!\!\sim$$

$$\mid X \qquad \mid Y$$
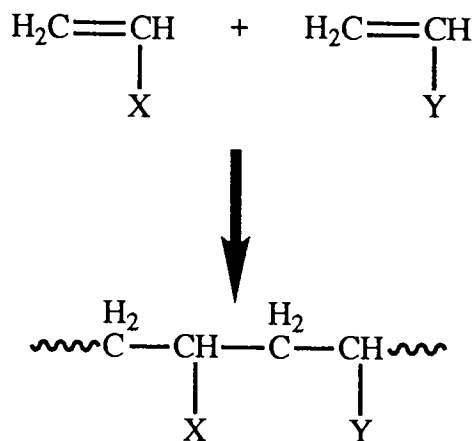
X and Y are determined by the monomers being used

Figure 1.7 Schematic diagram of a water-swollen hydrogel.
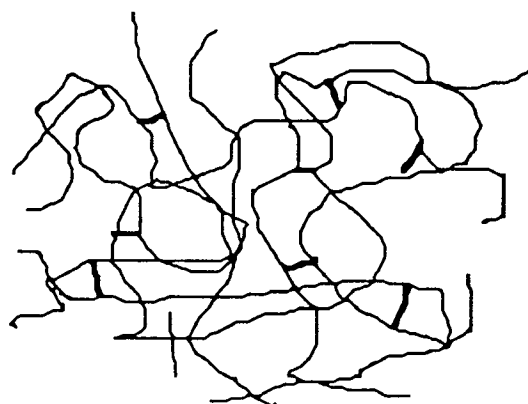
— Hydrogel chain

— Crosslinks

The water content of these hydrogels is a function of the monomers present and their relative concentrations, an illustration of this is shown in table 1.2. It has been found that as the ratio of VP goes up we see an increase in the EWC the same effect is seen with MA. As the ratio of MMA goes up EWC drops [8] reflecting its lower hydrophilicity.

Table 1.2 Equilibrium water content (EWC) of some commercial contact lenses [6,7].

Name	composition	EWC (%)
Soflens	HEMA	39
Hydrocurve	HEMA-VP	45
Tresoft	HEMA-MA	46
Sauflon 70	VP MMA	70
Permalens	HEMA-VP-MA	74

The lenses are manufactured by two methods; spin casting and lathe cutting. In the first of these techniques the monomers are spun in a mould that will give the outer curve of the lens, the inner or base curve is determined by the rate the mould is revolved, the initiator and catalyst are then added and the lens is formed, this then has its edge finished and polished and the lens is then hydrated. The second method is to cast the material in long tubes; these rods are then cut into buttons that are mounted on a lathe. The base curve of the lens is cut and then polished to remove lathe marks, the base curve is then stuck with bitumen onto a brass stub and the front surface of the lens is lathed. The front is also polished and the lens can, after cleaning, be hydrated. In both methods the lens is measured to ensure a correct back and front curve has been given after swelling the lens in water.

The base curve is chosen by the optician to fit the cornea of the patient comfortably, the front curve is determined by the optical correction that the patient requires.

Once the lens has been fabricated it is placed in saline or distilled water to allow any unreacted monomer to leach out of the lens. This is important as many of the monomers are toxic to body tissues. The lens is then placed in sterile saline in a sealed vial and this is then autoclaved at 121°C for 15 min, it is in this form that the lens is received by the optometrist.

The process as described is not particularly automated. Many lathes are not computer controlled and require a skilled operator. The machines are normally used in isolation and no automated production line as such is normally used. The very largest companies have automated some processes, and other methods of fabrication have been used to reduce production costs such as casting one or both of the faces rather that of producing plain buttons.

The understanding of the biocompatibility of contact lenses has been the aim of this project, but as well as the criterion of biocompatibility a material must show many other physical features. Hydrogels when hydrated are soft and rubber-like but have low tear and tensile strength. Much work is being done to improve the physical properties of hydrogels,[9] improvements have been made so that hydrogels can be used in other more physically demanding body sites.

The lens must be optically transparent and have a suitable refractive index if it is to function as an effective lens; it has been found [10] that refractive index decreases with increasing water content, lenses of 20% water content have a refractive index (RI) of 1.46 to 1.48 whilst lenses of 75% have a RI of 1.37 to 1.38. The material must show

dimensional stability with respect to time, temperature and pH.

The oxygen permeability of the material is a very important consideration, as we shall see later in this chapter the cornea derives its oxygen from the atmosphere *via* its dissolution in the tear film. Thus, any contact lens placed over this cornea must allow as much oxygen through as possible if it is not going to cause the cornea trauma and ultimately vascularisation. Work has shown [11] that oxygen permeability is governed by water content, as the water content goes up so does the oxygen permeability.

In conclusion for a material to be successful it must show a wide range of physical properties, when these physical properties are suitable it must then pass a range of biological tests before it is deemed suitable as a biomaterial.

1.6 The ocular environment

The contact lens when in use is placed in contact with the cornea and is bathed in tear fluid. In discussing the environment of the contact lens we are primarily concerned with tear fluid and the underlying cornea.

The contact lens lies upon the cornea, this is a transparent tissue consisting of 3 layers, see figure 1.8. The epithelium is 5 to 7 cells thick, the stroma makes up the bulk of the corneal tissue, most of it is made up of collagen fibrils with a few keratocytes interspersed. The cornea is avascular and is 0.5 to 0.57mm thick at the centre and 0.85 to 0.9mm [12] at the periphery. The cornea on a microscopic scale is covered with a network of microscopic ridges called microvillae, these irregularities are thought to provide a roughened surface which aids mucus adhesion and thus wettability.

Figure 1.8   Diagram of the cornea (at x150 magnification)[12]
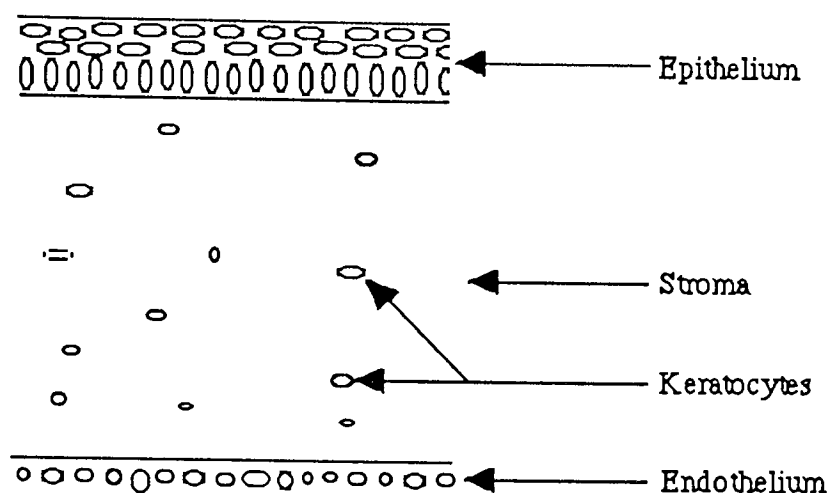
The cornea is covered by the eyelids which close during the blink reaction, these lids protect the eye, aid tear flow and help remove particulates from the cornea. It is within the eyelids and conjunctiva that the glands that secrete tears are situated, these are shown in figure 1.9. The conjunctiva can be described as the vascular membrane covering the posterior segment of the eyeball that carries through to the surface of the eyelids. The pouch formed by the lower eyelid is termed the conjunctival sack and is useful for drug delivery to the eye.

The role of the glands shown in figure 1.9 is believed [13] to be as follows; the main lacrimal glands are involved in the production of stimulated tears, whilst the accessory lacrimal glands (glands of Wolfring, Krause and Henle), are believed to produce the basal tears. The glands of Möll are modified sweat glands associated with the eyelashes. The glands of Zeiss are rudimentary sebaceous glands closely associated with an eyelash, they produce an oily fluid for the hair follicle. The Mebomian glands are found in the upper and lower lid, they have twisted long ducts, with openings at the lid margin, they are believed to produce the lipid layer found in tears.

Figure 1.9 Schematic diagram of the glands that produce tears (v.s. of the upper eyelid)
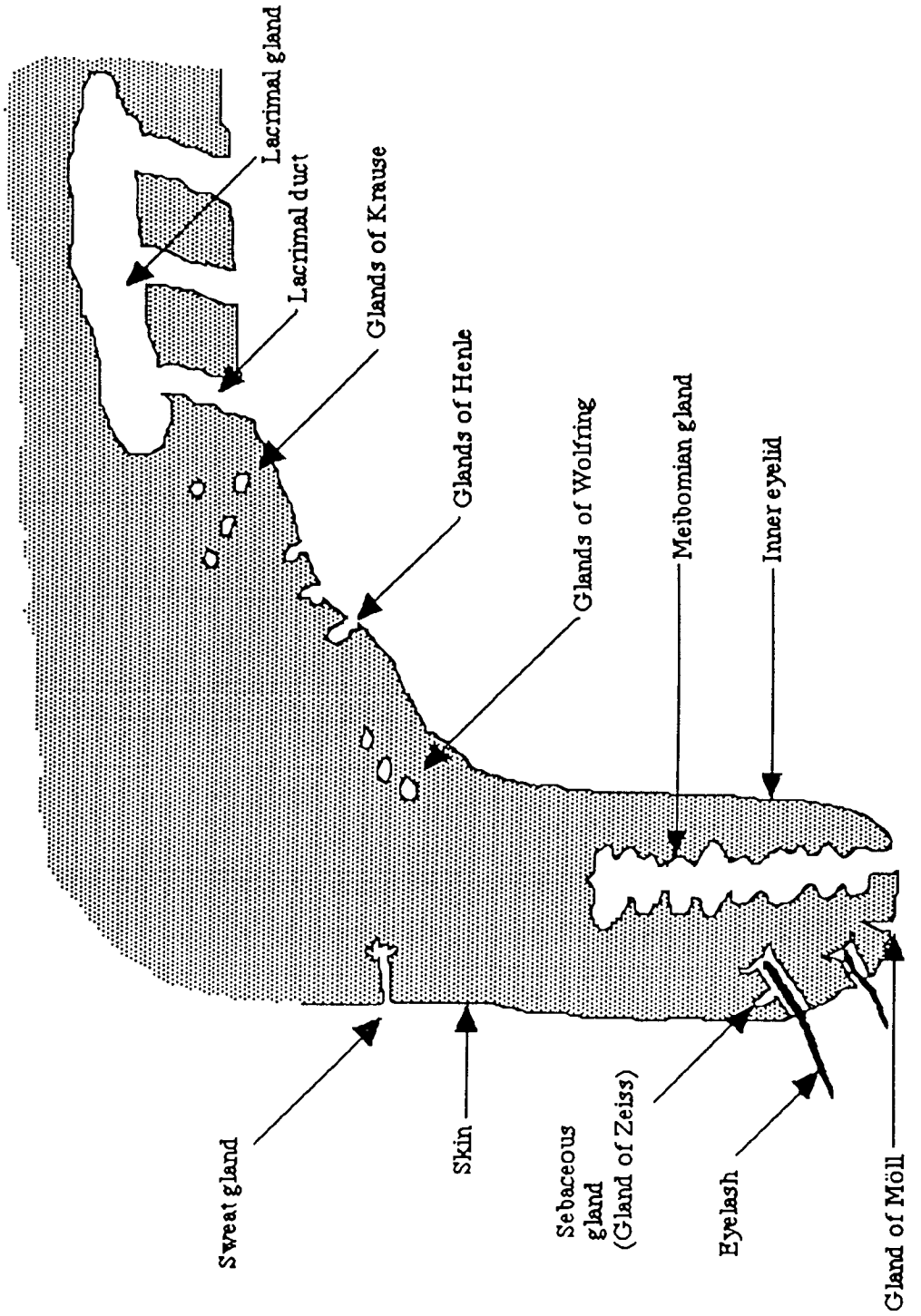
1.7 The physical properties of tears

The bulk features of tears are as follows; it has an average osmotic pressure of 300 mOsm/L (equal to about 0.95% NaCl) [14] a pH of 7.4 (7.3- 7.7), a refractive index of 1.357 [15], and a viscosity between 1.05 to 1.40 centipoise. The volume of tear fluid is about 5 to 10 µl [16] with a related tear flow of 0.3µl/min to 1.2µl/min [17].

The tear film is unlike most other tissue fluids in that it has a complex physical form [18]. A model based on observations has been suggested for the tear film, this model has 3 layers, see figure 1.10. The three layers are, the superficial lipid layer, thought to reduce evaporation, an aqueous layer and a less definite mucus rich layer next to the corneal epithelium. The thickness of each layer is as shown in diagram 1.10.

Figure 1.10   Schematic diagram of the tear film
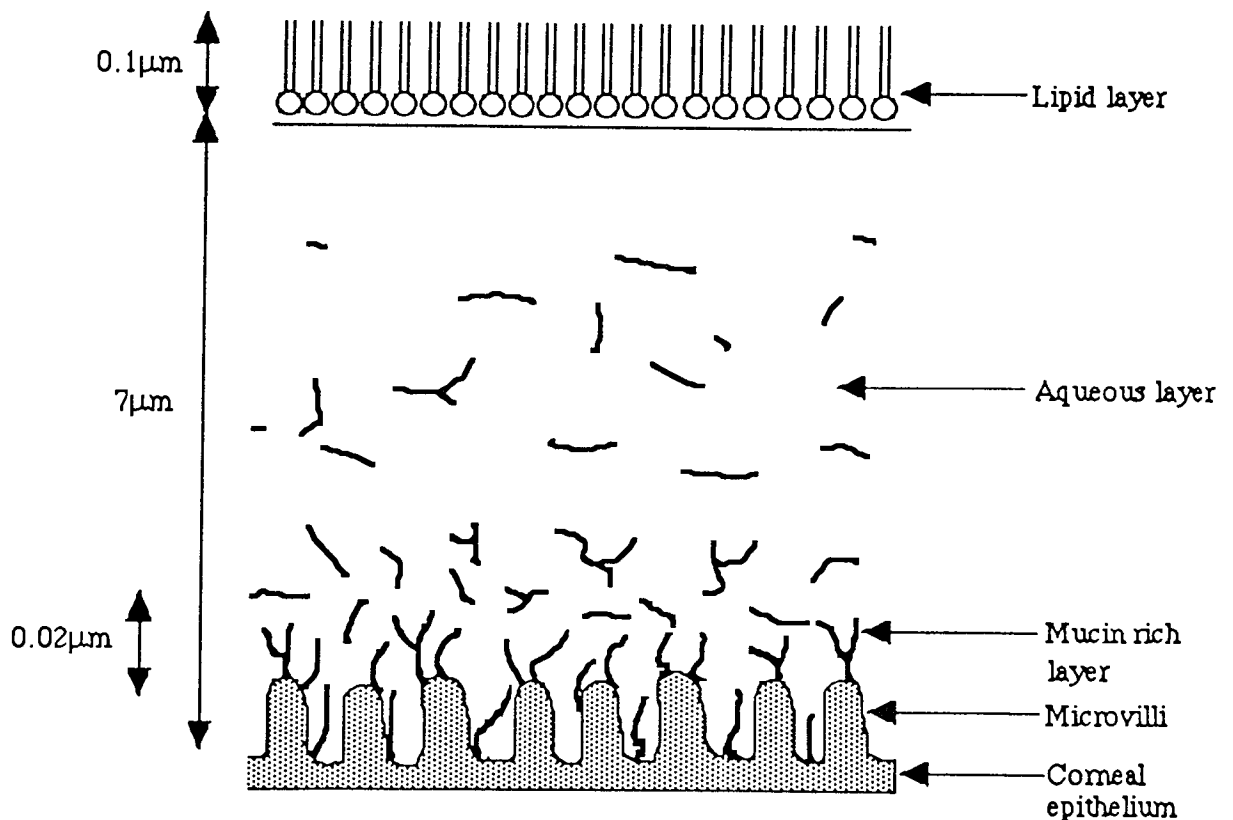
The lipid layer has been shown to be more stable than first thought, in that it remains complete during the blinking process [19,20]. The film is seen to thicken as the eyelids close during the blink reflex, then as the eyelids open the lipids rapidly spread, first as a monolayer, then to its full thickness. In the period between blinks the lipid layer is seen to thin.

1.8 The chemical makeup of tears

When interpreting the following information on tear composition, it should be pointed out that the method and conditions of tear sampling has a large affect on the result obtained. Many methods make use of adsorbent materials such as Schirmer filter paper, cellulose sponges or cotton threads. The tears are then eluted off the adsorbent material. The major flaws in this method were summarised by van Haeringen [21] as follows:

1   It causes irritation to the conjunctiva and stimulates reflex secretion from the main lacrimal gland.

2   Mucus and denuded cells adhere to the absorbent material.

3   It causes damage to the cornea and conjunctival epithelial cells, which results in the liberation of the cellular contents, thus components not naturally found in tears are detected.

4   The tear components can bind with the adsorbent material and are therefore not eluted off thus altering the apparent composition.

Glass capillary tubes have also been used to sample tears; this method gives very different values to that given with the absorbent material method [21]. The difference occurs because

in the capillary method only freely floating tear fluid is collected, while with the absorbent material, not only fluid is collected but also mucus and other cellular debris. A recently suggested method of tear collection is by means of placing a contact lens in the eye [22], this is then removed and the tear envelope washed off the lens. This method suffers from the points raised concerning sampling with other absorbent materials.

It should be remembered that as tear fluid only has a volume of $5-10\mu l$ withdrawing enough of this fluid for a valid quantitative or even qualitative analysis causes a large perturbation in the system. Some experimentalists simulate tear flow by various means including mechanical stimulation and chemical agents; this increases the tear flow, but the results of the composition gives information about reflex and not basal tears. Other workers use pooled tear fluid samples to enable components at very low concentrations to be detected. This solution to the problem of tear analysis causes an averaging effect of the individuals tear chemistry.

The analytical work done on tears has shown that tear composition varies between two individuals and also a single individuals' tear chemistry varies over a period of time. Thus a single correct tear composition will not be arrived at.

1.8.1.Electrolytes

Various electrolytes are present in tears, these are detailed in table 1.3.

Table 1.3 Ion concentrations in tears (averaged from refs in ref 23).

	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-
Tear concentration (mmol/ml)	140	24	0.8	0.45	126
Serum concentration(mmol/ml)	140	4.5	2.5	0.9	100

The levels of sodium ions in tears are near equal to that in serum, thus it is assumed that it has free passage. Potassium is found in tears at about four times its concentration in serum thus some active transport into tears must occur. Calcium and magnesium are at a lower concentration in tears than in serum, therefore a barrier to these ions must exist.

1.8.2 Metabolites

Various metabolites have been detected in tears, these are believed to pass into the tear fluid *via* the epithelial barrier from the blood. Glucose is believed to be at about 0.2mmol/l [23]. Lactate, pyruvate and urea have all been detected but these metabolites could be released by conjunctival damage during sampling.

1.8.3 Proteins

The analysis of tear protein is not a simple process because of the small volume of tears and the low concentration of tear protein. Protein concentrations have been assayed and reported with little agreement, see table 1.4 for details.

The number of protein species found in tears is very much open to debate; Gaucon *et al.*

[31] did an extensive study using a number of techniques, under poly-acrylamide gel electrophoresis (PAGE) they found 12 distinct bands, whilst under sodium dodecyl sulphate (SDS) PAGE 30 bands appeared. Under two dimensional electrophoresis, (isoelectric focusing in the first dimension, SDS PAGE in the second dimension), 60 individual species appeared. The large number of proteins reportedly found in tears could be due to several reasons: the proteins could be dissociated into subunits or even fragmented, especially after SDS treatment. Also a single protein could have a heterogeneous make up, Gauchon *et al.* [32] found that specific tear pre-albumin was made up of six distinct bands under two dimensional electrophoresis. The association between proteins also could cause confusion (it has been found [33] that lactoferrin binds strongly to IgA, IgG and serum albumin), also tear specific pre-albumin associates [34] with ocular mucus to give extra apparent bands. The calculation of tear protein concentration is also made more complicated by ambiguity in some papers as to the nature of sampling.

Table 1.4 Tear protein concentration

Author	Protein concentration (mg/ml)
Dohlman et al. [24]	20.0
	3.0-7.0
Berman [25]	2.0-6.0
McClellan [26]	6.7-8.0
Newell [27]	7.0
Callender [28]	10.2
Krause et al. [29]	3.56
Fullard [30]	
micropipet	
unstimulated	17.2
stimulated	6.8
Schrimer paper collection	
stimulated	13.1

There now follows a rundown of the major proteins found in tears and their physico-chemical properties.

1.8.3.1 Lysozyme

Lysozyme is a mucolytic enzyme discovered by Fleming [35] that catalyzes the depolymerization of sugars from peptidoglycan polymers from the cell walls of some gram

positive bacteria. It also, in the presence of complement, facilitates IgA bacteriolysis [36]. It is produced within a cellular organelle called the lysosome and has been proved [13] to be produced in the lacrimal gland by means of a ^{14}C pulsed labelling experiment on isolated lacrimal tissue.

The enzyme has a molecular weight (Mr) between 14 000 and 15 000 [32,37] as measured by SDS PAGE, an optimum pH range 6.0 to 7.4, and a high affinity, Km=6.15 x 10^{-6}, for its substrate. Its specific activity is the cleavage of the mucopeptide N-acetyl glucosamine(β1-4)N-acetyl muramic acid at the (β1-4) position [36].

Lysozyme makes up about 20-40% [30,38,39] of the total tear protein. Although found in most tissues, it is only present in concentrations high enough to be bacteriolytic in white blood cells, nasal secretions and tears. The concentration of lysozyme has been assayed in tears using a method similar to that of the antibiotic disc assay and a spectrophotometric (turbidimetric) assay, both depend on the clearing of suspension of *micrococcus lysodiekticus*. A value of 4.63 +/- 0.5 mg/ml was found by Fullard *et al.* [30], but Vinding *et al.* [40] found 2.22mg/ml using the same method. McGill *et al.* [41] who used an enzyme linked immunosorbent assay (ELISA) to determine lysozyme, rather than the kinetic assay, recorded a concentration of 1.10-0.72 mg/ml. Radial immunodiffusion a good technique for measuring protein concentrations gave a level of 2.95±0.27 mg/ml [42]. The concentration of the enzyme is seen to decrease in some disease states such as conjunctivitis and also with age.

Lysozyme is difficult to analyse as part of a mixture using electrophoresis since lysozyme binds to gel supports and other proteins [30] and can form bridges between immunoglobulins, also PAGE is difficult as lysozyme will not enter many gels because of its high isoelectric point [43].

1.8.3.2 Lactoferrin

Lactoferrin is an iron-binding glycoprotein. It makes up approximately 25% [44,39] by weight of the total tear proteins. Although found at high levels in tears lactoferrin is virtually absent from serum [45]. The level of lactoferrin in tears has been assayed by a number of workers, see table 1.5 for details.

Table 1.5 Reported tear lactoferrin concentration

Author	Lactoferrin concentration(mg/ml)
Jensen et al. [46]	1.12
Kijlstra et al. [44]	2.2
McGill et al. [41]	1.36
Fullard [30]	2.09
Rapacz et al. [42]	1.73

It was shown [13] that lactoferrin in tears is produced by the lacrimal gland. Lactoferrin binds

two molecules of iron reversibly with an affinity 300 times [47] greater than the other iron binding protein transferrin, and has been proved to be immunologically distinct from this protein.

Its bacteriostatic properties are often attributed to its ability to make iron unavailable for microorganisms, but workers [44] have shown that it could have some direct effect on certain bacterial strains. Lactoferrin also facilitates the action of lysozyme [48] by its iron chelation properties. Lactoferrin has also been attributed with the regulation [44,46] of the production of granulocyte- and macrophage- derived colony stimulating factors. Furthermore lactoferrin has been shown to prevent complement activation, these activities suggest that lactoferrin also may be involved in the regulation of the inflammatory response.

Lactoferrin binds to several proteins particularly acid macromolecules, [30,43] such as serum albumin and IgA, it also binds to gel support media [30] such as agar and agarose, as well as to solid supports [30] such as silica. Both of these non-specific bindings makes assay or identification by electrophoresis, immuno-electrophoresis [33], ELISA [44] or ultracentrifugation very difficult. It is because of these problems that molecular weight determinations give such diverse results (see table 1.6).

Table 1.6 Lactoferrin reported molecular weights

Author	Molecular weight determined(Daltons)
Broekhuyse [33]	82,000
Gachon et al. [32]	75,000
Janssen et al. [43] un dissociated	109,000 to 320,000
subunit	86,000

The heterogeneous values of Janssen et al. [43], in table 1.6 were explained by lactoferrin being present in tears in the form of complexes (with other proteins), of mixed molecular weight.

Under isoelectic focusing lactoferrin made a band between 5.3 to 7.1 pH units. An interesting point is that, with age [49], lactoferrin levels go down markedly; Jenssen et al. [43] reported levels of 1.48mg/ml in the 3rd decade, dropping to 0.81mg/ml in the 8th decade.

1.8.3.3   Immunoglobulin A (IgA)

IgA is the predominant immunoglobulin [30] in tear fluid. The IgA of external secretions, such as tears, differs from circulating IgA by having an additional antigenic fragment attached; this secretory component aids the IgA dimer to pass across biological membranes. The structure of the IgA monomer and the dimer with secretory piece in place is shown in figures 1.11 and 1.12.

Figure 1.11 Diagram of the IgA monomer
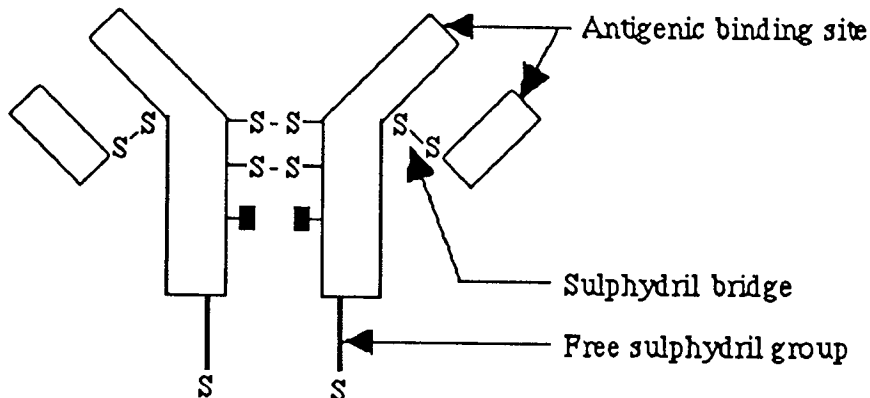
Figure 1.12 Diagram of a IgA dimer and secretory piece
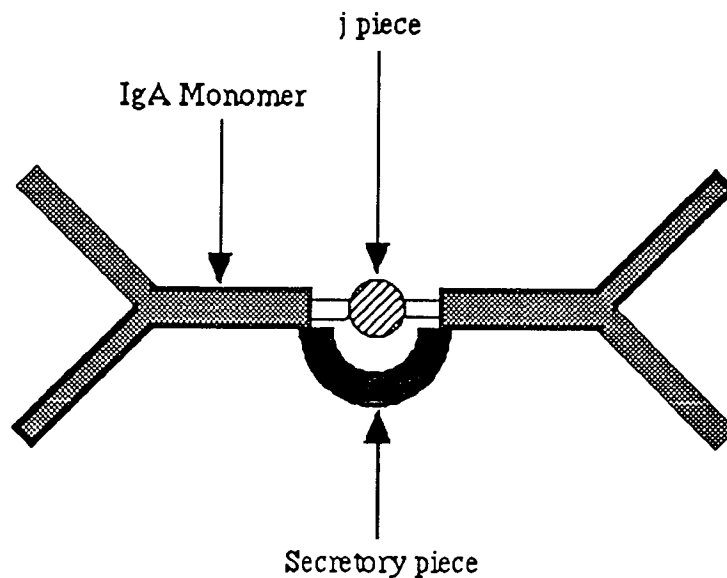
IgA represents about 14.8% ± 0.1 % of the total tear protein [39], it has been found to have a molecular weight of 260 to 360 kDalton [50]. IgA concentrations in tears have been worked out by number of techniques, 542+/-98μg/ml [30] 0.715μg/ml [40] and 490μg/ml all by ELISA [51], 511 μg/ml [52] by radial immuno-diffusion.

The ratio of secretory IgA to serum type IgA (with no secretory piece attached) was 50-90:1 [30]; the IgA is believed to be produced in the lacrimal apparatus and from there secreted into tears [41]. Some workers have suggested that IgA is only produced in low concentrations in the lacrimal glands [43], and that this is not the major source of secretory IgA in tears. IgA levels are found to drop with age [41].

1.8.3.4 Other Immunoglobulins

Although secretory IgA is the major immunoglobulin in tears, other immunoglobulins have been detected at low concentrations. IgG reportedly occurs at the highest concentration [53], reported values include 6.75mg/ml [53], 17mg/ml [41] (both by ELISA), 0.536mg/ml [30] (by radial immuno diffusion) and 0.17mg/ml (by an unspecified technique) [54].

IgG in tears is thought to originate from damage to the conjunctiva [56] during sampling; this is supported by reports that IgG levels go up with age [41] unlike most other tear components, this is explained by the break down in the blood/tear barrier with age. It is possible that some IgG is produced in the lacrimal apparatus [13].

The other immunoglobulins are detected in very low concentrations, see table 1.7

Table 1.7 Reported immunoglobulin concentrations in tears

Immunoglobulin	reported concentration
IgM	5.82mg/100ml [53]
	<1mg/ml [54]
IgD	none detected [54]
IgE	250ng/ml [54]

IgG levels are not found to alter significantly during disease states [53].

1.8.3.5 Tear Specific Prealbumin (TSPA)

Tear albumin is a unique protein never found in serum; electrophoretically it is a pre-albumin, migrating to a similar position to serum pre-albumin. However, antiserum raised to serum pre albumin does not react with tear pre-albumin [56]. It has been reported that TSPA occurs at a concentration of 1.23+/-0.4mg/ml [30] in basal tears. TSPA is heterogeneous [57] in make up. Gachon [32] reported 6 individual proteins with molecular weights of 15,000 to 20,000 under non-denaturing, two-dimensional electrophoresis. Janssen [43] reported between 2 and 4 individual species, a molecular weight of 24,000 and a subunit molecular weight of between 18,000 and 19,000 using various electrophoretic techniques. Under iso-electric focusing, TSPA formed a band between 4.85 and 4.94 pH units [43]. TSPA was shown to be produced in the lacrimal apparatus by means of pulsed labelling experiments [13] on isolated lacrimal gland tissue.

1.8.3.6 Serum albumin

Serum albumin is not present in the secretions of the lacrimal gland, but is mixed with the tear fluid in the conjunctival capillaries [13]. Trauma during tear collection can affect the levels of serum albumin in tears. Very little can be deduced from reported tear serum albumin levels, such as 360+/-35ng/ml [30](by ELISA) because of the non-reproducibility in the trauma caused during sampling. Serum albumin has a molecular weight of 62,000 [31] to 65,000 [43], and an isoelectric point of 4.85 to 4.95 [43] pH units at 4°C.

1.8.3.7 Epidermal Growth Factor

Epidermal growth factor (EGF), is a polypeptide that stimulates tissue growth including the cornea. It has been found in tears by two separate methods at a concentration of 0.7ng/ml to 9.7ng/ml [58,59]. As it has been shown that EGF is a normal component of human tears both basal and reflex [58] it must therefore be important for conjunctival and corneal epithelial integrity.

1.8.3.8 Other Proteins

Very many proteins have been identified in tears, some by physical properties, like electrophoretic or ultracentrifugal mobility; many more have been detected at very low levels by virtue of their enzymatic activity [41]. Immunological studies have also led to the identification of many species [20]. It would be a futile exercise to list all the proteins identified as most are detected only because of trauma to the conjunctiva during sampling, leading to leakage of cellular components.

1.8.3.9 Lipids

The lipids in tears occur mostly at the tear-air interface in a thin layer approximately 0.2μm thick. The composition of this lipid layer is not well understood; lipids, in common with all the components discussed, occur in small quantities in the eye making analysis difficult. Lipids also suffer added complications: lipids are very prone to oxidation and isomerisation, thus extracts analysed often contain artifacts. Many lipids occur as members of homologous sequences, differing only in alkyl chain length; this makes separation and identification difficult. Many analysis systems do not try and distinguish between homologs, only separating down to lipid families. Many lipids are un-charged thus electrophoretic techniques cannot be used, the exception to this are the fatty acids and the phospholipids. Many lipids have no characteristic chromophoric properties thus even if separation is afforded, detection is problematic.

The classical lipid separation technique of thin layer chromatography (TLC), using sulphuric acid followed by charring to visualize the bands, is unsuitable for tear lipids because of the small sample sizes. Pooling of samples is possible but work carried out on the subject [60] has shown great variation between individuals, thus this is not a suitable method.

The majority of lipids in tears are believed to be produced in the meibomian gland [61], a number of smaller ancillary glands are also implicated, but lipids produced by the skin are also found in tears as contaminants. Work carried out at Aston has lead to the development

of a HPLC system that can separate the extracted lipids from a single contact lens, which is a comparable sample size to that collected in a single tear sample of 10-15μl. The system suffers from the overlap of retention times due to homologue similarities, making unequivocal identification difficult.

The work on lipids has shown the presence of a wide range of lipid classes [60,62,63] including waxy esters, cholesterol esters, triglycerides, diglycerides, monoglycerides, free fatty acids and phospholipids.

1.8.3.10 Mucus and glycoproteins

Mucus makes up the inner most layer of the tear film. It is believed to be important because it lubricates the cornea by rendering its hydrophobic surface more hydrophilic [64] permitting spreading and stabilization of the tear film. It has been suggested [65] that the role of the mucin at the epithelial tear interface is not simply to lower the interfacial tension, but also has to maintain this low interfacial tension in a hostile environment where lipid molecules continuously bombard the surface. The mucus layer, it has been theorised, maintains hydrophilicity despite considerable quantities of lipid contamination by masking the hydrophobic nature of the lipids, only when lipid contamination is excessive does the mucus become hydrophobic. The contaminated mucus is then no longer stable in a thin layer, and aided by the shear forces of the moving lids, rolls up forming fine mucin fibrils that aggregate into mucus threads. These move down into the lower fornix and then into the corner of eye. The lipids that cause the contamination include meibomian lipids and epithelial

cell debris rich in phospholipids.

The mucin is believed to be produced in the conjunctival goblet cells [66, 67], but this rests on histological evidence only.

Mucus is made up of high molecular weight glycoproteins, each of the polypeptide chains has, at about every 10th residue, a carbohydrate chain, each chain is about ten saccharides long. A mucin glycoprotein can be compared with a bottle cleaning brush, with the carbohydrate chains protruding from the protein in three dimensions. Some regions of the chain do not have any carbohydrate and it is at these regions that covalent linkages occur [68]. Various other non-specific interactions occur between the chains, these help to maintain the mucus's three dimensional integrity.

The characterisation of mucins is fraught with problems as with other tear components, the levels of these compounds are low and the sample size is very small. Another problem is that of sample collection, as tears occur in a three layered structure, the position of the sampling device becomes crucial to the composition and quantity of the mucus sample obtained.

Work on the composition of mucus is hindered by its multi-molecular gel-like structure. To enable any analytical work to be done some dissolution must be carried out, workers have used proteolytic enzymes, reducing agents, high ionic strength solutions, urea or guanidine

and physical disruption.

The glycoprotein component of mucus has been separated by Moore and Tiffany [69], the principal mucin complex GP1 (molecular weight >2 x 10^{6}) and its subunit GP3M (molecular weight ≈200,000) were detected in an unreduced saline extract of mucus. Reduction of disulphide bonds in the extract gave rise to GP3 (molecular weight >1.3 x 10^{6}). Human GP2 and GP3M each contained a high proportion of carbohydrates, but a considerable variation between people was detected. GP2 was shown by means of immunohistological techniques [69], to be exclusively produced in the conjunctival goblet cells [69] and not the lacrimal apparatus. Another glycoprotein GP3T (molecular weight ≈200,000) was also discovered, this did not react with antibodies to GP2.

GP2 and GP3M had a high proportion of serine and threonine [70] as expected because in many glycoproteins the linkage between protein and carbohydrate is an O-glycosidic linkage between seryl or threonyl residues [71], and N-acetylgalactosamine.

Electrophoresis of the high carbohydrate glycoproteins has a major problem: the microheterogeneity [72] in the side chain structures leads to poorly defined broad bands especially under isoelectric focusing. The slight variation in carbohydrate sidechain makeup is brought about by its method of construction, unlike proteins, carbohydrates are not assembled using a molecular template. The sequence of saccharides depends only on the specificity of enzymes that produce them; as a rule of thumb, the longer the carbohydrate and

the more distal the saccharide unit the higher the chance of variability between chains.

The ocular mucus is not made up of purely mucin-type glycoproteins; many if not all tear components found in the aqueous phase are found in mucus. Some components such as IgA [73, 74] and albumin [73] are believed to be concentrated in the mucus; IgA, it has been argued, interacts non-covalently by means of a region between the F_{ab} and F_c regions [74]. Other workers have suggested [75] that some of the IgA could be in fact be bound covalently to the mucus and orientated outwards giving a defense against pathogens. Lactoferrin and lysozyme [73] were also found in the mucin: these too could provide improved bacteriostatic properties on the mucin.

A number of simpler methods, other than complete characterisation, have been employed to detect and quantify these glycoproteins. Versura *et al.* [67, 76, 77] and Wells *et al.* [78] have used lectins to detect specific carbohydrate residues. Lectins are proteins of vegetal origin that specifically bind glycosidic residues; the lectin can be conjugated to colloidal gold [67, 75, 77] and then used as an electron microscopy stain. Lectins also can be bound to a chromophore or a fluorophore [78] and used in optical detection systems, both methods have the advantages of good sensitivity but the information gained is hard to interpret: as the lectins binding depends on the accessibility of the carbohydrate chains and also other components can compete with the lectins, when a complex mixture is analysed. The technique gives good qualitative information but if the mucus is in a thick layer, quantitative information is very unreliable.

Another simple system to detect and quantify mucus is to digest fully the macromolecules into their component saccharides and amino acids, then analyse one or more of these components. By the use of known standards this value can be extrapolated backwards to give a value for the amount of mucus that was originally digested. Dohlman *et al.* [79] used this method to quantify tear mucus by analysing hexosamine concentrations after hydrolysis; they reported a hexosamine content in unstimulated tears of $0.6^{+}/-0.1\mu g/ml$.

The saccharides present in mucus glycoproteins include [70-72] fucose, mannose, galactose, glucose, N-acetyl galactosamine, N-acetyl glucosamine and the sialic acid N-acetylneuraminic acid.

The saccharide(s) chosen should occur in the glycoprotein at high concentrations, but not in any other tear component, e.g. glucose, galactose and mannose would be unsuitable because these are present as metabolites in the pre-corneal tear film.

The amino acids produced in the hydrolysation of the glycoprotein would be unsuitable for quantification because they occur in all the proteins found in tears. Serine and threonine could be assayed because these are at unusually high concentrations in mucus glycoproteins but in unpurified samples absolute quantification would be difficult.

Several good reviews [80, 81, 82] of mucus biochemistry have been published, these contain

much more detail about the nature and the characterisation of mucus than can be entered into in this account. These should be consulted to full appreciate the diverse nature of these molecules.

CHAPTER 2

Contact lens spoilation:- Occurrence and analysis

2.1 Contact lens spoilation:- General

This chapter sets out to look at the techniques used to study spoilation and the information obtained by these means.

The analysis of the components in contact lens deposits has proved more difficult than the analysis of tears, as the amounts of samples are smaller and often the species are very tightly bound, thus very sensitive analytical techniques are required.

2.2 Deposit morphology

A great deal of literature exists on the appearance of deposits on contact lenses; several reviews have been written on the subject [83, 84, 85].

Bowers and Tighe [83] have reiterated a system of classification of deposit types; these categories were suggested not as definitive, but rather as a system that matches the clinician's ability to categorize the samples observed.

Table 2.1 Proposed classification of contact lens deposits

1   Discrete elevated deposit:- These so called 'white spots' [86, 87] group together many deposits of a complex chemical makeup.

2   Lens coatings:

(A) Proteinaceous films.

(B)Inorganic films:- Mostly calcium phosphate covered with protein.

3   Granular deposits:- These are of unknown makeup.

4   Specific inorganic calcium deposit:- These are made up of crystalline needle like calcium carbonate covered in protein.

5   Microbial spoilation.

6   Deposits caused by extrinsic factors:- such as cosmetics and airborne foreign bodies.

7   Melanin particulates and yellow brown lens discolouration

The lines of demarcation between categories are indefinite, and there is a possibly that all deposit types are built up on the same foundation (i.e., initial events are the same) and develop according to their local environment.

Of the above groups the deposit with the most interesting physical form are those in group 1 in table 2.1. The white spots are large (up to 150μm), with a distinct lobular structure, they were well described by Tripathi *et al.* [88] as 'barnacles', these structures are normally attributed to calcium, but work [87] has shown that this is an over simplification. They are now thought to include many of the molecular species found in tears. The white spots show a high intrinsic fluorescence [87].

Many papers have studied the optical appearance of the films that build up on the lens surface, many of these appear similar [84, 89, 90], and thus many have been miss-attributed, or described by general appearance, e.g. inorganic films (if grainy) lipid or protein deposits (if smooth). A number of light microscopic techniques have been used to study this problem, phase contrast [91] and fluorescence [92], these have shown some additional detail but unequivocal identification of deposits by optical microscopy is not possible.

2.3 Chemical makeup

The study of contact lens deposit fall into two broad categories: invasive techniques where the deposit is removed from the lens and the extract is analysed, and destructive techniques where the deposit is studied *in situ* on the lens surface.

2.4 *In situ* analysis of spoilation

Techniques to study spoilation *in situ*, on and in the lens, include histochemistry, electron microscopy, biological probes, and various spectroscopic techniques. There follows a review on each of these techniques as used to investigate this phenomenon.

2.4.1 Histochemical examination

The simplest of the non-invasive techniques to determine a deposits' chemical composition is optical microscopy coupled with specific stains. The use of these histochemical stains not only enables deposit components to be identified but also their spatial distribution to be shown. A number of stains have been used [87], see table 2.2

Table 2.2   Stains used in deposit histochemical analysis [87]

Stain	Specific for	Location in deposit
Digitonin	lipids	throughout
Performic acid Schiff	unsaturated lipid	at the deposit interface
Bromine silver nitrate	unsaturated lipid	throughout
Periodic acid Schiff	carbohydrate	very little staining
Mucicarmine	mucins	very little staining
Millon's reagent	protein	very little staining

Mucins can be visualised by histochemical means [93]. Alcian blue/periodic acid

Schiffs' reagent indicates the presence of mucin components. Mucins were detected by

this method on heavily deposited lenses but not on normal or lightly deposited lenses;

this negative staining could be due to a lack of sensitivity in the stain rather than the

absence of mucins. From the histochemical evidence it has been suggested that the

initial interactions at the deposit contact lens interface involve unsaturated lipids [87].

This conclusion awaits further investigation.

2.4.2 SEM and TEM

SEM (scanning electron microscopy), and TEM (transmission electron microscopy),

are non-invasive but destructive techniques. The sample is dehydrated and then can be

gold or carbon coated, it is then placed in the microscope, which is then evacuated. A

beam of electrons is then shot at the sample, in SEM the reflected electrons are collected

whilst in TEM the sample is cut very thin and only electrons passing through the

sample are detected. Electron microscopy (EM) has the advantage in magnification

over light microscopy due to the wavelength limitation imposed in light microscopy.

EM does have a drawback: the sample must be treated very roughly, it must be totally

dehydrated and fixed by means of various harsh chemical treatments if the structures

are not to collapse. The interpretation of EM is difficult as much of the structural detail

seen could be artifactual.

The work carried out has shown in more detail the structures described under the light

microscope [86,94,95], white spots have been shown [86] to have far more detail than

first imagined.

When SEM is coupled with energy dispersive X-ray analysis (LINK), information about a deposits' elemental composition can be gained: it was found [87] that white spots contained calcium, potassium and chloride, but mostly at the surface. The technique suffers from poor sensitivity, especially from some elements with a small response factor, elements with low molecular weights cannot be detected at all and only surface concentrations can be deduced. The results are also open to misinterpretation because of buffer contamination of the surface; this layer of buffer is dehydrated leaving an inorganic film overlying the deposit surface.

2.4.3 Biological probes

The term biological probe encompasses a number of groups of molecules produced by living systems, their most striking feature is their high specificity in their binding to other certain molecules. The two most commonly encountered biological probes are antibodies and lectins, both groups have been used successfully in the study of worn contact lenses. The probe is coupled to a visualising agent to enable its location to be detected, the visualising agents used include colloidal gold, fluorescence, radio isotopes and enzymes.

In interpreting histochemical data using biological probes, it is important to realise that many factors, such as antigenic conformational integrity of the deposit, degree of penetration of the probe, availability of antigenic-binding sites and interference from other deposit constituents are all capable of interfering with a quantitative reaction. The result of the use of these probes should be viewed as reflective of the surface availability rather than the actual concentration of a given substance.

2.4.3.1 Lectin probes

Lectins are probes that are vegetable derived proteins, which bind to certain regions of polysaccharide chains.

Using various lectins labelled with fluorescein iso-thiocyanate, it was found [96] that the following saccharide residues were present on contact lens surfaces; α-linked or β-linked; D-mannose, D-glucose, D-galactose, L-fucose, N-acetyl D-glucosamine, N-acetyl D-galactosamine, N-acetyl neuraminic acid (a sialic acid). As all these are residues found in mucins it seems likely that mucus components have coated the lens surface. Distinct and isolated deposits need to be studied using the lectin technique.

2.4.3.2 Immunological probes

Immunoglobulins (IG), are the most useful and flexible biological probes. The various classes of IG make up part of the body's normal defense mechanism, when a higher organism detects a foreign object within it-self it responds by the production of highly specific proteins that will bind with the foreign moiety. Many reviews [97,98] of the immune response and the part played by immunoglobulins are to be found, in this discussion no more will be said about this mechanism.

As has been said, antibodies have a high affinity for one specific molecule termed an antigen; this high binding affinity and high specificity makes them ideal biological probes. Antibodies can be raised against nearly any compound by injecting the antigen of choice into an animal, such as a rabbit, repeatedly. The antibodies can then be extracted after a period of time from the serum that is taken regularly from the animal.

The antigen needs to be a large molecule, small molecules, termed haptens must be bound to large carrier molecules before an immune response can be elicited.

The antibody can be bound to a labelling agent directly or a cheaper and more sensitive system can be used: an antibody to the required moiety is raised or purchased, e.g., rabbit anti-lysozyme. A labeled antibody is purchased commercially, this antibody should be one against immunoglobulins from the first animal, e.g., in our case camel anti-rabbit. The system gives two advantages: only one expensive conjugated antibody need be purchased or prepared, and an amplification step takes place, where more than one anti rabbit can bind with a single rabbit anti-lysozyme.

Wedler [99] used immunoglobulins bound to fluorescein as a marker for deposit components. By means of this technique it was found that albumin, lysozyme, γ-globulins, and α_1-Lipoprotein were all present in the deposit. Other workers [100] have shown lysozyme to be the major available component on the lens surface; some other tear components were detected, these were in decreasing order of abundance, IgA, lactoferrin and IgG.

In addition to fluorescent labels the antibodies can be bound to an enzyme such as horseradish peroxidase. The activity of the enzyme can be utilised to develop a colour reaction, this gives a very good sensitivity as each enzyme can produce very many coloured molecules thus acting as an amplification. Using this system [101] deposits were found to contain lysozyme, lactoferrin, various immunoglobulins and albumin, but the latter only in very low concentrations.

Antibody labelling can be utilised in SEM and TEM by binding an electron-dense particle, such as colloidal gold, to the antibody. By means of this approach [102], in decreasing order of concentration, IgA, IgG and IgE were found on spoiled lenses. C_{1q}, one of the components that make up the body's complement system [103], was found in the deposit. This suggests that the hosts' tissue has staged an immuno-defense against the prosthesis.

2.4.4 Spectroscopic techniques

The analysis of spoilation by spectroscopic means appears to be the ultimate in non-invasive investigation, as electromagnetic radiation is the only probe, but often sample preparation can be harsh, also the spectra obtained are very difficult to interpret due to the very complex nature of the multi-component systems studied. Two techniques that show the above shortcomings are U.V. and NMR spectroscopy.

2.4.4.1 TIRF

Total internal reflection fluorescence (TIRF) is a method for probing protein adsorption on solid surfaces [104] by virtue of their intrinsic fluorescence. The technique is complex and has only proved successful at studying adsorption on glass or quartz surfaces that make up part of the optical system of the apparatus [104, 105]. Though not used on contact lenses specifically, it has been used to study other biomaterials with some success: haptoglobin [105] adsorption has been monitored by this technique.

2.4.4.2 FTIR

Multiple internal reflectance Fourier transform infrared spectroscopy (MIR-FTIR) was one of the few spectroscopic techniques to have been used to study lens spoilation at

the outset of this project.

MIR-FTIR spectroscopy involves the collection of an infrared spectrum from a surface. It is not constructed by beam transmission but by reflection of the beam off the sample *via* a crystal of high refractive index. For detail of the theory and practice of MIR-FTIR see chapter 4.

By the use of MIR-FTIR, Castillo [106-108] has attempted to study the infrared properties of spoiled soft contact lenses, the major problem in doing this is that hydrogels contain large quantities of water: water absorbs strongly in the infrared masking many of the bands of interest [106]. For a useful spectrum to be constructed, spectral subtraction of the contribution made by water must be carried out; this subtraction can be carried out by eye , i.e., the disappearance of the water peaks, but for a reproducible result to be produced a least squares computer program must be utilised [106].

In the infrared spectrum of a complex molecule such as a protein all the bands seen cannot be attributed to particular structural features, but by looking at particular absorption bands, information about the quantity present and the conformation of the molecule can be gained.

The conformational integrity can be judged by the position of the two major amide bands in the infrared spectrum, compared to the native protein. Details of the group frequencies of proteins and other biological molecules can be found in chapter 4.

The adsorption of lysozyme to contact lenses in an *in vitro* experiment [107, 108] was followed by MIR-FTIR. It was found that lysozyme adsorbs both reversibly and irreversibly; the reversibly bound lysozyme exhibits only minor changes in its secondary structure whilst severe conformational change occurs in the irreversibly adsorbed protein. As a function of time [108] the irreversibly bound lysozyme appears to bind first, with subsequent reversibly bound layers on top of this. Lysozyme adsorption was found to decrease on pHEMA / MAA lenses after silanization.

Conformational changes in human serum albumin on adsorption to various soft contact lenses [109] have also been monitored by this technique. It showed a change in the secondary structure, similar to heat denaturation, on adsorption to the hydrogel. HEMA/MAA lenses were shown to denature albumin much slower than pHEMA lenses. Also the effect of lens fabrication technique on adsorption showed [109] that spin cast pHEMA lenses denatured albumin faster than the lathe cut pHEMA lenses.

Mucin adsorption has also been investigated [110] by MIR-FTIR: bovine submaxillary mucin (BSM) was adsorbed onto various soft contact lenses with interesting results. It was found [110] that the BSM adsorbed in a three layered structure:

1   A thin layer of strongly bound and conformationaly altered mucin in a random ß sheet configuration.

2   Minimally changed BSM.

3   BSM that is not changed at all.

(99% of the adsorbed BSM is found in the upper layers 2 and 3)

Surface morphology and chemical composition of the lenses only appeared to affect the

reversibly adsorbed BSM.

2.4.5 Radio isotope labeled protein adsorption

The information on work done on radio labeled proteins is placed here but it should be noted that this is not a technique to study deposits formed *in vivo*, it is rather a method by which we can study in a model environment the rates of protein adsorption.

The technique of labelling proteins with radio isotopes has been widely used in many areas of biological research, the study of biomaterials is no exception. The most common radio label is ^{125}I, this isotope has high activity and is a gamma emitter. The protein is reacted with the ^{125}I so that the label is tightly bound [111]. The labeled protein can then be added to various other proteins to study competitive adsorption, the polymers are then washed and counted in a gamma counter.

Royce [111] looked at ^{125}I labeled protein adsorption on a range of pHEMA-MMA co-polymers: he found that protein adsorption increased as pHEMA concentration in the gel went up. This work has now been discredited [112] as it was discovered that the high apparent adsorption of protein to high pHEMA hydrogels was due to the irreversible binding of free ^{125}I to the higher water content gels. If the free ^{125}I is removed by dialysis, a minimum protein adsorption is observed on co-polymers containing 50% or more HEMA. Albumin was found to be the most heavily adsorbed protein on all pHEMA-MMA polymers. It was also shown that commercial contact lenses, made from HEMA/N-vinyl pyrrolidone(NVP) or acrylamide(AAM) adsorbed

smaller amounts of protein (especially lysozyme) when compared to the pHEMA-MMA co-polymers.

The effectiveness of detergents was investigated, but this was as part of the less reliable study [111]; it suggested that SDS was far more effective than urea or NaCl, and that SDS removed protein from the high MMA more successfully than from the high HEMA lenses, suggesting that hydrophobic interactions are more important in the MMA-protein binding.

In another study [113] lysozyme and albumin adsorption was followed by means of radio labeled proteins, lysozyme was found to be taken up at a faster rate and in greater amounts than albumin. A most interesting result was obtained [114] when tear fluid at 1/5th dilution was added to the protein solutions, the labeled proteins were found to adsorb quicker and at a higher level than when no tear fluid was present.

The results of these radio-tracer experiments have been cast into doubt because the ^{125}I can dissociate from the proteins, this dissociated ^{125}I can then be absorbed onto the polymer. The method is further hampered by the fact that the adsorption characteristics of a protein could be changed by having ^{125}I bound to it. The question should also be asked, is the nature of the protein altered during the chemical coupling of the [125]I ? The coupling technique is harsh and involves covalent linking the protein to the ^{125}I often using harsh chemical reagents.

2.5 Techniques involving removal followed by analysis

These techniques depend on solubulisation of the deposit from the lens surface, this extract is then analysed. Invasive techniques suffer from a number of problems these are summarised in table 2.3.

Table 2.3 Intrinsic flaws in solubulisation of the deposited material

1 Not all deposited components are removed

2 On removal, components can be damaged and fragmented leading to artifacts.

3 On removal, components can form aggregates leading to artifacts.

4 By removal of the material we get no information on spatial arrangement.

5 Removal normally destroys important information about conformational integrity of the molecule when *in situ*.

6 No removal regime removes all of the deposit, the residue is likely to be the deposit foundation and therefore the most important to elucidate.

The value of the subsequent analysis depends very much on the effectiveness of deposit removal and this depends on the interactions involved between the lens material and the deposit and also between the various deposit components. The regime used in deposit removal, depends on the type of component we want to analyse; for lipid extraction the use of an organic solvent to give a good extraction, whilst for proteins an often complex extraction medium is needed to break down the numerous types of interaction possible.

The use of extraction of lens deposits and subsequent analysis had at the outset of the

project only received limited attention. The majority of papers in this field are published by clinicians and because of unavailability of suitable equipment many of their studies are descriptive of the manifestation of this problem rather than its analytical study. Of the papers published some have attempted to identify the macromolecular components, whilst others use hydrolysis and then analyse the fragments formed.

2.5.1 Hydrolysis and amino acid analysis.

Some of the original work on contact lens deposit compositional elucidation was done by Karageozian [115], who used an amino acid analyser to look at hydrolysates of contact lens deposits. It was found at the time that the amino acid composition was very similar to that of lysozyme, thus it was presumed that lysozyme coated the lens. The study contains a number of flaws, the source of lysozyme, used as a control, is not stated, and as many workers use egg white lysozyme for adsorption studies (for reasons of economy), it is reasonable to suppose that Karageozian did so also. There is no evidence of high cross species retention of amino acid sequence, thus egg white lysozyme and human lysozyme could have very different amino acid sequences. The other flaw in the work is that a combination of other protein species, could on hydrolysis, yield a hydrolysate with an amino acid composition identical with that of lysozyme, though no lysozyme need have been present.

Sack et al. [101] used amino acid analysis and found some similarity between the lens hydrolysate and a lysozyme hydrolysate, but the fit was not as good as Karageozian [115] suggested, and Sack did not attempt to read such a definite conclusion into his results.

2.5.2 Electrophoresis

A full description of the theory and methodology of gel electrophoresis can be found in chapter 5. The very recent work done in this field is also discussed in chapter 5.

Wedler [99] has carried out extensive studies on which chemicals give the desired extraction, both singly and in combination, to yield samples suitable for gel electrophoresis. It was found that optimal extraction was observed with a combination of SDS, heat and the thiol reagent, dithiothreitol (DTT). The other solubulisation agents investigated included the chaotropic reagents urea, guanidine hydrochloride, potassium thiocyanate, potassium perchlorate hydroxylamine and EDTA; all proved less effective than SDS and DTT. The result of this study suggests that apolar interactions and disulphide bonds may be important in stabilising the deposit structure. Using this extraction procedure it was found [99] that a single lens contained 5-10µg/lens protein, 1.0-1.2µg/lens carbohydrate and 0.01-0.05µmole/lens phospholipid. Cholesterol and glucose were not at detectable levels.

Wedler [93] using a similar but slightly modified extraction procedure (i.e., 2% SDS, 5mM dithiothreitol and 1mM EDTA in a total volume of 1.5ml for 10min at 95°C), analysed the proteins extracted by SDS-PAGE and found that all proteins present in the lens deposit were found also in human tears. Table 2.4 compares the proteins that were detected on the lens compared to levels in tears. Wedler's [93] figures have been averaged to make comparison simpler.

TABLE 2.4 Protein levels in tears and deposits

	Protein				
	Lactoferrin	Albumin	35Kd	TSPA	Lysozyme
Tear Fluid	13	47	7	19	15
(%w/w of tear protein)					
Normal lenses					
µg protein	3.6	5.8	5.3	7.5	10.5
As a % of deposit	11.0	17.7	16.2	22.0	32.1
Heavily deposited lenses					
µg protein	5.7	25.0	7.3	24.4	23.4
As a % of deposit	6.6	29.1	8.5	28.4	27.3

Sack *et al.* [101] carried out extraction from various lens types, which were classified as nonionic and anionic depending on declared monomer composition. On the nonionic polymers, the lens-bound protein layer was invariably thin and largely insoluble. The extractable protein proved to be devoid of active lysozyme; amino acid analysis showed a variable composition. On the anionic pHEMA co-polymer lenses, the lens bound protein layer was much thicker and composed primarily of loosely bound protein, which on electrophoresis was shown to be lysozyme. The activity of the lysozyme proved to have been retained. Amino acid analysis partly supported the hypothesis that the layer was made up of lysozyme. The specificity of deposition was attributed to the ionic affinity of lysozyme, the retention of activity is due to the stable nature of lysozyme under extreme conditions. Sack [117] in further studies found that the white cloudy deposits found on high water content hydrogels was lysozyme, the published

electrophoretograms were far from equivocal in this. The study [117] also involved the use of amino acid analysis to confirm this result, thus a doubt is cast on its validity.

In a general study [116] the protein adsorption properties of the groups of polymers that make up the FDA system of contact lens material classification, it was found that high water content ionic lenses adsorbed the highest amount of protein, these lenses are of a high water content ionic material

Bilbault *et al.* [117] and Lin *et al.*, both reported lysozyme as the major component of the deposits found on worn contact lenses, Lin *et al.* [118] reported also that the other proteins detected did not increase after longer wear periods, whilst lysozyme did. Bilbaut *et al.* [119] found again that lysozyme was present, but also large numbers of proteins or degraded peptides.

Mucin, when studied after extraction, [93] was found only on heavily deposited lenses, and not on normally or lightly deposited lens. Alcian blue/periodic acid Schiff reagent was used to stain the gels to show mucins. This stain has a relatively low sensitivity and thus could explain the apparent absence of mucin on normal and lightly deposited lenses. The mucin proved to be a complicating factor in electrophoresis as it caused smearing and formed elemental silver mirrors on silver stained SDS-PAGE gels.

The study [93] makes an interesting comparison with Castillo's work using FTIR on mucin (see section 2.4.2.2), with which he found that mucus formed a complex three layered structure that quickly built up after the lens was placed in the eye. Castillo [110] and Holly [65] both suggested that mucin adsorption to the lens was advantageous in that

it aided wetability and biocompatibility. Wedler suggests that mucin only builds up on heavy deposits and is only then a problem. He found a high correlation between heavily irritating lenses and deposition of mucin components on top of the tear protein aggregate. Wedler even suggests that a mucin removal component should be included in lens cleaning fluid. This subject needs much more work to determine the role of mucus in deposit formation, then it can be decided whether its adsorption should be encouraged or avoided at all costs.

2.6 Other Contaminants

Some work has been carried out looking at environmental contaminants in contact lens deposits. Broich [120] found detectable levels of nicotine on contact lens surfaces of people who smoked: the nicotine could be derived from the exhaled smoke, but is more likely to come from the blood where nicotine levels in smokers are ten times higher than in non-smokers.

2.7 Microbiological Studies

The study of the microbiology of contact lenses is a large subject. Of the wide range of microorganisms that are found on soft contact lenses, most are believed to be derived from the patients handling of the lens and inappropriate maintenance regimens [121]. Soft contact lenses have been used successfully to sample microorganisms from the eye [122], the lens is then placed in blood agar and the microbes identified on the agar.

A new trend is the study of microbe attachment to different lens types. It was found in one of these studies [123] that the lens material and water content had little effect on the attachment of *Pseudomonas aeruginosa*, the causative agent of bacterial keratitis. The

study found that worn lenses bound less microbes than unworn lenses, this suggests that the adsorption of some tear-derived species is inhibiting the microbial binding.

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CHAPTER 3

Materials and methods

3.1 Materials used in contact lenses

The monomers used in contact lens fabrication are discussed in section 1.5 of this thesis.

3.2 General reagents and solvents

All chemicals were of reagent grade and were obtained from Sigma Chemicals Ltd. Pool, Dorset, except where stated otherwise.

3.3 Glassware and plasticware

Where possible disposable plasticware was used, for small sample handing stoppered Eppendorf tubes were used (Fisons Ltd.). Where glassware was used it was cleaned before use with a phosphate free detergent (Decon Labs. Ltd.), then rinsed in distilled water and dried in a oven before use.

3.4 Liquid dispensing

All small volumes 4µl to 1000µl were dispensed with an electronic air displacement pipette (Finnpipette Ltd.). Disposable plastic tips were used at all times and disposed of after a single use. Volumes between 1ml and 5ml were dispensed with a manual air displacement pipette (Finnpipette Ltd.).

3.5 Electrophoresis

Electrophoresis is the separation of a mixture of molecules according to their charge size and to a lesser degree shape. This process is facilitated by the application of a voltage across the system which induces the mobilities we witness. The process is

normally carried out on an inert gel support matrix which stabilises the separation and acts as a filter medium for the molecules.

A Hoefer SE600 vertical slab electrophoresis tank was used, supplied by Hoefer Scientific, California. A Hoefer PS 1500 power supply, which is capable of operating in constant current, constant voltage and constant power modes. The power supply also allows change-over of operating modes during a run, an automatic timer is also included. The gels were cast in the laboratory using the gel casting stand supplied with the SE600 tank. Gradient gels were formed using a Pharmacea gradient maker GM-1 . The gel is run immersed in the tank buffer to ensure no heating effects are seen. The temperature is further regulated by the use of a cooling coil system with tap water running through along with a magnetic stirring flea in the lower tank. The progress of the electrophoresis is followed by the addition of a coloured dye which is added to the upper tank, the dye has a higher mobility than any component in the mix, when it reaches the end of the plate the run is deemed to be complete. The current is switched off and the gels are removed from the plates by the use of a soft plastic wedge which opens the plates without causing the gel damage.

3.5.1 Electrophoresis Reagents

Sigma Electrophoresis grade reagents were used through the study. Stock solutions of acrylamide/bis acrylamide mixture were made up (see appendix 1) and stored at 4°C in darkness. The treatment buffer and gel buffers were all made up in advance and stored at 4°C. The solutions have a typical shelf life of 4 weeks, this should be bourne in mind when the solutions are made up. The tank buffer was made up at working strength, this was stored at room temperature. Appendix 1 details all the solutions

required for PAGE.

Acrylamide is a potent neuro-toxin and is cumulative in nature, all procedures involving these solutions or gels should be carried out in fume hood were possible. Disposable rubber gloves should be worn when the solutions are dispensed and the equipment that has been in contact with acrylamide is handled. Acrylamide is at its most dangerous when in a dry powdered form, thus it should be dissolved as soon as it is possible.

3.5.2 Electrophoresis methodology

The procedure for the preparation of polyacrylamide gels is discussed in detail in section 4.3.2.

The gels were run at the following currents

0.75mm gels	15mA per gel
1.0mm gels	25mA per gel

This gave a starting value of 40V per gel, the voltage is seen to go up during the run. It is worth recording the voltage at time periods during the run as any problems with the gel or buffers is quickly detected by anomalous voltages.

3.5.3 Protein Extraction for Electrophoresis

The worn contact lens was washed with distilled water from a wash bottle for a few seconds to remove residual storage solution. The lens was then placed in an Eppendorf tube with 1ml of 1% SDS solution, in some instances 1% 2-mercapto ethanol was included. The tube then had its lid punctured to stop pressure build up. The tubes were then placed upright in boiling water for 2 min and were periodically agitated. The

lens was then removed from the solution and retained for further examination. The solution was cooled rapidly on ice and stored at -20°C if not to be used immediately.

3.5.4 Sample preparation for Electrophoresis

The samples were prepared by mixing equal volumes of a treatment buffer of the composition shown below, with an equal volume of the protein solution under study in a plastic Eppendorf tube. This was mixed by vortex and the lid of the Eppendorf was punctured to allow pressure release. The tube was the placed in boiling water for 2 min with periodic gentle agitation. The solutions were then rapidly cooled on ice, if the samples were not for immediate use they were stored at -20°C until required.

3.5.5 Protein Standards

The protein standards used were purchased from Sigma Chemical at the highest purity available. As well as the use of single known standards, solutions of mixed standards were used on a day to day basis for general molecular weight determination. Two were commonly used termed, 6H and 7, the composition of the two is shown below.

Table 3.1 Molecular weight standards used in PAGE

SDS 6H Molecular weight markers (Sigma Chemicals product code SDS-6H)

Component	Molecular Weight (Daltons)
Carbonic anhydrase (Bovine)	29 000
Albumin (Egg)	45 000
Albumin (Bovine)	66 000
Phosphorylase (Rabbit)	97 000
β Galactosidase (*E.Coli*)	116 000

Myosin (Rabbit)   205 000

SDS 7 Molecular weight markers (Sigma Chemicals product code SDS-7)

Component	Molecular Weight (Daltons)
Albumin (Bovine)	66 000
Albumin (Egg)	45 000
Glyceraldehyde-3-phosphate dehydrogenase (Rabbit)	36 000
Carbonic anhydrase (Bovine)	29 000
Trypsinogen (Bovine)	24 000
Trypsin inhibitor (Soyabean)	20 100
α Lactalbumin (Bovine milk)	14 200

3.5.6 Staining procedures

The gels after the electrophoresis are stained according to one of three methods depending on the sensitivity required. The simplest staining method is the use of Coomassie blue in methanol and acetic acid, this single solution fixes the protein in the gel and also develops a colour where the proteins are present. A destain of aqueous acetic acid and methanol is used to reduce the background staining. The stain is cheap and quick but is not the most sensitive, this is the normal stain used in this study. A higher sensitivity is obtained by the use of colloidal Coomassie blue, this reagent is available in kit form only from Sigma Chemicals. It is a little more time consuming than the first method described, a separate fixation step is required. The fixer is a mixture of 5-sulphosalycilic acid and trichloroacetic acid. The gel is then washed and then the stain is added, after a period of time a destain of aqueous acetic acid is used, again to reduce the background.

The third method of staining is a multi-step silver staining procedure, the method used in this study was a kit supplied by Sigma Chemicals. The procedure is time consuming and was only used when high sensitivity was required.

3.5.7 Electroblotting

Electroblotting is the transfer of molecules from an electrophoresis gel onto another medium, normally nitrocellulose. The transfer is facilitated by means of an electric field, in much the same way as the original molecular mobility is obtained in the electrophoresis gel. The advantage of the proteins being attached to the nitrocellulose sheet is that further work can be now done on the sheet using relatively small volumes of reagent. Also the protein is concentrated somewhat by the process.

After an electrophoresis run the gels are removed from the glass plates. Gloves were worn throughout so that no protein was transferred from hands to gel. The gels were placed in contact with a sheet of nitrocellulose membrane (Sigma Chemicals), this was sandwiched between sheets of blotting paper (Whatman 3MM grade filter paper). This was placed into the plastic cassette supplied with the the electroblotting tank (Hoefer Scientific, Ltd.). The blotting buffer was added to the tank. The tank buffer was thermostated by the means of a tap water cooling coil. A significant amount of heat is developed due to the high current (~250mA), and the long blot times (~12 Hours).

The nitrocellulose sheet is then removed and can be used with biotin avidin reagents to show the position of any proteins present, or highly sensitive and selective antibody reagents can be used to locate specific proteins.

3.5.7.1 Biotin Avidin stain

This method makes use of the high affinity of biotin for avidin. The proteins bound to the membrane are reacted with NHS biotin. An avidin horseradish peroxidase(HRP) conjugate was then added. This binds the biotin, and the activity of the HRP is used to develop a colour reaction. All the proteins present on the membranes show up as strongly coloured bands. The method used is supplied by Bio-Rad as a kit (number 170-6512). Full details of the methodology of this stain is detailed in chapter 4 of this thesis.

3.5.7.2 Antibody stains

Highly sensitive antibodies were used to selectively detect proteins bound to the nitrocellulose membrane. A primary antibody to the protein of interest was purchased. A labeled secondary antibody which was antigenic to the primary antibody was then added. The label was alkaline phosphatase, the colour reaction developed by the enzyme allowed the detection of the protein of interest. Full details of the antibodies used and the general method of antibody staining of membranes is discussed in chapter 4.

3.6 Optical Microscopy

In this study a Leitz Dialux 20 microscope, which has the facility for bright field, darkfield phase contrast and fluorescence, was used, photographs were taken with a Wild MPS 15/11 photo system.

3.7 Scanning Electron Microscopy

The wavelength limitation of white light microscopy has lead to the development of scanning electron microscopy (SEM), this allows for much higher magnifications. The sample must be dehydrated before it can be mounted in the microscope. The sample is mounted in the microscope and a high vacuum is developed within the apparatus. A beam of electrons is directed by means of magnetic lenses at the sample, the reflected electrons are detected and an image is formed on a cathode ray tube.

A Cambridge Instruments Stereoscan microscope was used during this study, photographs were taken of a slow raster monitor using a SLR camera.

3.7.1 SEM sample preparation

The preparation of samples for SEM has two aims, to maintain the structures integrity and to remove all the water present. A number of methods of dehydration can be used, the simplest is dehydration in a vacuum oven at 40°C and reduced pressure. A commercial freeze drier can be used to remove all the water. The third method is to use a critical point drier, in this method the sample is first dehydrated in a concentration series of alcohol. This is then replaced by Freon (ICI Chemicals), the sample is then mounted in the critical point drier, and flooded with liquid CO_2, the pressure and temperature is then manipulated so that CO_2 is at its critical point. The CO_2 is then allowed to escape and the sample is left dry without a freezing/ non-freezing interface passing through the sample.

The sample is mounted by means of double sided sticky tape to an aluminium stub and firmly pressed down. The specimen is then gold or carbon coated in a sputter coater

depending on the analysis that is to follow. Many samples, if small and well attached to the stub, proved to SEM satisfactorily if sprayed with a fine coating of an aerosol antistatic spray.

3.7.2 EDXA

When the sample is mounted in the SEM the nature of the X-rays emitted may be analysed. The energy of the X-rays is characteristic of the elements present, within certain limitations this can be a useful facility. The EDXA was a LINK analysis system linked to a Cambridge Stereoscan microscope.

3.8 Confocal Microscopy

In confocal microscopy the positioning of a focusing aperture and the use of a monochromatic laser source leads to a very shallow depth of field. Thus by means of motorised focusing control and digital image storage a large set of images in the XY plane through a three dimensional object can be collected. This data can be manipulated to give an image as if taken as a section through the object of interest in the Z plane.

A Bio-Rad Lasersharp MRC-500 optical system mounted on a Nikon research grade microscope was used to collect the confocal images. The data was manipulated on a Compaq IBM compatible computer, the huge volume of data collected was stored on a Bernoulie box disk drive system. A video printer gave instant colour pictures, high resolution black and white images were taken on a photo monitor using a 35mm SLR camera. An output to a U-Matic video system was also used to store the images in a dynamic manner, and to make real time animation playback.

3.9 FTIR

A number of Fourier transform infrared spectroscopic techniques were used during this study, these are detailed below. The infrared spectrum gives us information about the compounds present. In the case of proteins, subtle changes in the spectrum can give information about the conformation and tertiary structure.

3.9.1 Vertical FTIR MIR

Multiple internal reflection accessories allow the collection of infrared (IR), spectra from the surface region of objects. The IR beam is directed along a crystal of high refractive index, it suffers internal reflection along the inside of the crystal. At each reflection point a small proportion of the beam exits the crystal and interacts with the sample which is in close contact with the crystal. This portion of the beam then re-enters the crystal and passes out into the spectrometer, it includes the IR information of the sample.

MIR infrared microscopy was carried out using a Perkin Elmer 25 reflection horizontal multiple internal reflection accessory. This was fitted in the beam path of a Perkin Elmer 1710 optical bench, the bench was controlled by a dedicated computer. Data storage and manipulation was carried out on a Perkin Elmer 3600 data station.

3.9.2 Horizontal ATR

Horizontal attenuated total reflection (HATR) spectroscopy works on the same principle as MIR spectroscopy. The major difference is the orientation of the crystal which is horizontal facilitating easy sample removal. The HATR accessory was used exclusively with the Nicolet 310 bench and Macintosh IIci microcomputer.

3.9.3 Photoacoustic Spectroscopy

In photoacoustic spectroscopy (PAS), an infrared spectrum is collected from a sample not by transmission or reflectance of an IR beam but by means of the acoustic signal generated when the IR beam interacts with the sample. The sample is placed in a sealed chamber purged with helium, the IR beam is directed onto it, a sensitive microphone is mounted in the chamber it picks up the acoustic signal caused by the heating effect of the IR beam. The spectrum obtained appears as an absorbance IR spectrum.

The PAS cell used in all these studies was a MTEC 200, which is mounted by means of a special kit into the sample chamber of a Nicolet 310M optical bench, this was controlled as before with an Apple Macintosh IIci computer. The cell has two cup sizes depending on the sample available.

3.9.4 FTIR Microscopy

FTIR microscopy allows the collection of infared spectra from very small samples. This is accomplished by means of beam condensers that act as glass lenses but are in fact precision reflecting mirrors, this is necessary as glass is itself a strong IR absorber. The microscope allows viewing and photography of the sample, when an area of interest is observed by means of movable apertures an IR spectrum is collected.

Initial work on FTIR microscopy was carried out on a Bio-Rad 150 and 300 microscope and an 8 series bench. Some of the very early work utilised a Spectra Tech Research grade microscope and a Nicolet data station.

FTIR microscopy was carried out primarily on a Spectra Tech analytical grade

microscope linked to a Nicolet 310M optical bench. The optical bench was controlled by an Apple Macintosh IIci microcomputer operating under system 6.0.6 *via* a dedicated Nicolet controller card fitted to its NuBus slot. The microscope was fitted with two X15 all reflecting Cassegrain objectives.

3.9.4.1 Sample preparation for FTIR Microscopy

To obtain the infrared spectrum of a contact lens it was found to be necessary to section the lens. The lens under study was mounted in water on a 1320 Leitz freezing microtome (Leitz Ltd.), the lens was held in a vertical position and the water was frozen by means of compressed CO_2. The microtome blade was also cooled with compressed CO_2. The optimum section thickness was found to be 20μm, the sections were collected off the microtome blade and placed in water. A drop of this water was placed on a clean glass microscope slide, this was dried in a vacuum oven. The sections can be seen on the glass slide, a scalpel blade is used to remove a number of lens sections, these are mounted on a CaF window for viewing under the FTIR microscope.

CHAPTER 4

Electrophoretic techniques

4.1 Electrophoresis:- General

In electrophoretic techniques compounds are separated by means of an electric field acting upon the charges on the molecule. The separated molecules can then be detected by a number of methods depending on the system that has separated them.

Two main methods were employed, isotachophoresis (ITP) and poly acrylamide gel electrophoresis (PAGE). ITP is a little used but potentially powerful method, as the equipment was available in the laboratory it was decide to invest some time into investigating its possibilities. PAGE is very widely used in biological research, it was not available in the laboratory but the capital outlay was small compared to other alternative protein separation systems such as fast protein liquid chromatography (FPLC).

4.2 Isotachophoresis (ITP):-Theory

Electrophoretic techniques have been widely used by other workers: most have used PAGE although, an alternative but little used technique is isotachophoresis. As the name implies, all ions under observation are given the same velocity. In isotachophoresis the separation is affected not within a solid medium, but in a small bore capillary. ITP is carried out in a discontinuous electrolyte system made up of the three components shown in table 4.1

Table 4.1 Electrolyte components necessary in ITP

1   a leading electrolyte, which must contain only one ion species, the leading ion L^-, having the same sign as the sample ions to be separated and an effective mobility higher than that of any of the sample ions.

2   a terminating electrolyte, which contains one ion species, the terminating ion, T^-, having the same sign as the sample ions to be separated and an effective mobility lower than that of any of the sample ions.

3   the sample to be separated.

(1,2 and 3 all must have a common counter-ion.)

An electric field is placed across the above system so that the leading ion migrates to the electrode that is placed on the same side of the sample as the leading electrolyte. When the system reaches equilibrium all the ions move with the same speed but individually, they separate into a number of consecutive zones in immediate contact with each other, and arrange in order of effective mobility. A number of reviews as to the theoretical considerations behind isotachophoresis and the selection of a suitable leading and terminating electrolytes have been published [124-127].

The first commercial ITP device was the LKB Tachophor 2127 produced in 1974 by LKB Produkter AB of Bromma Sweden, it was this apparatus that was used throughout this study.

The LKB Tachophor 2127 used has two detection systems to monitor the separation of ions during the run; a thermal detector which responds to the rise in temperature caused

as the sample passes the detection zone, and a U.V. monitor which is next to the thermal detector, see figure 4.1. The U.V. detector has a number of filters so that various wavelengths can be monitored. Later versions of the Tachophor 2127 were equipped with a conductivity detector rather than the thermal detector, this was alleged to improve general detection of bands. Unfortunately, the model used was not fitted with the new detector arrangement.

Figure 4.1 Diagram of layout of the capillary isotachophoresis apparatus
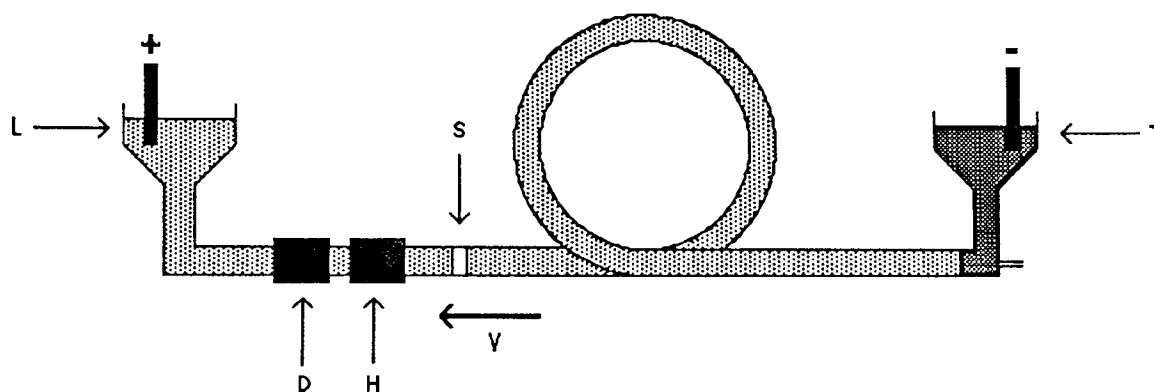
Key

T=terminating electrolyte   H=u.v. detector

L=Leading electrolyte   v=velocity of sample

D=thermal detector   s=sample band

The u.v detector has very good sensitivity and detects very narrow bands, whilst the thermal detector although universal has poor sensitivity, a slow response time and needs zones to be relatively broad. As the u.v. detector has the facility for different filters to be used, this gives the detector a degree of selectivity of detection , saying that the choice of filters is limited. The tachophor capillary tube is bathed in a silicone oil thermostated by means of Peltier elements so that separations can take place at any temperature between 3-29°C. The thermostating of the capillary leads to more stable

separating zones and better reproducibility due to reduction in the thermal disturbance of the zones.

The tachophor with a simple electrolyte system gives good results with small molecules where the effective mobilities are suitably different so that detection of the individual bands is possible. When separating complex mixtures of large macromolecules such as proteins is attempted, it is found that the protein bands have similar U.V. absorbances and their net mobilities are very similar, thus the bands are narrow and detection between them is impossible. The addition of non-U.V. absorbing ions with net electrophoretic mobilities in the same range as those of the proteins under study creates a spacer mobility gradient [128]. The compounds used are small synthetic polypeptides termed ampholites, these were primarily developed for use in iso-electric focusing, but are suitable for this role. Isotachophoresis has had some success in the separation of protein mixtures by the use of spacer gradients [129-131], but the use has been limited to non routine analyses thus far.

The addition of an agent to increase solution viscosity (e.g.hydroxy propyl methyl cellulose) is common and helps stop convection currents from disturbing the boundaries.

Gravity has been found to have an effect on boundary stability within the capillary. Because of this, isotachophoresis was carried out in space as part of the NASA Skylab mission, with improved separation performance.

4.2.1 Methodology

Samples were centrifuged to remove any particulate matter and $5\mu l$ to $10\mu l$ of the sample was introduced through the sample port, at a point between the leading and terminating electrolyte. The voltage was then applied at a fixed current of $50\mu A$, with the 610mm column this gives a separation time of 60-80min. The thermal and U.V. detectors were then monitored by means of a paper chart recorder, or a computing integrator which has the advantage of being capable of integration below peaks for quantification. The electrolyte system used was basically that of Gower and Woledge [130] with some minor modifications. The leading electrolyte was 5mM HCl with 18mM β-alanine as the counter ion, 0.5% carboxy methyl cellulose (CMC) was included to increase the viscosity of the electrolyte. The terminating electrolyte was 5mM n-caproic acid. The sample was injected by means of a Hamilton syringe at a point between the two electrolytes at the outset of the run.

4.2.2 Results

The tachophor was run using a number of simple electrolyte systems for familiarization with its operation. Various amino acids and organic acids were separated successfully. Complex protein mixtures were attempted to be separated with no spacer mobility gradient present, FCS gave two broad peaks with very little definition so it was obvious that a spacer gradient was necessary to separate these proteins. Ampholites, a mixture of synthetic polyamino acids, can act as this spacer gradient; these mixtures are designed for use with iso-electric focusing systems. It was found that these compounds are very expensive and only sold in large quantities (50ml of a 40% solution) and as only ~5μl of a 0.2% solution was needed for each run, the expense

involved was great. For optimum performance in ITP we need spacer gradients of narrow range so that a small portion of the ITP run is 'magnified' as much as possible. In ideal conditions we would purchase a large number of narrow range ampholites and then select from these. Because of the high costs it was found necessary to buy two wide range ampholites, these in theory should give some separation of the protein bands but not in its optimum form. The second problem of using a spacer gradient is that the signal from the non-discriminatory detector, the thermal detector, becomes meaningless. The thermal detector picks up the molecules that make up the gradient, and unless at high concentration, sample bands do not appear. The ampholites are synthesised so as to have as low a U.V. absorption as possible, thus the output from this detector is monitored during these runs. The ampholites used were supplied by Bio-Rad and were of the range 6-8pH to 8-10pH and were sold under the trade name Bio-Lytes. In protein separation runs, a spacer gradient was used that comprised 4mg glycine, 4mg valine, 3.6mg ß alanine and 0.167ml Bio-lyte 6-8 and 0.333 ml Bio-Lyte 8/10, all made up to 1ml in distilled water. 8µl of the spacer solution was used with a sample of 2µl.

Figure 4.2 Shows a typical separation achieved with the LKB tachophor 2127. The sample was foetal calf serum (1/10th dilution), a good model sample as it contains similar molecules to those found in tears. As can be seen from the trace, the separation yields a large number of species, many of which appear as spikes. This is due to the inefficiency of the spacer gradient used, it is better to use narrow ranged ampholites to magnify a small section of the run. The upper line shows the output from the thermal detector, the use of this detector when a spacer gradient is used is very limited as it detects the passage of the spacer molecules.

Figure 4.2 Isotachophoretogram of FCS (see text for sample details and run conditions)

Thermal Signal

UV Signal

Knauer Berlin 37

Knauer Berlin 37

◄— Time
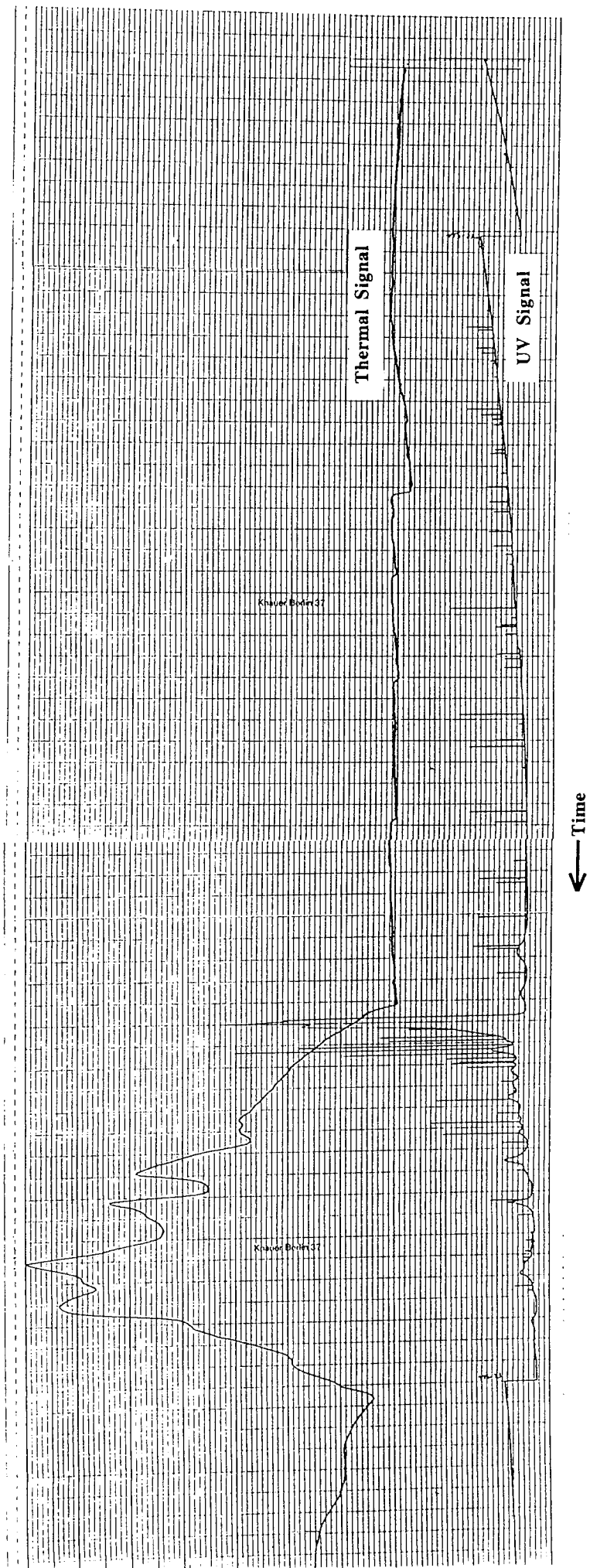
4.2.3 Problems with ITP

ITP at first appearance offers advantages over conventional electrophoretic techniques, is requires a small sample size, it has high sensitivity, it gives 'on-line' results which can be recorded electronically for further manipulation. Sample preparation is minimal, but treating of sample with SDS is not possible as all proteins would be given the same mobility and would stack as a single band. As extraction techniques that involve this reagent are common in contact lens work, its use is severely limited to the proteins that can be removed by other means. As discussed earlier it is likely that the easily removed fraction of the adsorbed protein would be the portion of the least interest. ITP is not a fast technique, a typical run can take up to hours. This is for a single sample, whilst a run of 2-3 hours in polyacryalmide gel electrophoresis can separate 60 samples.

The single most important problem with ITP is that of interpretation of results. If we take two scenarios, in the first the ITP run contains four components A,B,C and D, if the conditions have been optimised, a good separation is obtained as depicted schematically in figure 4.3. In the second instance a sample is run that contains all the compounds found in the first run but in addition contains X and Z. If X and Z have intermediate mobilities to A,B,C and D they will cause the shifting of these bands as shown in figure 4.4.

Figure 4.3 Schematic diagram of an isotachophoretogram of a four component mixture.
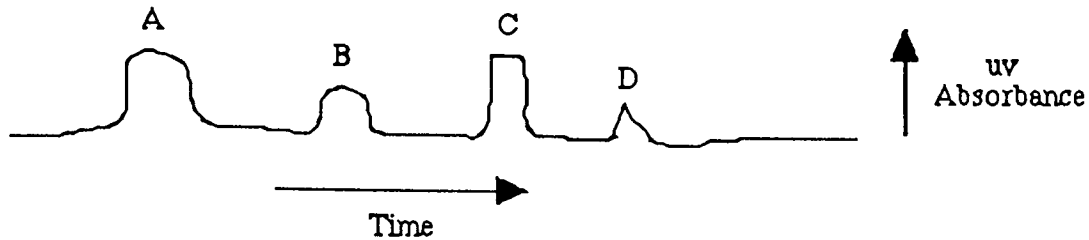
Figure 4.4 Schematic diagram of an isotachophoretogram of a six component mixture.
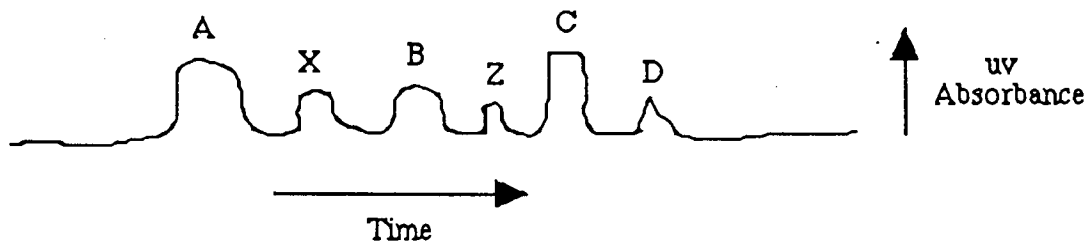
If the bands were not labeled in figure 4.4 it would be impossible do determine by position alone the identity of each compound. Thus unlike PAGE the position of the bands is not a function of the compound that causes it alone, it is dependant on all the other components in the sample being separated. A second similar problem to the above is that, given that the width of a band is a function of its concentration, if relative concentrations differ widely the other peaks in a separation will be compacted. The result of these effects is that even under optimum conditions real life sample of proteins from contact lenses would be impossible to interpret due to the highly variable nature of the level of compounds present.

The paper trace is the only record of an isotachophoretic run, the compounds separated cannot be further analysed, by means of antibody or lectin probes. The LKB tachophor does have an accessory called the Tachofrac. This allows the collection of the separated compounds by dripping then onto a cellulose acetate strip, this can then be analysed further. The addition of this device makes the operation of this piece of apparatus even more difficult. LKB has now merged with Pharmacea, one result of this merger was the discontinuation of the LKB Tachopor 2127 and its accessories. The Tachofrac was no longer available at the time of this investigation. Given the shortcomings of the apparatus and the difficulty of interpretation of results, it was decided to target polyacrylamide gel electrophoresis as the possible solution to our analysis problem. The information obtained from ITP is unique and complimentary to that obtained from gel electrophoresis. Isotachophoresis is a useful technique and should be borne in mind but it is hard to envisage it as a routine laboratory task, the time and care required in its use is excessive. Its position in soft contact lens spoilation analysis would to be run in tandem with gel electrophoresis on particularly interesting or intractable samples.

4.3 Gel Electrophoresis

The technique of gel electrophoresis is widely used in biochemical research, it involves the separation of molecules as a function of their charge, size, and to a lesser degree their shape. A gel acts as a support and often as a selective retardation medium, the molecules of interest are placed on the gel and a voltage is placed across the gel. The molecules that posses a charge will respond to this applied voltage by moving. The rate of movement is proportional to the charge the molecule possesses, the more charges on the molecule, the faster it will travel. This condition assumes that the medium that the molecule passes through exhibits no physical resistance to the passage of the molecule.

The gels commonly used, agarose, starch and polyacrylamide, do cause retardation. The gel can be likened to a vast three dimensional network, in polyacrylamide gels, the gel density can be altered easily and thus the retardation properties altered at will. The resistance a molecule encounters will be a function of primarily its size. An added complication is that of a molecules shape; globular proteins behave as we would expect but the mobilities of long thin molecules such as DNA are heavily influenced by shape. This phenomenon is seen in undenatured electrophoresis, when proteins have shapes far removed from globular.

An important modification of gel electrophoresis is that the treatment of proteins with the detergent sodium dodecylsulphate which masks the native charge that proteins posses. All proteins are given a charge proportional to their size thus their mobilities can be assumed to be a function of size alone. This treatment also denatures the protein and considerations of molecular shape become less important.

4.3.1 Gel electrophoresis and its use to investigate contact lens spoilation

Gel electrophoresis has been used by a number of workers to investigate the proteins that deposit onto soft contact lenses. Other workers [101,116], have used agarose as the gel support medium, this has some intrinsic problems. Agarose is a natural polymer and thus suffers from batch to bath variability. The physical properties of agarose gels is poor thus particularly with thin lenses the handling of gels becomes a problem. In poly acrylamide gel electrophoresis (PAGE), we can alter the gels retardation properties by altering the acrylamide and bis acrylamide concentrations. In agarose gels this alteration is not available. Some workers [101,116], have used equipment designed for

routine pathological lab work. This equipment is highly reliable when it is used on the samples it is intended for. The gels come ready-cast and the electrolyte buffers are supplied in ready-to-dissolve sachets. The system is not an ideal vehicle for research work where it is necessary to alter a large number of variables to obtain a good separation. With a good research grade PAGE system the operator can alter the gel type and density. Buffer type and concentration along with voltage current and power used to effect the separation can all be optimised. Work done on tear protein analysis [31,43,50], a project with interest and parallels to our study, have used a far more extensive range of electrophoretic systems, including isoelectric focusing, immuno electrophoresis and gradient gel electrophoresis.

It was decided that it was a worthwhile exercise to attempt PAGE on our samples, as the quality of the separations that have been published leave a lot to be desired. As well as improving the separation technique, the methods of detection on the gels after the run has been limited. Many workers have used little more than Coomassie blue stain. Other staining methods are available these include silver staining and the use of colloidal Coomassie blue stain. The gels can also be blotted onto nitrocellulose membranes and these used in further investigation using probes such as antibodies, lectins and biotin-linked stains. It could be said that this technique has been used in this field but has not been optimised.

4.3.2 PAGE Method development

The most suitable electrophoretic technique was believed to be disk electrophoresis, in this system a lower concentration acrylamide gel is placed on top of the normal

separating gel. The overlaying gel acts to concentrate the sample into a tight band. The band passes into the separating gel, the resulting bands in the separating gel are consequently sharp. The improvement over non-disc gels is significant and well worth the extra time and complexity involved.

The system for analysis that follows was the result of many experiments. A number of parameters were varied to achieve the best separation. The most important of these was altering the acrylamide concentration in the gels and the cross-linker (bis acrylamide), density. Alternative extraction techniques were attempted, the problem being with this that if a number of regimes are to be tested a number of lenses are required. No guarantee can be given that the lenses all contain the same amount of adsorbed material. Direct comparisons are thus not possible between extraction techniques, the relative merits can at best only be estimated. A further problem is that the commonly used Lowry total protein estimation is badly interfered with by many of the chaotropic agents commonly used. The agents tried included guanidine, CHAPS, (3-(3-cholamidopropyl)-dimethylammonio)-1- propanesulphonate), urea , cetylpyridinium chloride and Triton X-100. The detergents were chosen as they represent a spectrum of those available and can be classified as in table 4.2.

Table 4.2 Detergent classifications

Detergent	Type
SDS	anionic detergent
cetylpyridinium chloride	cationic detergent
Triton X-100	non ionic detergent
CHAPS	zwitterionic detergent

It was found that the presence of some of these agents can affect the electrophoretic separation, many can be removed by dialysis but this would entail loss of sample. Thus 1% SDS became the extraction of choice because of its ease of use, relative effectiveness and compatibility with the analysis to follow.

Other parameters such as gel thickness were altered to see the effect on sample capacity and sensitivity of detection. 1mm gels are adequate for most separations, but if the highest sensitivity is required the gel thickness can be reduced to 0.75mm. The 0.75 gels are very fragile, especially if low acrylamide gels are in use, thus they should only be used when absolutely necessary. The 0.75mm gels can be easily overloaded thus sample sizes of the standards should be reduced accordingly. The optimised conditions developed are detailed in the following sections.

4.3.3 Preparation of gels

The gels were cast in the apparatus supplied with the Hoefer SE600 gel tank. Two glass plates are sandwiched together with two spacer strips in-between, using the clamps supplied. This sandwich is then fixed onto a casting stand that has a rubber bottom, by means of eccentric cams the gel sandwich is pressed into the rubber base to seal the lower side. A small amount of grease is used to seal the corners of the gel assembly. The casting stand is then placed on a level bench and is ready for the gel solution to be added.

The gels were made up from the solutions detailed in table 4.3, these are numbered 1 to 8 overleaf; these codes will be used in later descriptions. Full details as to composition,

storage and shelf life of these reagents is detailed in appendix 1.

Table 4.3 Solutions required for PAGE

1   Stock monomer solution (30% w/v total acrylamide, 2.7% of which is bis

     acrylamide)

2   Running gel buffer (1.5M tris, pH 8.8)

3   Stacking gel buffer (0.5M tris, pH 6.8)

4   10% SDS solution (10% w/v sodium dodecyl sulphate)

5   Initiator solution (10% ammonium persulphate)

6   Running gel overlay (0.375M tris, pH 8.8, 0.1% SDS)

7   Tank buffer (0.025M tris, pH 8.3, 0.192 M glycine, 0.1% SDS)

8   Treatment buffer (0.125 M tris, pH 6.8, 4% SDS, 20% glycerol, 10% 2-

     mercaptoethanol)

The composition of the separating gel is as shown in table 4.4; this gives a 10%

acrylamide separating gel and 4% acrylamide stacking gel. The separating gel solution

was made up from the ingredients in table 4.4, except for the SDS, the TEMED and the

ammonium persulphate. The SDS should not be added as the detergent tends to foam

during degassing. This solution was degassed by slowly bubbling oxygen-free

nitrogen through it for 5 min. To this solution the ammonium persulphate and the

TEMED was added, which act as initiator and catalyst respectively. This solution was

taken up in a 50ml luer tip syringe making sure that no bubbles were introduced as

oxygen is a potent inhibitor of polymerisation and its introduction must be avoided at all

costs. The syringe was then slowly discharged between the glass plates of the casting

stand, the solution was run down the sides of the plates so as not to trap any air

bubbles. The plates were filled to the appropriate level (about 5cm from the top of the plate). The gel was then carefully overlayed with 2ml of distilled water which was added 1ml at each end of the gel. The water overlay ensures that no turned down meniscus will be produced by the surface of the monomer solution as it polymerises.

The gel was then left for 1 hour to allow polymerisation, if the stacking gel was not to be added immediately the special running gel overlay buffer replaced the water used during the polymerisation.

Table 4.4 Composition of the separating and stacking gel

	Separating gel	Stacking gel
Acrylamide solution 1	20.00ml	2.66 ml
Buffer 2	15.00ml	---------
Buffer 3	---------	5.00 ml
H_2O	24.10 ml	12.20 ml
Ammonium persulphate solution 5	300μl	100μl
10% SDS Solution 4	600μl	200μl
TEMED	20μl	10μl

The N, N, N^1, N^1-tetramethylethylenediamine (TEMED), is a liquid and was used as supplied.

The stacking gel was prepared as in table 4.4, this is degassed as with the separating gel. The initiator and catalyst was added, the water that overlays the separating gel was then removed and the top of the gel was washed twice with a small volume of the

stacking gel solution. The stacking gel solution was added down the glass sandwich until about 1 cm from the top of the plates. The comb that will form the sample wells was then slid between the glass plates. This is done with great care because if any air bubbles are introduced, the gel in that region will not polymerise to completion and irreproducible results will be obtained.

The gels are ready for use in 1 hour or more. If the gels are to be stored overnight some tank buffer (solution 7) should be added after the combs have been removed and a strip of Nescofilm (or clingfilm) should be placed along the top of the gel plates. The casting stand can be placed in a refrigerator at 4°C which protects the gels, particularly in hot weather conditions.

The gels can be stored for up to 2 weeks by overlaying with tank buffer. The gels are then removed from their clamps and the casting stand and wrapped in cling film. To stop drying out, distilled water-soaked blotting paper is wrapped around the gels and this is again wrapped in clingfilm. The whole is stored at 4°C, and before use the gel tops should be washed with fresh tank buffer.

4.3.4 Sample preparation and loading the gels

The samples were prepared in stoppered Eppendorf tubes, these were stored on ice until use. A glass Hamilton syringe was used to careful introduce the sample into the wells formed at the top of the gel during casting. If 1mm gels were being used 25µl of the sample was used, 0.75mm gels required samples no bigger than 15µl. As only one syringe was available because of cost, it was necessary to flush the syringe at least 6 times with distilled water between each injection. The samples were loaded in as little

time as possible as the sample, if left in the sample wells with no applied electric field for long periods of time, will diffuse sideways. This results in cross-contamination between sample lanes. The end lanes were whenever possible not used or were used for duplicates of samples run in other lanes on the gel. The reasoning behind this is that the samples in these wells do not undergo electrophoresis in a straight line due to edge effects in the gel. Gels take on the appearance of a smile if the centre of the gel heats up, expands, and then allows the molecules less resistance. The centre lanes thus run further than the edges, this makes comparisons of distance travelled difficult. If tap water is used through the cooling coil and the buffer is agitated by a magnetic flea this should not occur at normal operating currents. If the gel is run in these thermostated conditions the problem of gel 'smiling', should not be seen.

4.3.5 Running the gels

The power supply was operated in constant current mode, with a current per gel of 25mA for a 1mm gel and 15mA for 0.75mm gels; this gives an approximate run time of 3 hours. The course of the run is monitored by the addition of 2-3 drops of phenol red. The phenol red has a higher mobility than any of the compounds being separated, thus a red line is seen to pass down the gel in front of the sample. The red band should be allowed to reach the bottom or run off the gel before the current is switched off. The gel cassette should be split and the gel carefully removed using gloves. The fixing, staining or electroblotting should be started as soon as possible, in a matter of minutes at most. If this period is prolonged the protein bands will blur as the proteins diffuse from their position.

4.3.6 Results of PAGE

The gels were run as described in section 3.5.2 of this thesis. The lenses used in the early studies were worn lenses returned by opticians to the manufacturers with various problems, often not relating to spoilation ,e.g. torn or damaged lenses. Often no clinical data as to length of wear or cleaning regime was available. As the technique was still in its development phase this was not a problem. These early experiments allowed the many conditions of electrophoresis to be optimised for the separation and detection of proteins off a single soft contact lens. The conditions that it is the extract off a single lens is important if inter lens and inter patient variation is to been studied.

The 10% rather than 15% acrylamide gels gave the better separation of the major proteins found in tears and thus by implication on contact lenses. The buffers are as described in section 4.3.2 and appendix 1. The typical run time is three hours but some gel to gel variability was encountered. The gels should thus be monitored to ensure that no proteins are allowed to exit at the bottom of the gel. A useful method to ensure that no low molecular weight proteins exit the end of the gel is to use gradient gels. A gradient gel maker (Multiphor II 2117-910 manufactured by Pharmacia), was purchased. The gradient maker has two chambers linked by a small channel, the output from the gradient maker is from one of the chambers. Figure 4.5 shows the gradient maker in schematic form. A 5-20% acrylamide gel was cast using this apparatus. The outflow from the gradient maker was not augmented by a peristaltic pump, gravity was used to feed the solution to the gel. This resulted in a very slow overall filling of the casting stand causing two problems. The amount of initiator and catalyst was reduced as the solutions were beginning to polymerise in the casting stand. The second problem was that the solution in the mixing chamber was having time to flow

backwards into the low density solution. The upshot of this was that the gradients formed were far from linear.

Figure 4.5 Schematic diagram of a gradient maker and gel casting stand
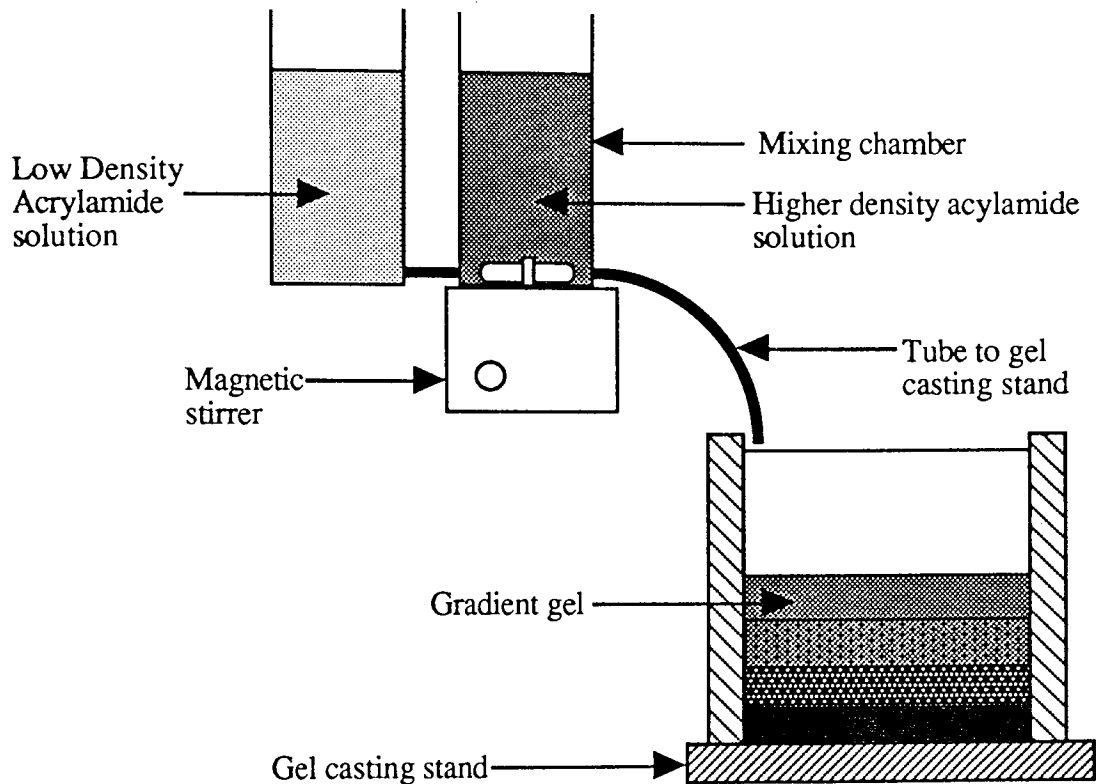
The fronts of the proteins on these gels were not straight, this was believed to be due to the method by which the solutions were introduced between the glass plates of the casting stand. The solutions were run down a tube that ended at the top of the plates, the solution was allowed to run down the plates from this point. This must have allowed some mixing of the gradient which would alter its composition. The effect would not be linear and the further away from the point of entry of the solution the less the effect. To get around this problem a long hypodermic needle was used, this allowed the solution to be introduced at a point just above the level of the gel solution. The problem with this was that the back pressure caused by the needle was high thus

the flow rate was decreased and thus the earlier problems were exacerbated. The obvious solution to these problems is to not rely on gravity and to use a peristaltic pump. Many such pumps are available but most lab peristaltic pumps are far from being pulse-less. The pump needs to be pulse-less if we are to obtain a good gradient. Pulseless pumps are available, but their cost is large, and as it was only a speculative exercise this route was considered inappropriate. The use of gradient gels thus proved to be time consuming and unsuccessful and their use was discontinued. This was unfortunate as the separations across a large molecular weight range obtainable with such gels can be very good.

The linear gel used with a stacking gel gave excellent results when standard tear proteins were separated. Lysozyme lactoferrin and albumin were found to give good, cleanly resolved bands. The move from these standard proteins to the separation of proteins derived from a single contact lens was not a simple as first thought. The initial problem was one of detection of the bands after electrophoresis.

4.3.7 Staining techniques

A number of different staining techniques are available and these were investigated to ascertain the one with the optimum sensitivity.

4.3.7.1 PAGE blue stain

The PAGE blue stain used has only a limited sensitivity and is used only when sample size is large. The stain is rapid and simple it only requires a single solution of water:methanol:glacial acetic acid in the ratio 5 : 5 : 1. containing 5%(w/v) page blue.

The gels are placed in this solution for 1 hour and gently agitated. Full details of this stain and its use can be found in appendix A 1.3 The gels are destained in a solution of aqueous methanol 20% (v/v) and 5%(v/v) glacial acetic acid. The stain when used on sample derived from a single contact lens showed up only two or three separate bands. The sensitivity was much less than required. A note about the nomenclature of this product should be made at this point, the same compound is know by a number of different names; if supplied by ICI it is known as Coomassie blue (which has become the generic name), Page blue if supplied by BDH and brilliant blue G by Sigma, often the names are used interchangeably.

4.3.7.2 Silver staining

The only alternative at the outset of the project was silver staining. The published methods suffered variable sensitivity and problems with the formation of elemental sliver mirrors in the gel. Sigma Chemical supply a silver staining kit which claims to avoid these problems. The kit, as supplied is still very time consuming to use. This may not seem an important drawback but if we are to develop a method for general analysis of a large number of samples we must ensure that the throughput of samples can be as high as possible. It is likely that the system will be used by an individual so any savings in labour would be advantageous.

The silver staining protocol was based on that of Heukeshoven and Dernick [132] as modified in the Sigma Chemical Company, silver stain kit AG-25. The information as to the use of this staining method is detailed in appendix A 1.4. The gels were fixed in 30% ethanol 10% glacial acetic acid. The solution was changed 3 times, 300ml was used each time and the solution was changed every 20 min. The gels were then washed

three times with 300ml of distilled water, each wash lasted 10 min. The details of the

following solution are not given as they were used as supplied with the kit. No

information as to the exact composition of the solutions is given by Sigma Chemicals

for commercial reasons. The procedure is described to give some idea as to the time

and complexity involved.

The gels were brought to equilibrium with a silver solution for 30 min. The gels were

then rapidly washed for 10-20 seconds. 150ml of the developer solution was added to

the gels and then discarded after 5-8 min. The remaining 150ml was then added, the

gel should be monitored closely so as to give the darkest bands possible with minimal

background staining. When the gel is sufficiently dark the developer solution is poured

off and a stop solution added for 5 min. The gels are the washed three times with

300ml of distilled water. The gels are then treated with a reducer solution for 10-30

seconds and are then rinsed under running tap water. It should be remembered that the

gels are fragile and that if the water hits the gels directly they are likely to be broken into

many pieces. The gels can be recycled through the procedure for reportedly higher

sensitivity; recycling was in fact found to increase background staining with little

increase in sensitivity. The total procedure takes in the region of 3 hours and 30 min,

as no step takes more than 30 min it is difficult to carry out whilst other procedures are

in progress.

The results obtained with this stain can be seen in figure 4.6, this shows a silver stained

gel. The samples used were extracts from lenses worn as part of a short clinical trial of

2 weeks. These were run in the lanes marked 4, 5, 7, 8, 11, 12 and 13, the other lanes

run are standards. The figure does not do the gel justice, it is difficult to

photographically reproduce these gel types because of the subtle colour densities between the background and the protein bands. Many more bands were visible to the naked eye than can be seen in figure 4.6. The lenses analysed could be described as in the hardest class to analyse because of the very short wear time The lenses had also been stored at 4°C from soon after removal from the patients eye minimising the risk of microbial contamination. An electroblot stained with biotin avidin is shown in figure 4.7, this is complimentary to the gel shown in figure 4.6. The procedure and success of blotting and subsequent staining will be discussed later in this chapter. The figure is placed here for ease of comparison. The two results are derived from gels that were loaded and run under identical conditions.

A schematic diagram of a typical gel after staining is shown in figure 4.8, this is shown for purposes of clarification. The diagram shows the sample wells in the stacking gel, the stacking gel is often removed from the separating gel. It is very fragile and is prone to physical damage during handling. It is a simple task to scrape it off the top of the tougher separating gel. The sample bands are shown as a set of parallel lines, this is a good result. If heating had taken place during the run or the gel had been badly cast the gel would show signs of curvature over a number of lanes. The Rf value for each individual band is worked out by dividing the distance travelled by the distance travelled by a standard band or the dye front. In the case of band z, the Rf would be given by y/x. It should be noted that comparisons between gels should not be made, the preferred method of expressing the results is to construct a standard curve from the molecular weight markers run at the same time. Two sets of markers were used during this study, termed 6H and 7. The composition of 6H and 7 is given is section 3.5.4 and in table 3.1. A standard curve can then be constructed from this information, and the molecular weight of unknowns can be estimated.

Figure 4.6 Polyacylamide electrophoresis gel stained with silver stain.
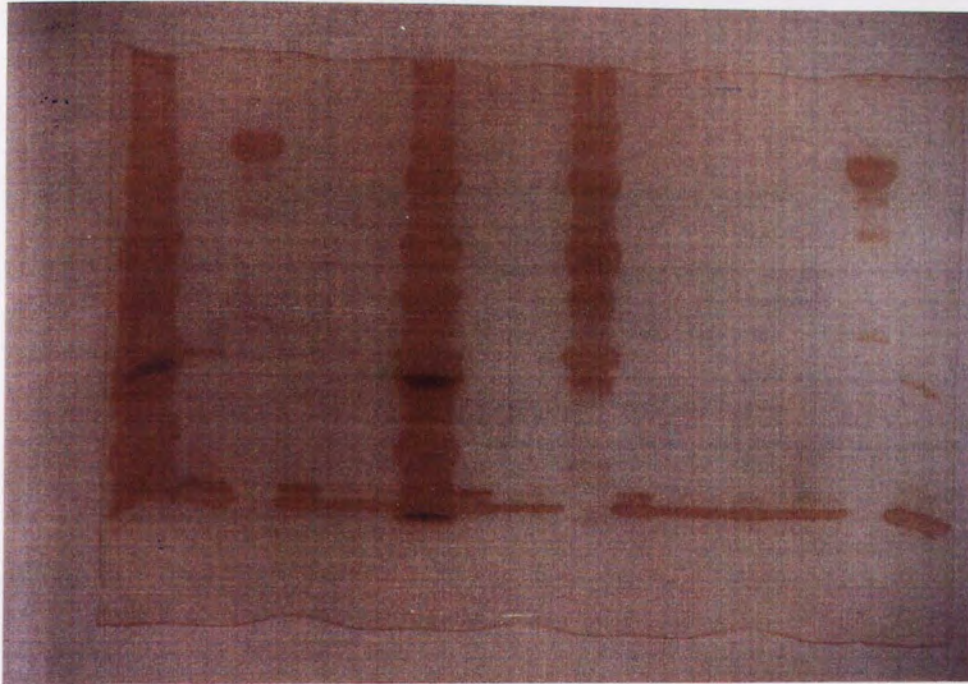
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 4.7 Biotin avidin stained electroblot of same samples as in figure 4.6
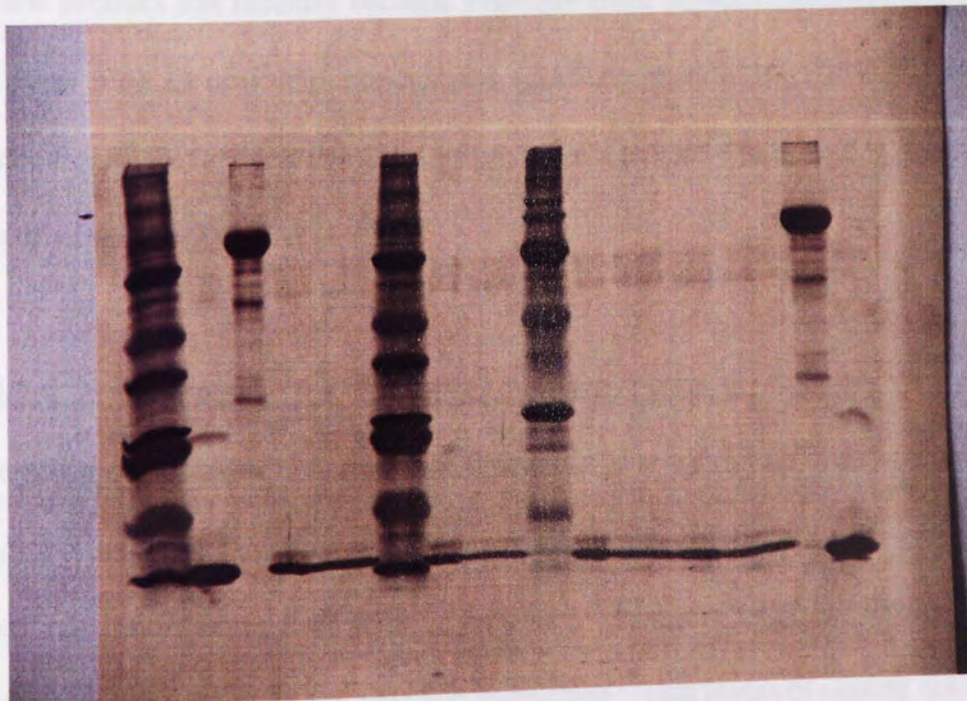
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 4.8 Schematic diagram of a stained electrophoresis gel
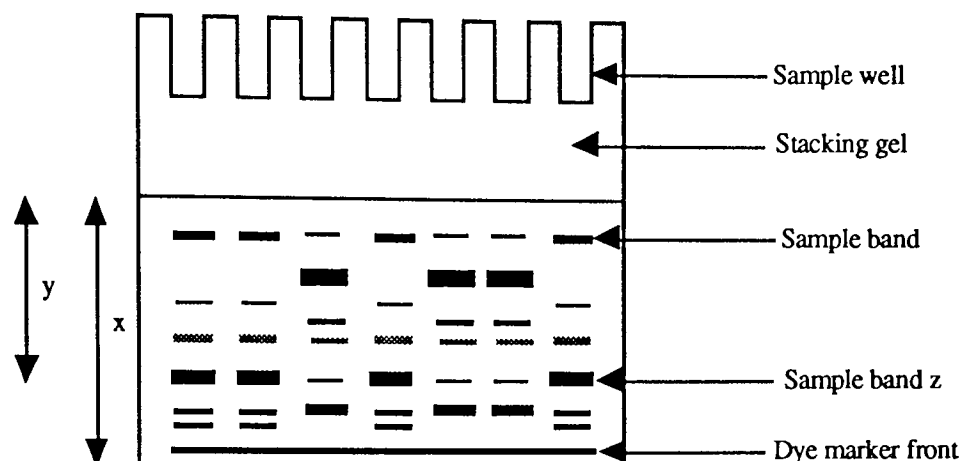
4.3.7.3 Colloidal brilliant blue G

A new product has recently become available from Sigma Chemicals and is sold as Brilliant blue G colloidal concentrate (product number B-2025). The reported sensitivity of this stain is ten times greater than coomassie blue, and is comparable to that of silver staining kits.

4.3.7.4 Methodology for Colloidal brilliant blue G staining

The gels are fixed with 12% (w/v) trichloroacetic acid and 3.5% (w/v) 5-sulphosalicylic acid, this crosslinks the protein and stops diffusion out of the gel. The gels should be fixed with 200ml of the fixer solution for 1 hour with gentle agitation. The stain should be prepared according to the supplied instruction. This involves adding 800ml to the Brilliant blue G colloidal concentrate. This should be mixed by inversion only. Immediately before use 4 parts of the stain solution should be mixed by vortex for 30

seconds with 1 part methanol. The solution formed is stable for 4 hours only. Typically 100ml of this solution was made up. The gel is placed in the stain solution for 1 to 2 hours with gentle agitation. The gels were destained with 10% acetic acid in 25% methanol for 10 to 30 seconds. The gel should be washed then with 25% aqueous methanol, this should be discarded and the gels should be destained in 25% aqueous methanol for up to 24 hours. The gels can be stored for a few months in distilled water in the dark. The gels can be scanned at 600nm if required.

4.3.7.5 Results of Colloidal brilliant blue G

Examples of gels stained in this manner are shown in figures 4.9 onwards. The comparison with the silver stained gel was good. The colloidal brilliant blue gel shows many of the bands seen in silver stained gels that were loaded under identical conditions. This method has the advantage that a single gel can be stained in approximately 2 hours, also the number of steps and correspondingly the attention required during this process is much less than the silver staining system. This stain system should become the method of choice to study the routine protein extracts from contact lenses. If particularly difficult samples are encountered then it is necessary to resort to the silver stain system. This stain was used in the enzyme digestion studies that follow. The gels run of samples removed from soft contact lenses contain much more information than will be extracted at this point. Molecular weight and concentration estimates could be made by scanning densitometry. This was not done because of the nature of these samples used during this stage of the method development. The samples were not on the whole well documented, samples with full information on wear and care regimes are rare and valuable. Such samples could not be squandered until complete confidence was obtained in a system of analysis. The next

section details some of the problems associated with PAGE and has some bearing on

the issue about what information should be extracted from gels and it relative value

4.3.7.6 Problems with PAGE

PAGE has been much used by other workers in this field, it is undoubtedly valuable. It

is relatively cheap to buy the necessary equipment, the running costs are also low. The

time involved in analysis is relatively high but by means of productivity aids such as

running a system of club sandwich gels, up to 60 samples can be run at any one time.

The primary problem is one of incomplete removal of the biological film from the lens,

this is not a problem with the technique *per se*, but rather with the logic behind using it

in the first place. If this drawback is put aside other difficulties linked with the

technique come to light. How are the bands detected to be quantified? The normal

method of quantification is to use scanning densitometry. An LKB densitometer was

used for this purpose, the apparatus gives accurate Rf values and also peak height and

peak area determination for individual bands. At first sight the trace given by such an

apparatus looks impressive. The inclusion of a group of concentration standards

should allow us to construct a standard curve, and thus absolute concentration values

can be obtained. The drawback in this is that Beer Lamberts law is only seen to be

linear over a relatively short concentration range in the gel. The second and bigger

problem is that different proteins exhibit different staining efficiencies. It is necessary

to construct standard curves for each individual band. This is not a possibility for

proteins that are unknowns or are not commercially available. The third problem

affects even proteins that are known and are commercially available. The staining

efficiency is variable between gels thus assumptions as to optical density and

concentration between gels are not necessarily true. This problem can be surmounted by the inclusion on each gel of a number of standards to ensure reproducibility.

The gels then become cumbersome, we have only 15 lanes per gel, the lane at each end is not used because of edge effects. It is necessary to run at least three molecular weight standards. We are left with 10 bands, if a range of five standards are run we can quantify five samples. It should be remembered that we have then only quantified one band in each of these five samples. If this process was to be repeated for each component, the gels required would be huge. For many bands no standards are available thus they cannot be assayed by this means. Many other workers have made assumption as to staining efficiency etc, these results have been accepted by the wider scientific community and have passed into the general consensus on contact lens work. For the purposes of quantification the use of scanning densitometry is of limited use in it-self and can add little to what can be judged by the human eye.

As for the problem of removal of the biological material from the contact lens, electrophoresis may hold the key. It could prove possible to electrophorese the deposited material off the contact lens. A preliminary trial of this method was attempted, 4 worn contact lenses were cut into strips of 0.5cm by 2.0cm and 0.5cm by 0.5cm. These strips were placed into the sample wells of the the gel slab. An extract from these lenses was also made in the normal way. The gel was stained with conventional page blue stain. The results are shown in figure 4.9. The XPR lens coded B showed the highest amount of removable material. The lane of both the lens fragments show a long tail. The two X-10 (A and D), lenses show some material has passed into the gel The XPR lens C has no evidence of adsorbed material passing into

the gel, as the lenses were not of known wear history it is possible that the lens had only been worn for a short period of time. Lens B shows the possibility of this type of transfer, the blur in the tracks it due to the protein not all leaving the lens material at the same time. As the protein did not leave the lens and enter the gel as a discrete band the separated bands would have the same properties. This drawback could be overcome by introducing a time element into the protein removal step. If the lens is placed in the sample well and the current turned on protein should pass into the stacking gel. If the power was then switched off and the lens fragment removed, the stacking gel should, if large enough, concentrate the protein to a tight band before entering the separating gel. It would be necessary to experiment with different time periods and size of stacking gel. Another alternative would be to make the lens fragment be in better physical contact with the separating gel. This could be achieved by casting the gel around the fragments, how this could be integrated with the removal of the lens fragment would of course be a problem.

Figure 4.9 Electrophoresis of lens fragments

7 Molecular weight markers

X-10 lens (A) 0.5cm x 2.0cm

X-10 lens (A) 0.5cm x 0.5cm

X-10 lens (A) extract *

XPR lens (B) 0.5cm x 2.0cm

XPR lens (B) 0.5cm x 0.5cm

XPR lens (B) extract *

XPR lens (C) 0.5cm x 2.0cm

XPR lens (C) 0.5cm x 0.5cm

XPR lens (C) extract *

6H Molecular weight markers

7 Molecular weight markers

X-10 lens (D) 0.5cm x 2.0cm

X-10 lens (D) 0.5cm x 0.5cm

X-10 lens (D) extract *
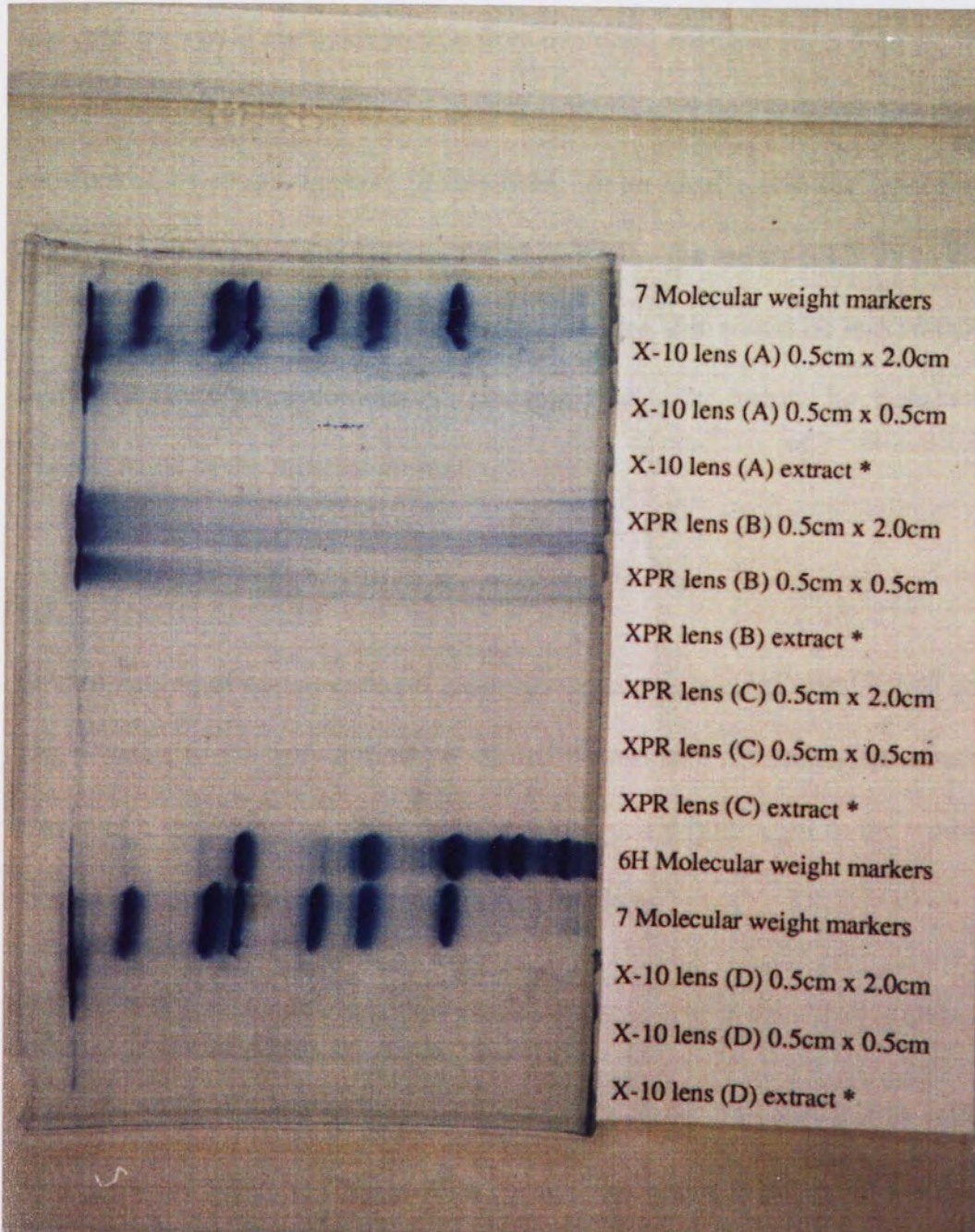
4.3.8 General note of sample collection and storage

Many lenses that find their way to laboratories for analysis have been stored in non-sterile conditions at room temperature. It is likely that if lenses of this type were analysed we would see more protein from microbial contamination than we would see from patient deposition. This is a general problem with lens analysis, one that is often overlooked by many workers in this field. In an ideal world the lenses would be removed from the patients eye and stored at -20°C. The lenses could as an alternative be sterilised in an autoclave or by chemical means, this would be less suitable as the treatment could compromise the analysis that is to follow by denaturation or fragmentation of the proteins present.

4.3.9 Electroblotting

Electroblotting of compounds off electrophoresis gels is a well established technique. The proteins in the acrylamide gel are transferred to a nitrocellulose membrane by means of a small current. This transfer of molecules from a gel to the membrane by means of an electric field is termed Western blotting.

4.3.9.1 Methodology of electroblotting

A Hoefer Transphor TE 52 tank was used in this study. The acrylamide gel is placed on 3 pieces of Whatman 3MM filter paper. The whole is placed in a photographic development tank filled with the transfer buffer. The transfer buffer was 20mM tris, 150mM glycine and 20% v/v methanol. The nitrocellulose membrane was placed on top of the gel and 3 more pieces of Whatman 3MM paper are placed on top of the membrane. It is important that no air bubbles are introduced during this process. The

whole assembly is placed in the cassette supplied with the TE52 tank. This cassette is lowered into the transfer tank and the transfer buffer is topped-up so as to ensure that the membrane is not exposed to the air. A current of 150mA achieves transfer overnight whilst 250mA is required for a 6 hour transfer. The cooling coil must be used as a considerable amount of heat is developed during the transfer. Gloves should be used throughout the process otherwise the operators fingers will be the most notable feature of the blots made. More details of this process is given in appendix A 1.6

The proteins migrate out of the gel, and because of the nature of the membrane they bind tightly to its surface. The membrane used was 0.45μm pore size Nitrocellulose supplied by Sigma Chemicals. The proteins bound to the membrane, are now available for probing with highly sensitive reagents. Many reagents are too large to enter the gel matrix, such as antibodies, these reagents are also very expensive and as much less of them is required on a membrane rather than a gel the advantages are twofold.

The blot can be treated with a number of reagents to detect various components. The most important of these are antibodies that are specific for components that are believed to be present. A second method is a biotin/avidin stain that detects all protein bound to the membrane. This is normally used to determine the position of the potential bands for antibody analysis. The membranes are often cut in two with one half being stained with biotin/avidin whilst the other half is probed with antibodies. The biotin/avidin blot could be important to us because of its reportedly high sensitivity (nanogram level), for tiny amounts of protein. The blot procedure is time consuming but may have a role with difficult samples.

4.3.10 Biotin avidin blot

A biotin avidin blot kit supplied by Bio-Rad (Cat. No. 170-6512) was used. The reagents can be purchased individually but for general ease and higher reproducibility this commercial kit was utilised. The blot is dependant on the high affinity that biotin has for avidin ($K_D \approx 10^{-15}M$)[133]. The membrane is reacted with NHS biotin, all the proteins bind to this, the excess is removed and the avidin is reacted with it. The avidin used has been conjugated to horseradish peroxidase (HRP). The HRP acts upon a colourless reagent to give a coloured product. This is how the proteins present are visualised.

4.3.10.1 Methodology for biotin avidin blot

The following solutions were made up, (the code in bold is used in later instructions).

Borate-Tween solution (**BT**) (0.05M sodium borate 10-hydrate, 0.2% Tween 20 pH 9.3)

Tris buffered saline (**TBS**) (0.02M tris, 0.05M NaCl) pH to 7.5 with HCl

Tris Tween solution (**TTBS**) (0.02M tris, 0.05M NaCl, 0.2% Tween)

Avidin horseradish peroxidase conjugate solution (**A-HRP**) 0.001% (v/v) in TTBS

Phosphate buffered saline (**PBS**) (0.01M phosphate buffer) adjust to pH 7.2 with HCl

NHS Biotin solution (75mM N-hydrosuccinimide biotinate in dimethyl formamide)

HRP colour development solution (**HRP-C**) 4 chloro-1-naphthol 0.003% (w/v) in methanol (ice cold) then just before use add 0.003% (v/v) H_2O_2 (30%) and 5 parts TBS for every 1 part methanol.

Full details of the above solutions, their formulation and storage can be found in

appendix A 1.7.

Procedure for biotin avidin blot is described below, the codes for the appropriate solutions are as above.

1 The membrane is washed in 100ml **BT** for 10 min this is repeated 3 times

2 Discard the wash solution and replace with 100ml **BT**

3 Agitate the membrane in the solution and add 200µl of the **NHS biotin**

4 Incubate for 15 min

5 Wash the membrane in 100ml of **BT** for 5 min repeat

6 Wash the membrane in 100ml of **TTBS** for 5 min repeat

7 Prepare the **A-HRP** solution as described above

8 Incubate the membrane in the above solution for 1 hour

9 Wash the membrane in 100ml of **TTBS** for 5 min repeat

10 Wash the membrane in 100ml of **TBS** for 5 min repeat

11 Prepare the **HRP-C** solution as described above

12 Incubate the membrane in the **HRP-C** solution until the bands become visible, normally about 15-30 min

13 Stop the development when the bands are visible and the background staining is not excessive by washing repeatedly in distilled water.

The membranes can be dried between two sheets of blotting paper, the membrane can be stored for a long period of time without fading if it is kept in the dark.

The result of a blot of a separation of contact lens extracts is shown in figure 4.7. The high sensitivity of this method can be seen, many more bands are visible than were

seen even with the silver stain (figure 4.6). This is the most sensitive method to visualise the proteins separated on a gel.

4.3.10.2 Problems with the Biotin / avidin blot.

The biotin avidin blot has an important role when used in conjunction with antibodies, it allows the total proteins present in a run to be visualised and then to see the bands that react to the antibodies chosen. Its role as a general protein detection method must be limited, the cost and time involved in this procedure makes it unsuitable for screening large numbers of samples. It would be necessary to prepare the samples, cast and run the gels, electroblot overnight and then go through the lengthy procedure described above. It may have a role in specialised analysis when it is important to detect all the components in a mixture.

Blotting followed by biotin avidin detection has another major drawback, it has been found that different proteins have different electroblotting properties. Some proteins do not exit the separating gel easily and thus are under represented on the blot. Other proteins do not bind well to the nitrocellulose membrane (particularly true for small proteins), these pass through the membrane giving only a small contribution to the final blot. The nitrocellulose membranes have only a small finite binding capacity for proteins, thus we have only a narrow window over which we get linearity between concentration in the gel and colour density on the membrane. The reasons given above brought us to the conclusion that densitometry of blots was a non starter, as all the problems associated with gels are multiplied when we come to work on membranes.

4.3.11 Antibody detection on blots

The power of this technique is huge. A blot can be treated with an antibody to any component we are interested in detecting. The first antibody used is termed the primary antibody, it is raised in an animal, e.g. a sheep. The blot is then treated with a secondary antibody, this is an antibody to our first antibody, in our case donkey anti sheep immunoglobulin would be used. The second antibody is conjugated to a visualising agent such as a fluorescent compound, a radiolabel or an enzyme. The enzymatic tag is considered advantageous as the enzyme develops the colour by acting on many colourless molecules to produce a coloured compound. The use of this two antibody system also enhances sensitivity, a single primary antibody can be bound to more than one secondary antibody.

The antibodies used were supplied by The Binding Site Ltd (Birmingham, England). The antibodies purchased are detailed below.

Sheep anti human prealbumin (immunocytochemistry grade)

Sheep anti human lysozyme (immunocytochemistry grade)

Sheep anti human lactoferrin (immunocytochemistry grade)

Sheep anti human Human serum albumin (immunocytochemistry grade)

Anti sheep IgG (H+L)(donkey host) labeled with Alkaline phosphatase conjugate

The antibodies used were all polyclonal, the secondary antibody had been tested out against the primaries by the manufacturer.

4.3.11.1 Methodology for antibody blots

The solutions required for this procedure are detailed below.

1 **TBS** (20mM tris, 150mM sodium chloride)

2 **TTBS** (20mM tris,150mM sodium chloride, 0.1% (v/v) Tween 20)

3 Blocking buffer **(B)**(50mM tris, 500mM sodium chloride, 0.5% Tween 20)

4 Ponceau red stain **(P)**(0.2% Ponceau red in 100mM sodium acetate pH 5)

5 **NBT** (Nitro blue tetrazolium 50mg/ml in methanol)

6 **BCIP** (5-bromo 4-chloro 3-indolyl phosphate 50mg/ml in dimethyl formamide)

7 Stop solution (20mM tris, 5mM EDTA pH 8.0).

The nitrocellulose membrane is prestained with Ponceau red, a non specific protein stain. This has low sensitivity but major protein bands will show up, this facilitates the cutting of the gel into separate lanes for different antibodies.

The procedure for antibody staining used was as follows.

1 Remove the nitrocellulose sheet from the sandwich, NB do not allow to dry out during these procedures.

2 Soak membrane for 5min in TBS at room temperature with gentle agitation.

3 Stain with Ponceau red for 5-10min or until some bands become visible.

4 Wash extensively in distilled water, the bands should become clearer.

5 The membrane can be cut into the necessary pieces for separate analysis.

6 Block the unused binding sites on the membrane with 4% w/v milk powder for 15-30 min or overnight at 4°C. If lactoferrin is being analysed use 1% (w/v) bovine serum albumin (BSA) as the block protein.

7 Incubate with primary antibody in TBST(1:4000), for 2 hours at room temperature.

8   Wash with TBST three times for 10min each

9   Incubate with secondary antibody in TBST(1:7500) for 30min at room temperature.

10 Wash with TBST six times for 10min each.

11 Blot on dry filter paper

12 Make up colour development substrate solution, to make 5ml use 33µl NBT solution, 5ml AP buffer, 16.5µl BCIP substrate. This solution is stable for 1 hour put should be protected from light.

13 Incubate membrane with colour development solution until bands appear.

14 Stop the reaction with stop solution S

This was the optimised method developed for the analysis of extracts. The primary antibody can be used at higher concentrations but 1:4000 gave adequate results without unnecessary wastage of an expensive reagent.

4.3.11.2 Results of antibody work

Some of the results of these investigations are shown in figure 4.10 and 4.11. The strips of nitrocellulose in figure 4.10 are labeled 1 to 4, lane 1 and 2 are biotin avidin total protein stained lanes, whilst 3 and 4 have been reacted with the anti lactoferrin antibody. The lactoferrin can be seen as a mark in the lane, it has been necessary to enhance the band so that it would reproduce photographically. In figure 4.11 we see 5 lanes, lane 1, 2 and 3 have been treated with the biotin avidin total protein detection system, lanes 4 and 5 have been treated with anti lysozyme antibodies, the lysozyme shows clearly as a coloured band, no enhancement was necessary for lysozyme.

The other antibodies used did not detect their respective proteins, it is possible that the

proteins were present but were badly denatured making them antigenically unrecognisable to the antibody.

4.3.11.3 Conclusions on antibody work

The use of antibodies in our investigations leads to the unambiguous identification of the protein bands in an electrophoretogram, if the protein is one commercially available. In the case of tear specific pre albumin this is not the case, it would be necessary to prepare mg quantities of this protein if the antibody was to be raised in a host animal. It is a far from impossible task, it could prove a worthwhile task if this detection was felt necessary. At the present it is possible to identify two of the adsorbed proteins removed from soft contact lenses by SDS.

Figure 4.10 Anti Lactoferrin antibody blots of contact lens extracts.
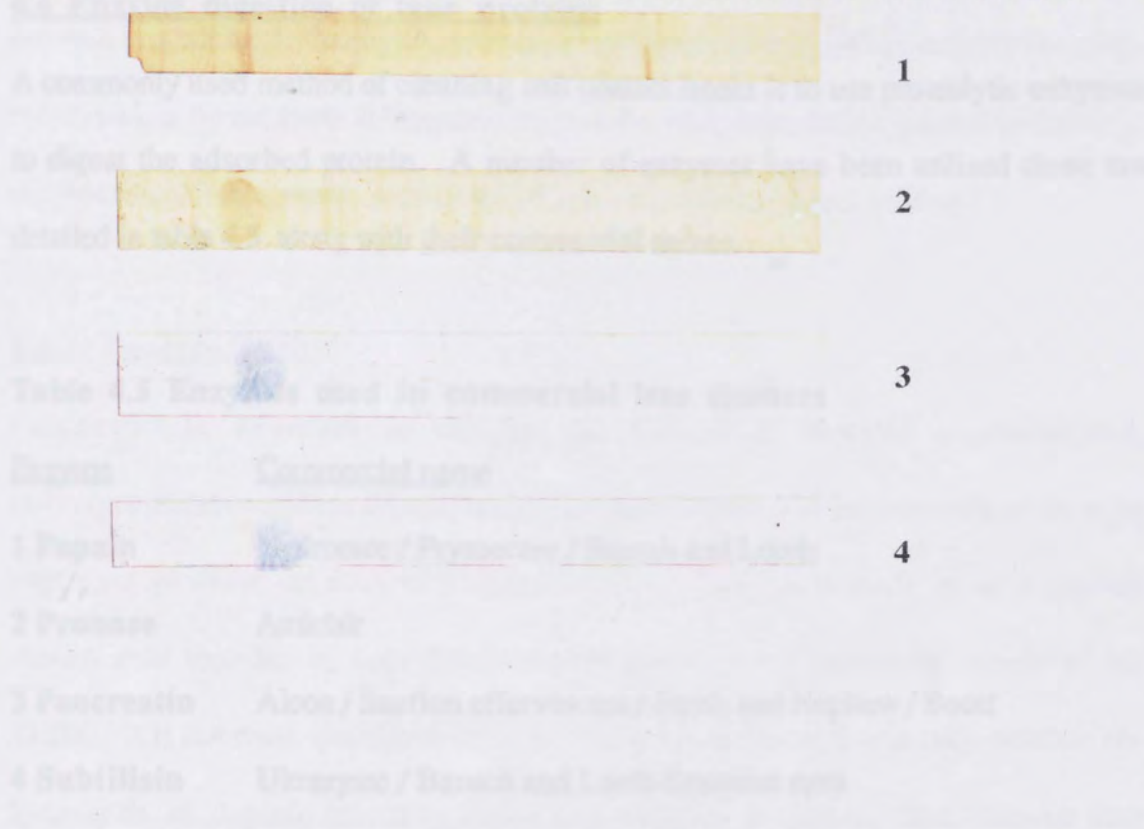
Figure 4.11 Anti Lysozyme antibody blots of contact lens extracts.

-131-
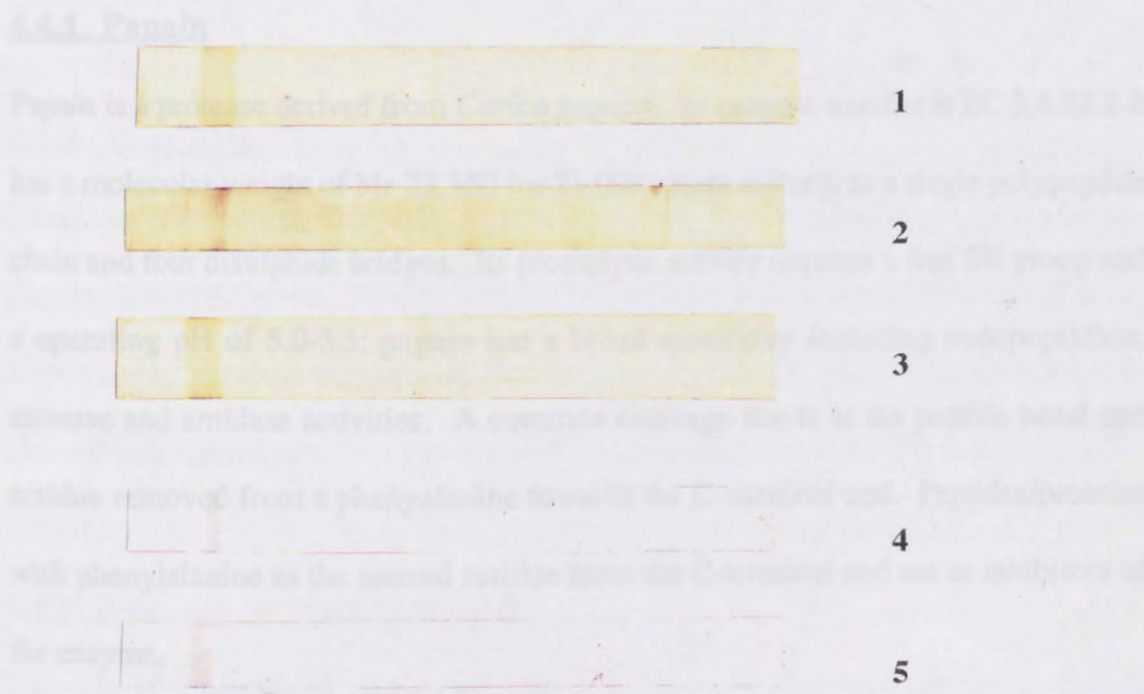
4.4 Enzyme digestion of tear proteins

A commonly used method of cleaning soft contact lenses is to use proteolytic enzymes to digest the adsorbed protein. A number of enzymes have been utilised these are detailed in table 4.5 along with their commercial names.

Table 4.5 Enzymes used in commercial lens cleaners

Enzyme	Commercial name
1 Papain	Hydrocare / Prymecare / Bausch and Lomb
2 Pronase	Amiclair
3 Pancreatin	Alcon / Sauflon effervescent / Smith and Nephew / Boots
4 Subtilisin	Ultrazyme / Bausch and Lomb-Sensitive eyes

The above enzyme types have the following origins, compositions and properties.

4.4.1 Papain

Papain is a protease derived from *Carica papaya*. Its enzyme number is EC 3.4.22.2 it has a molecular weight of Mr 23,350 (or 21,000 values differ), in a single polypeptide chain and four disulphide bridges. Its proteolytic activity requires a free SH group and a operating pH of 5.0-5.5; papain has a broad specificity including endopeptidase, esterase and amidase activities. A common cleavage site is at the peptide bond one residue removed from a phenyalanine towards the C-terminal end. Peptides/proteins with phenylalanine as the second residue from the C-terminal end act as inhibitors of the enzyme.

4.4.2 Pronase

Pronase is a cocktail of enzymes including a protease from *Bacillus subtilis* (possibly subtilisin), a lipase from *Rhizopus arrhizus* var. *delomar*, and a second proteolytic enzyme from *Streptomyces griseus* which it is claimed will digest mucins.

4.4.3 Pancreatin

Pancreatin is expected to contain the following; trypsin, chymotrypsin, carboxypeptidase amylase deoxyribonuclease and a lipase. Of these trypsin is the most important protease, its enzyme number is EC 3.4.21.4 .It is made up of 3 peptide chains, held together by four disulphide bridges to give a molecular weight of Mr 23,300. It is the most specific of the digestive endopeptidases it will only catalyse the hydrolysis of peptide bonds at lysine and arginine residues. The enzyme also undergoes autolysis, at pH 9.0 and 30°C, activity is totally lost in 24 hours, but it has been reported that Ca^{2+} will protect from self digestion. The reported optimum pH is in the range 7.8-8.5

4.4.4 Subtilisin

Subtilisin is a protease from *Bacillus subtilis* or *B. licheniformsis* both of which are alkaline serine proteases. The enzyme is very thermolabile with a optimal pH of 8-11. The Alergan product that utilises this enzyme also has N-acetylcysteine which is claimed to be mucolytic.

4.4.5 Why use enzyme cleaners?

The role of these agents in the build up or removal of adsorbed protein is open to debate. We are adding a protein to our contact lens, it is thus possible that the enzymes

themselves attach to the lens surface. As these are alien proteins to the eye they are potentially more harmful than adsorbed tear proteins, the eye could launch an immunological attack. The second problem is that these proteins are potent proteases thus if the adsorbed enzyme maintains activity we will digest the tear proteins in solution. The implication of this is that the protective role of the tear proteins may be compromised.

A very fundamental question is, "Do we want to digest the proteins adsorbed on a contact lens?" It is possible that the partial digestion makes the fragments that remain more prone to interact with further tear proteins.

As the electrophoresis equipment was available during the development of a suitable method to analyse contact lens extracts, it was decided to study the digestion product of these enzymes.

4.4.6 Methodology to investigate enzyme action

The enzyme under study was made up as per the instructions of the manufacturer this normally involved the dissolution of the tableted enzyme preparation in 10-15ml of sterile saline. A standard solution of the protein to be digested was made up at a concentration of 1mg/ml. 50μl of the enzyme solution was mixed with 50μl. The solutions were mixed by vortex. The digestions were allowed to proceed at room temperature. The digestion was stopped by the addition of an equal volume (100μl) of the SDS PAGE treatment buffer detailed in the previous section. The solutions were immediately heated to 100°C for 2min. The resulting solutions were cooled on ice until electrophoresed. The gels were run as described for the contact lens extract analysis.

25μl of the digestion solution was placed in each well, samples of the enzyme under study and of the undigested protein were also run along with molecular weight markers (6H and 7 as detailed in section 3.5.4). The resulting gels were stained with colloidal brilliant blue G stain and are presented in figures 4.12 to 4.20.

4.4.7 Results of enzyme digestions

The gels of the enzyme digests are shown in figures 4.12 to 4.20. The first three gels show the effect of the various enzymes on human albumin. Human albumin was chose as it is a protein found in tears (admittedly in small amount), and it is a typical globular protein with no enzymatic function itself. It was believed that is would make an ideal model substrate. The enzymes used are designated by their commercial name, table 4.5 shows the enzyme used where known, the enzymes used were those that were available in the laboratory at the time of the study. The time period used was much shorter than that suggested by the manufacturers, this was to ensure that complete digestion had not taken place. The shorter incubation time makes an effort to mimic the reduced availability of the protein substrate to the enzyme when on and in the lens matrix. Human albumin was found to be digested most effectively to small fragments by Sauflon enzyme cleaner (see figure 4.13), the enzyme cleaner itself is the only large protein visible even after short periods of time. Ultrazyme was relatively successful (see figure 4.12), with little of the undigested protein remaining. The third enzyme Clenzyme was not very effective (see figure 4.14), it caused some fragmentation of the albumin but no further digestion took place even after 10 min.

It was decided to look at the effect of these enzymes on a typical tear protein, lysozyme was chosen as it is often, as shown in Chapter 2, implicated in the spoilation process.

The Clenzyme once again proved to be very unsuccessful (see figure 4.17), with little evidence of any digestion. Sauflon was not effective against lysozyme (see figure 4.16), this is in direct contrast to that seen with human albumin. Bauch and Lomb's Sensitive eyes subtilisin-based cleaner showed some variable digestions as a function of time (see figure 4.18), this could be due to ineffective mixing of the solutions prior to incubation. The undoubted winner against lysozyme was Ultrazyme, this showed the best digestion of this protein (see figure 4.15).

A second tear protein was tried with two enzymes, Clenzyme and Bauch and Lomb's Sensitive eye preparation. The clenzyme once again proved very ineffective whilst the B&L product digested the lactoferrin effectively.

This short study has shown the variability in the effectiveness of enzyme cleaners. It was interesting to note the variability from protein to protein. The temptation for manufacturers is to use cocktails of enzymes, but if the enzymes are derived from widely different sources it is likely that they will digest one another rather than the adsorbed protein.

Before we look for the best digestive enzyme available we should ask if we want a lot of half digested proteins and peptides around on and in the lens matrix? Until we know more about these fragments and their properties it is hard to formulate an answer.

Figure 4.12 Electrophoresis gel showing the effect of Ultrazyme on human albumin
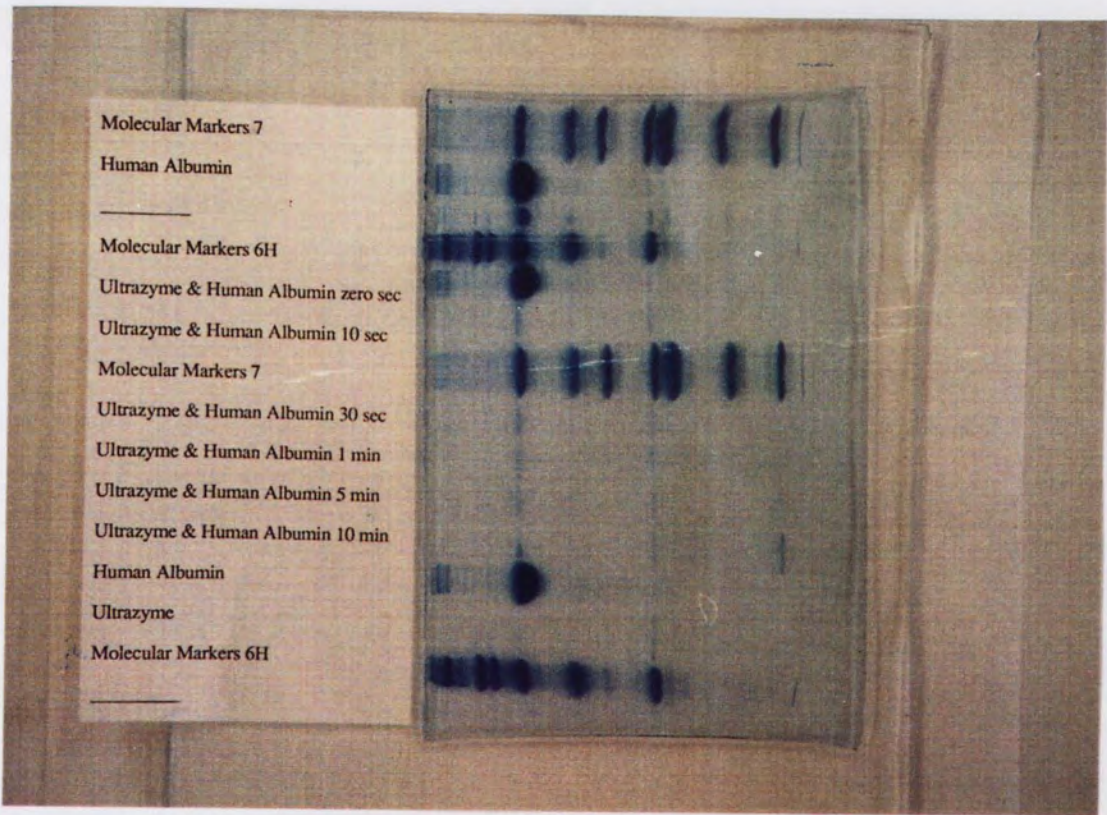
Figure 4.13 Electrophoresis gel showing the effect of Sauflon on human albumin
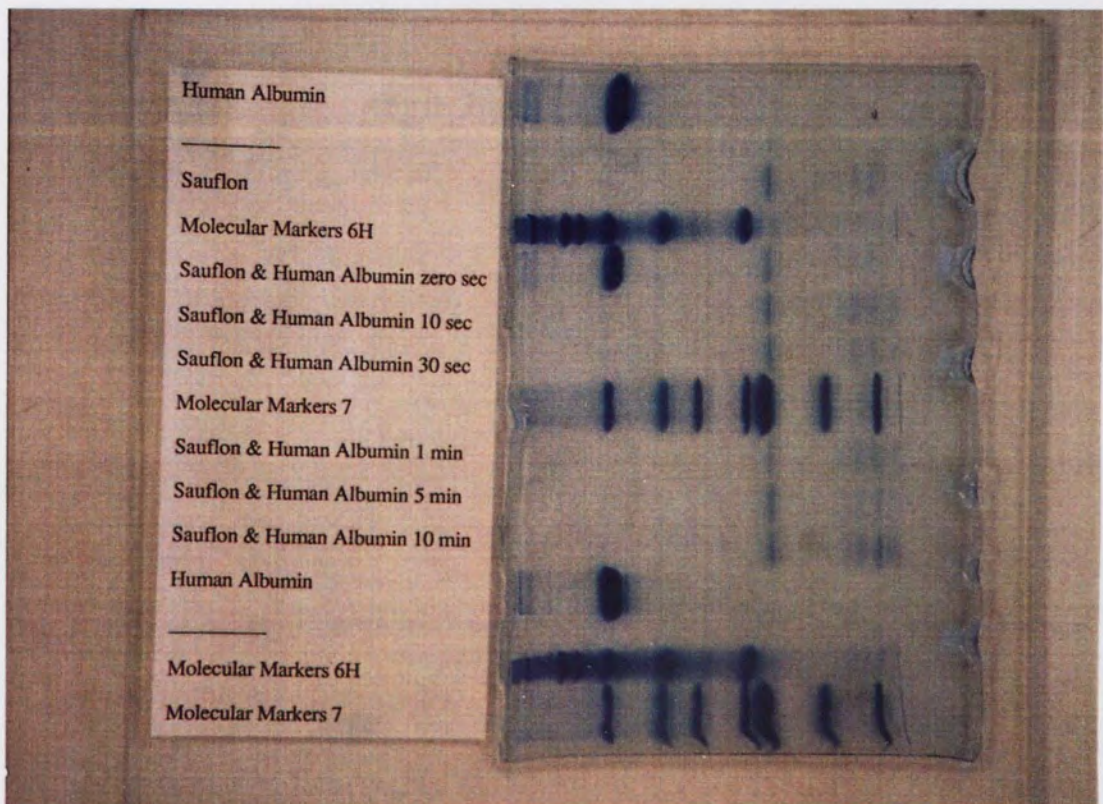
Figure 4.14 Electrophoresis gel showing the effect of Clenzyme on human albumin

Human Albumin :
Molecular Markers 6H
Clenzyme & Human Albumin 10 sec
Clenzyme & Human Albumin 30 sec
Molecular Markers 7
Clenzyme & Human Albumin 1 min
Clenzyme & Human Albumin 5 min
Clenzyme & Human Albumin 10 min
Human Albumin
Clenzyme
Molecular Markers 6H
Molecular Markers 7
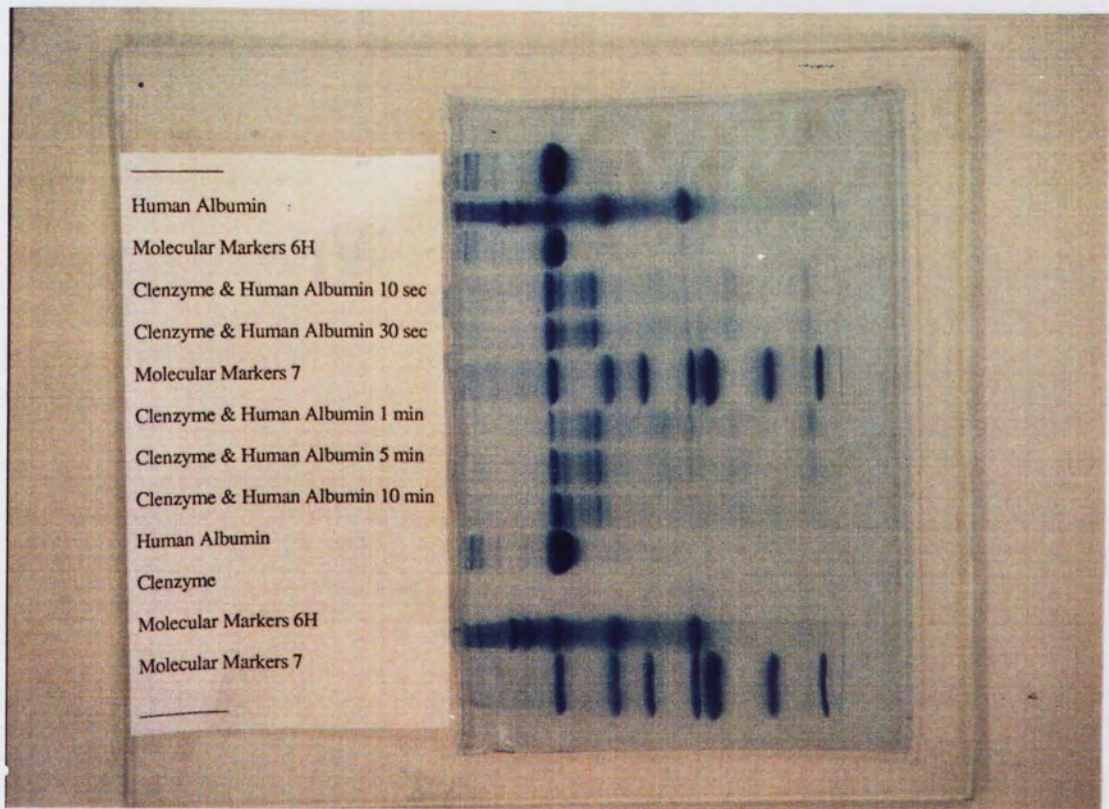
Figure 4.15 Electrophoresis gel showing the effect of Ultrazyme on lysozyme

Molecular Markers 7
Lysozyme

Molecular Markers 6H
Ultrazyme & Lysozyme zero sec
Ultrazyme & Lysozyme 10 sec
Molecular Markers 7
Ultrazyme & Lysozyme 30 sec
Ultrazyme & Lysozyme 1 min
Ultrazyme & Lysozyme 5 min
Ultrazyme & Lysozyme 10 min
Lysozyme
Ultrazyme
Molecular Markers 6H
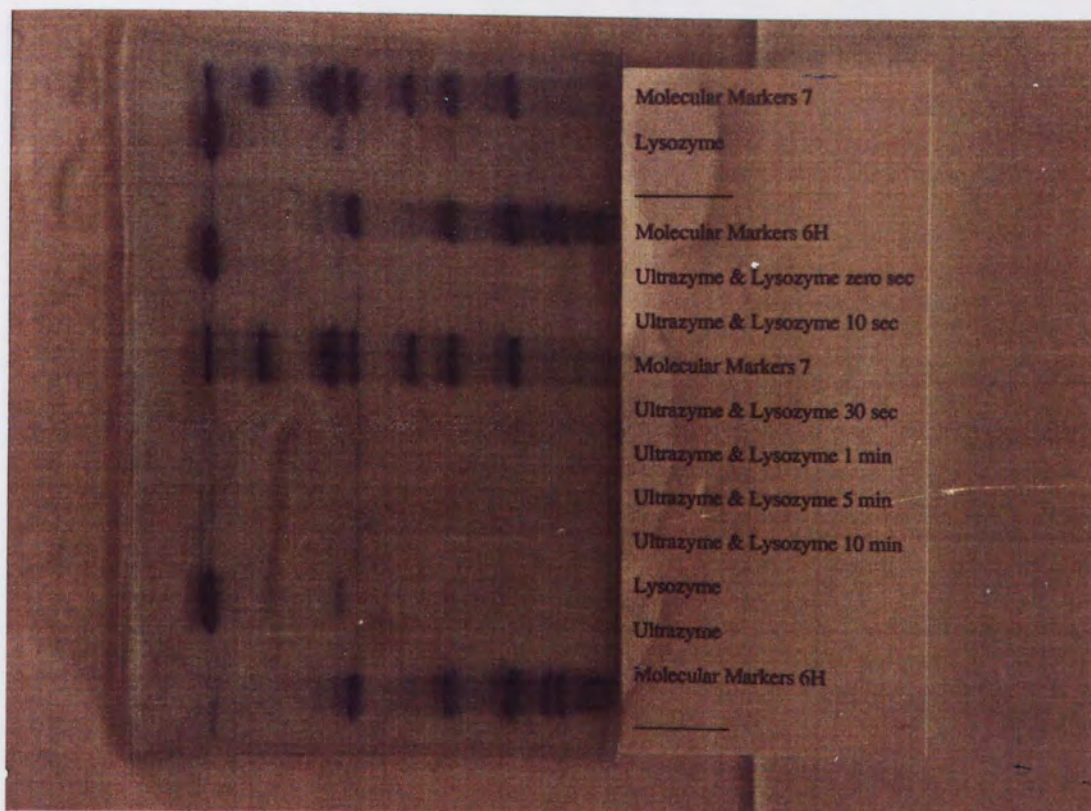
Figure 4.16 Electrophoresis gel showing the effect of Sauflon on lysozyme

Lysozyme

Sauflon
Molecular Markers 6H
Sauflon & Lysozyme zero sec
Sauflon & Lysozyme 10 sec
Sauflon & Lysozyme 30 sec
Molecular Markers 7
Sauflon & Lysozyme 1 min
Sauflon & Lysozyme 5 min
Sauflon & Lysozyme 10 min
Lysozyme

Molecular Markers 6H
Molecular Markers 7
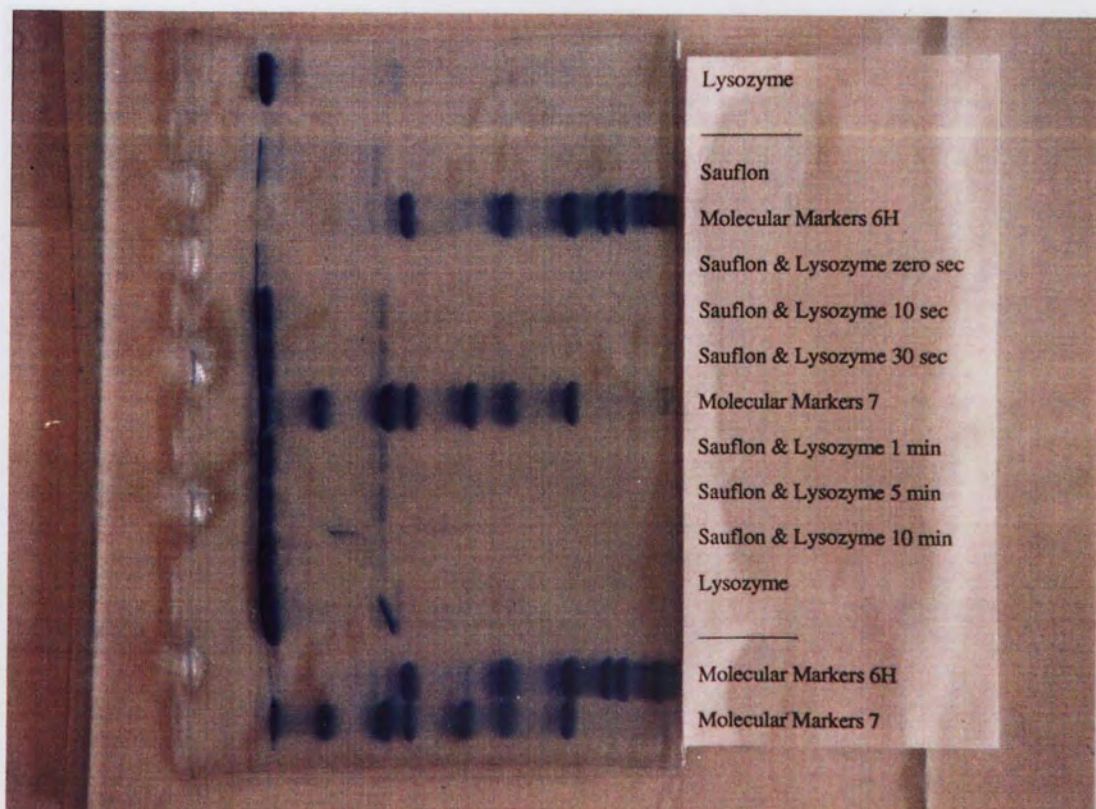
Figure 4.17 Electrophoresis gel showing the effect of Clenzyme on lysozyme
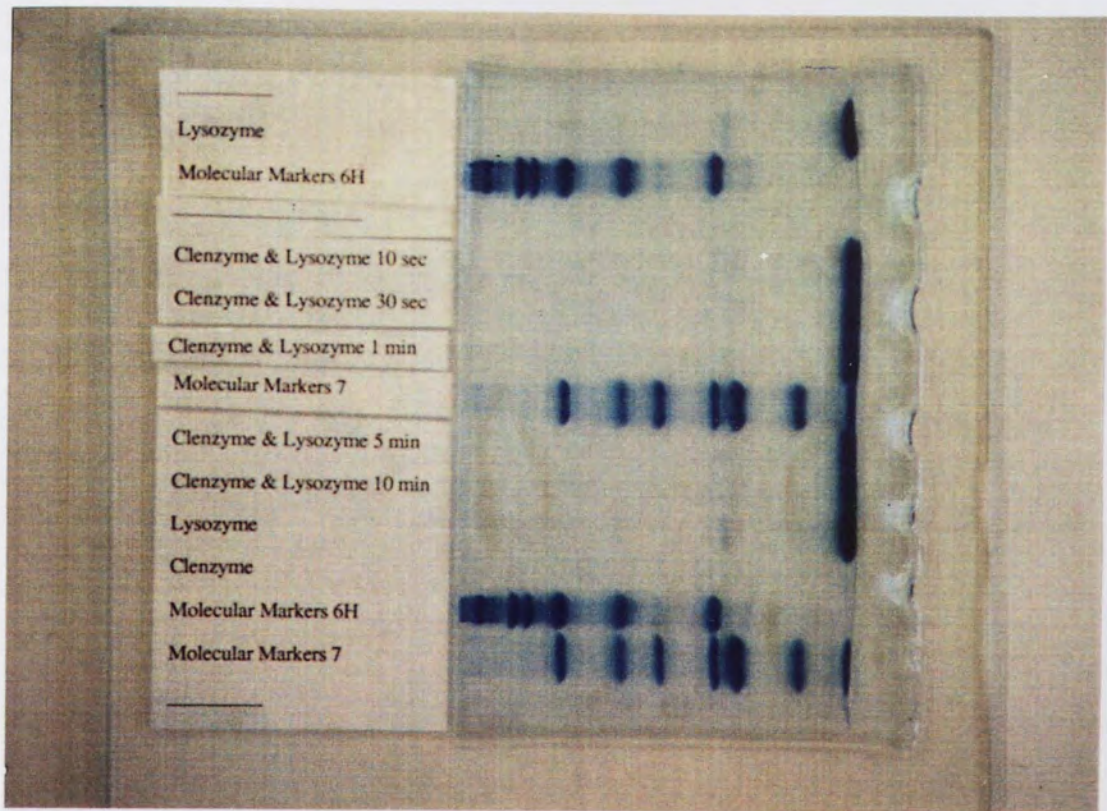
Figure 4.18 Electrophoresis gel showing the effect of B&L sensitive eyes on lysozyme
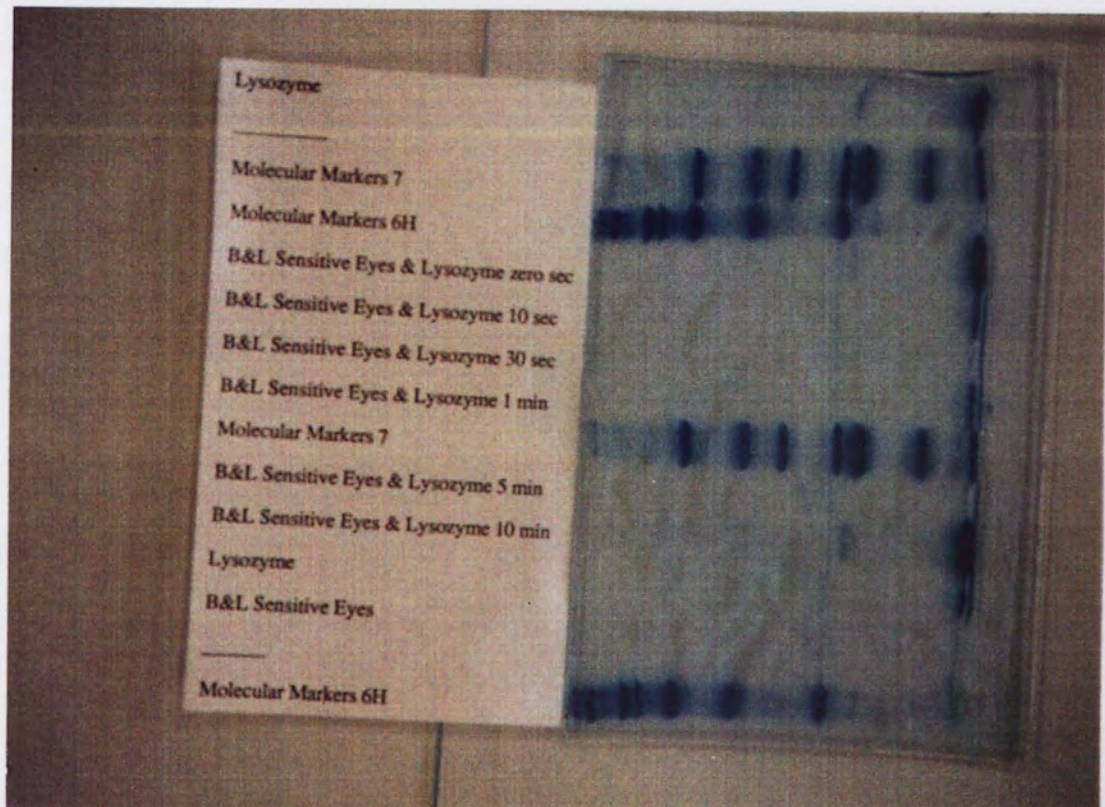
Figure 4.19 Electrophoresis gel showing the effect of Clenzyme on lactoferrin

7 Molecular weight markers

Lactoferrin

Clenzyme

6H Molecular weight markers

Clenzyme+Lactoferrin 10sec

Clenzyme+Lactoferrin 10sec

Clenzyme+Lactoferrin 10sec

Clenzyme+Lactoferrin 10sec

Clenzyme+Lactoferrin 10sec

7 Molecular weight markers

Lactoferrin

Clenzyme

Clenzyme+Lactoferrin 10sec

6H Molecular weight markers

6H Molecular weight markers
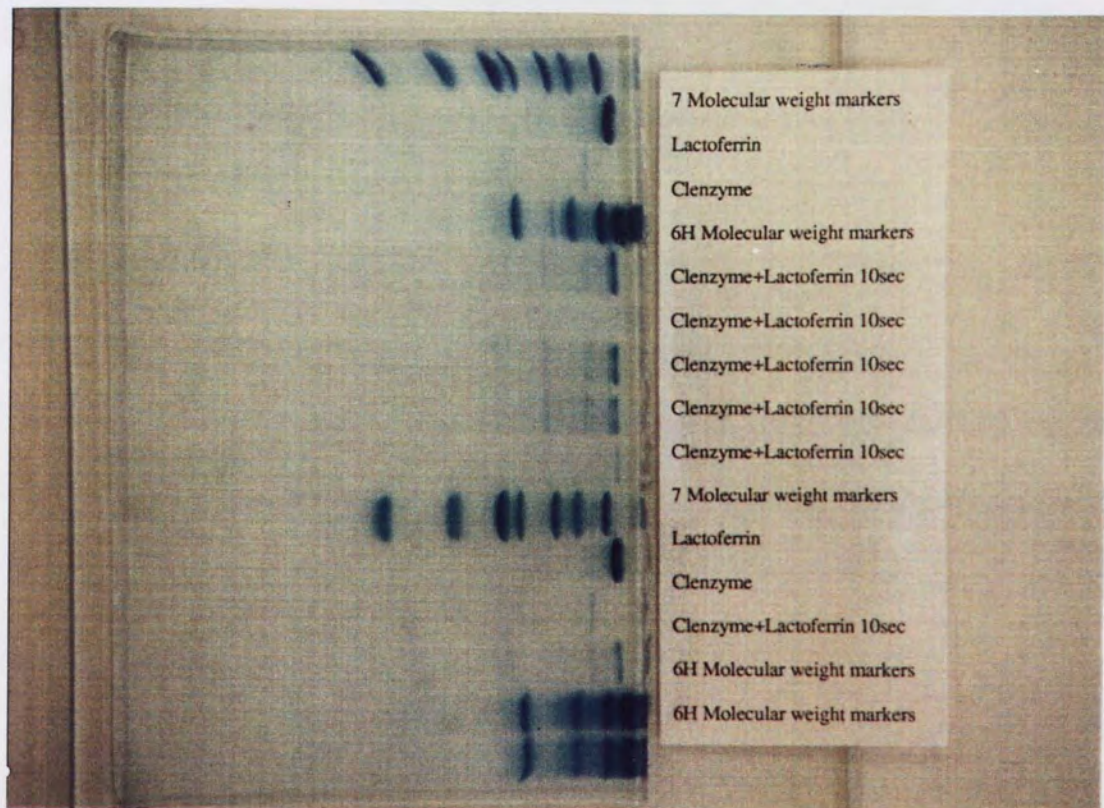
Figure 4.20 Electrophoresis gel showing the effect of B&L sensitive eyes on lactoferrin

6H Molecular weight markers

Lactoferrin

B&L Sensitive Eyes Effervescent

6H Molecular weight markers

B&L Sensitive Eyes Effervescent +Lactoferrin 10sec

B&L Sensitive Eyes Effervescent +Lactoferrin 30sec

B&L Sensitive Eyes Effervescent +Lactoferrin 1min

B&L Sensitive Eyes Effervescent +Lactoferrin 5min

B&L Sensitive Eyes Effervescent +Lactoferrin 10min

7 Molecular weight markers

Lactoferrin

B&L Sensitive Eyes Effervescent

B&L Sensitive Eyes Effervescent +Lactoferrin zero sec

6H Molecular weight markers

7 Molecular weight markers

CHAPTER 5

Spatial studies

5.1 Spatial Studies: Introduction

A full understanding of spoilation will involve elucidation of the chemical events that take place. In addition to this the physical form of deposits contains information as to how the materials build up on the lens and their relative spatial arrangement.. White spots, the gross deposits visible to the naked eye, do appear to contain a great deal of structural information. If this structure could be observed in its natural milieu we might learn something as to the deposits history. It has been postulated that if we could see to the bottom of the white spot we may find the nature of the nucleation sites that trigger the formation of such deposit.

In the previous chapter we saw the problem of incomplete removal of the molecules that build up on the lens surface, this is not a problem in these studies as the structures are studied *in situ* and it is unnecessary to disturb the system.

All samples used in other analytical work done during this project were examined under the optical microscope to see if any general observations could be made. In these examinations many of the manifestations described in the literature were observed. The information gained can be useful in formulating the route of investigation that each lens would undergo.

5.2 Optical Microscopy

Light microscopy is, and should remain, the first routine examination that can be carried out on a contact lens. The sample, if handled carefully, is not damaged and can be used in further investigations.

For these studies the lens was mounted in a drop of saline from its storage vial, a coverslip was placed on the lens and lowered slowly so that the lens is flattened and no air bubbles are trapped.

A number of micrographs are shown, these depict the film like deposits often seen on soft contact lenses, little information can be gained as to their nature by examination alone, see figures 5.1 to 5.3. Details of figures 5.1 to 5.3 are detailed below

Figure 5.1 Film like deposit on a contact lens (Bright field illumination)

Figure 5.2 Film like deposit on a contact lens (Bright field illumination)

Figure 5.3 Grainy film on a contact lens (Bright field illumination)

These film like deposits are often seen on lenses and are often reported in the literature. Some authors make deductions as to the chemical nature of the films purely from their appearance. This is obviously an oversimplification, often films are labeled as calcium but if SEM and EDXA are carried out little or no calcium is observed. Other workers have claimed that the films are proteinacious in nature, undoubtedly protein is present but without further analytical investigation it would be a dangerous assumption to make that protein was all that was present. To sum up it would be foolish to make deductions as to a films chemical makeup from its appearance alone.

Figure 5.1

Figure 5.2

Figure 5.3

White spots make far more interesting objects to study under the white light microscope, even at limited magnification a great deal of structural information can be seen. The white spot shown in figure 5.4 shows up well under bright field illumination. Note the barnacle like arrangement and the concentric rings radiating from the centre. It was found that these deposits fluoresce strongly under the microscope. Figure 5.5 shows the typical auto fluorescence exhibited, the deposit is the same as that in figure 5.4. The deposit shown in figure 5.4 and 5.5 is typical of many that were seen. An interesting deviation from this barnacle like deposit is seen in figure 5.6 and 5.7 this structure shows a more doughnut like appearance. In later studies this doughnut like appearance was seen again so these figures are include for comparison.

What can be deduced from these figures is that white spots appear to have a complex physical form, and the similarity between deposits of this class points to a ordered accumulation of material rather than a random aggregation. Attempt to section these structures proved to be very difficult. The white spot is very physically hard, whilst the lens material is a soft gel, thus as the microtome blade cuts the deposit it is often pushed through the lens material or detached. In the sections obtained if the deposit is seen embedded in the lens is this a true reflection of its natural state? If we are to see the lowest layers of the deposit another method is required.

Figure 5.4 White spot under bright filed microscopy
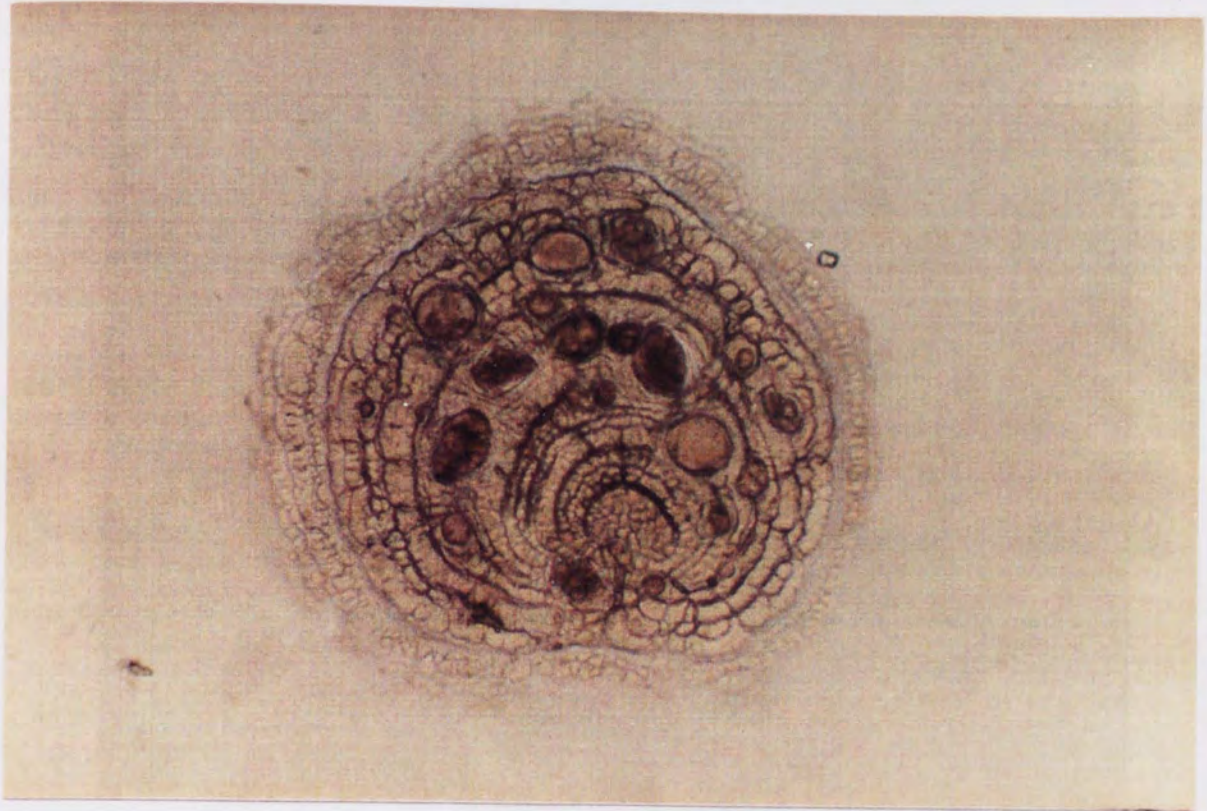
field

Figure 5.5 White spot under fluorescent microscopy
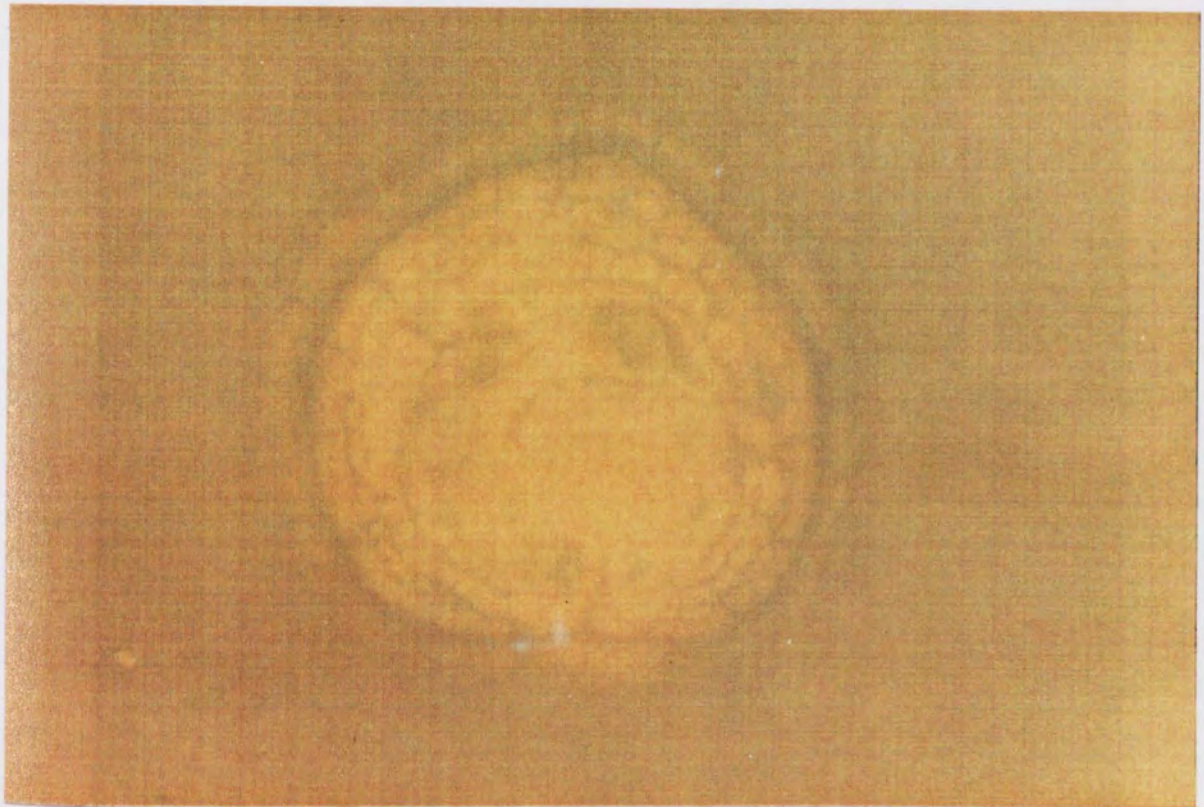
field

Figure 5.6 Doughnut like white spots (bright field)

Figure 5.7 Edge of doughnut like deposit (bright field)
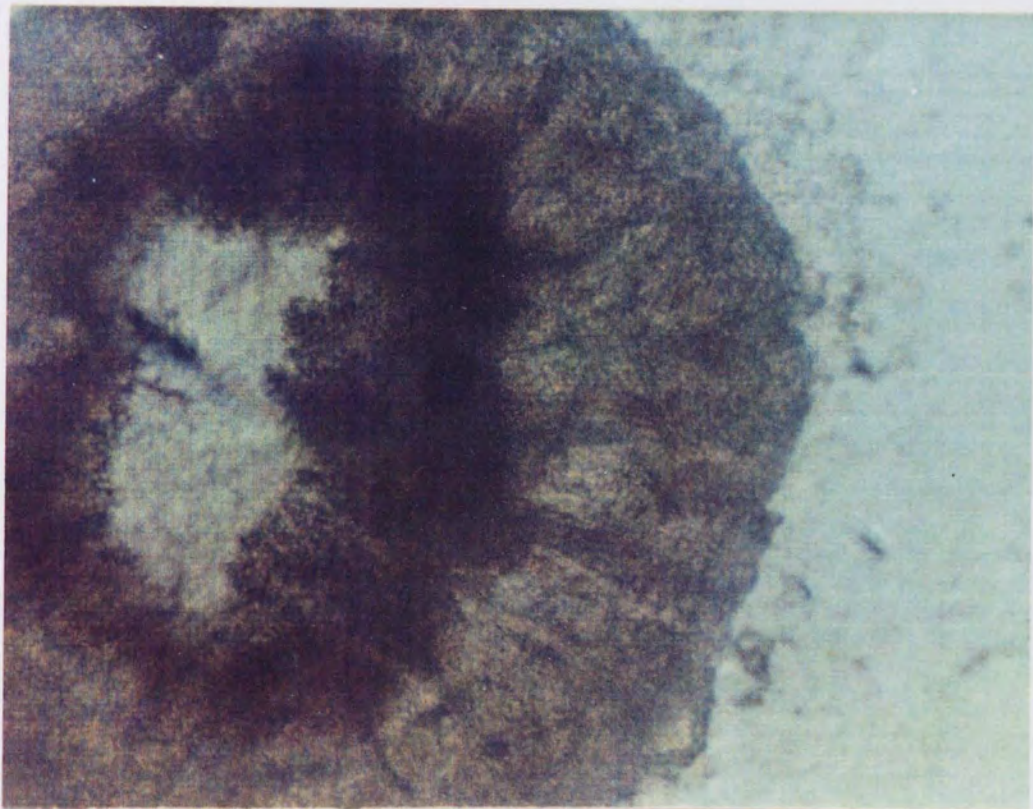
5.3 Scanning electron microscopy (SEM).

Samples for scanning electron microscopy were prepared as described in section 3.7.1. A small selection of the electron micrographs taken are shown below. A film like deposit is shown in figure 5.8, this is only a light film, figure 5.9 shows a heavy film of material on a contact lens surface as seen under the electron microscope. Damage to the lens is often seen as in figure 5.10 these are thought to be caused by mishandling by the patient, what role such defects have in initiating the deposition of biological material is the subject of much conjecture. As can be seen in figure 5.10 it appears as if a layer of biological material is beginning to accumulate around the defect.

The information gleaned from these studies have the same problems as those with the white light microscope, physical appearance cannot be used to recognise chemical composition.

Figure 5.8 SEM of a light film like deposit on a soft contact lens
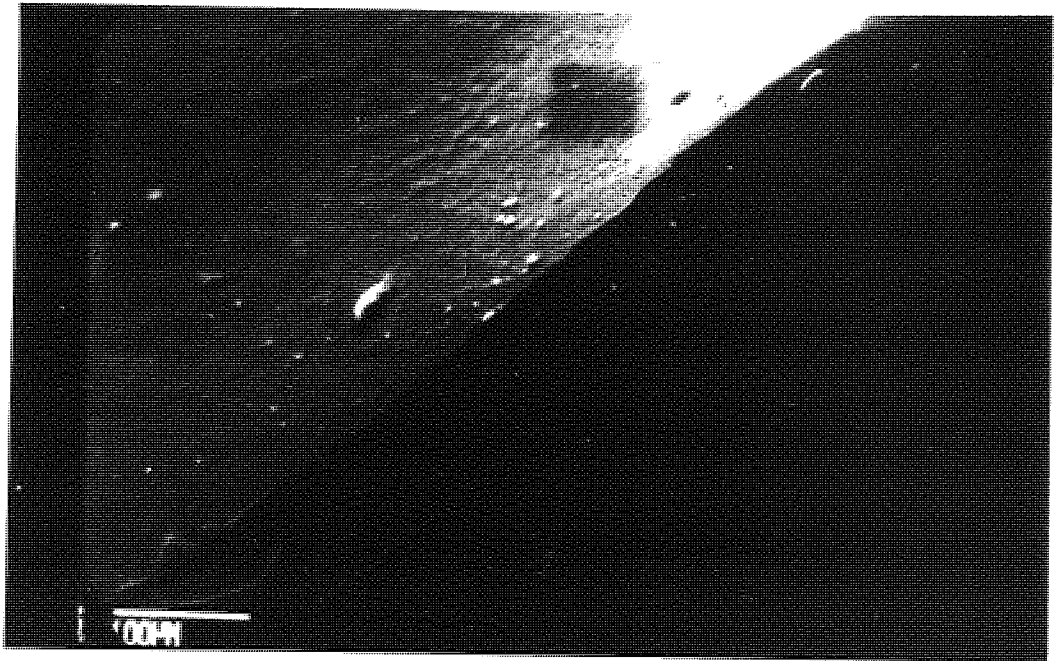
Figure 5.9 SEM of a heavy film on a soft contact lens
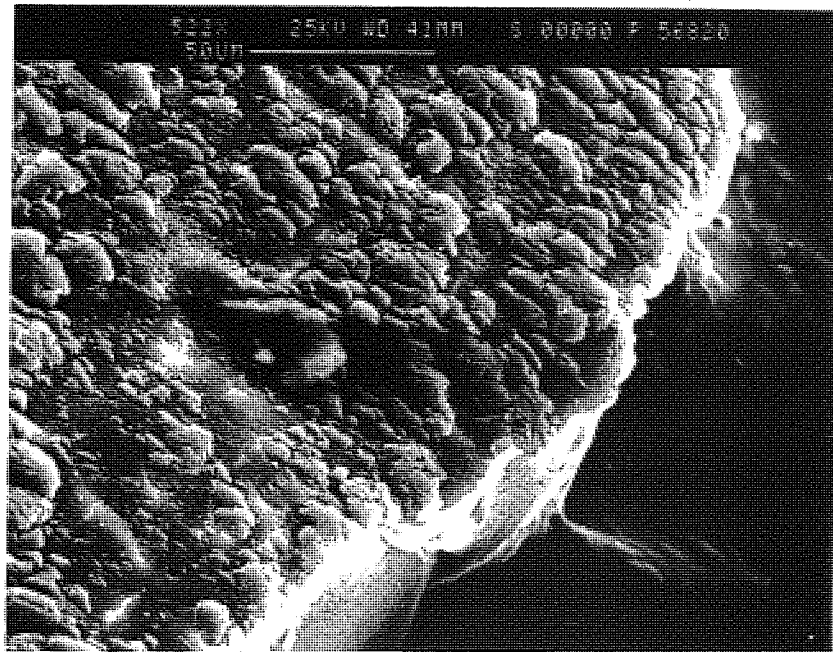
Figure 5.10 SEM of a physical defect seen in a soft contact lens
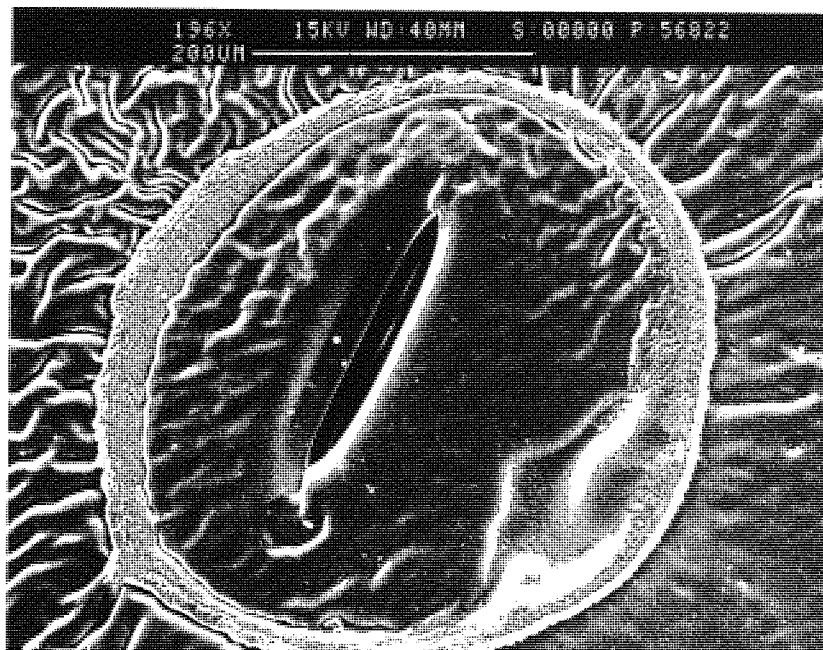
5.3.2 SEM of White spot deposits

SEMs of a number of white spots were taken, they show the characteristic structure as seen under the white light microscope. In the case of SEM we obtain some information as to the structures three dimensional form, the deposit is seen as a structure that rises above the surface of the lens, a central hole is seen at the pinnacle of the deposit. The white spot appears to have a 'volcano' like structure. Figure 5.10 and 5.11 show these features clearly. How much of this structure is a true representation of the deposit and what is artifactual is hard to determine from these images alone. Later work in this chapter using the confocal laser microscope casts some light as to the true nature of these structures before sample preparation for SEM. The dehydration of these samples undoubtedly has an effect as can be seen by the surface of the lens material shown in figures 5.10 and 5.11. The surface is seen to be creased, obviously due to sample dehydration.

Figure 5.11 SEM of a typical white spot
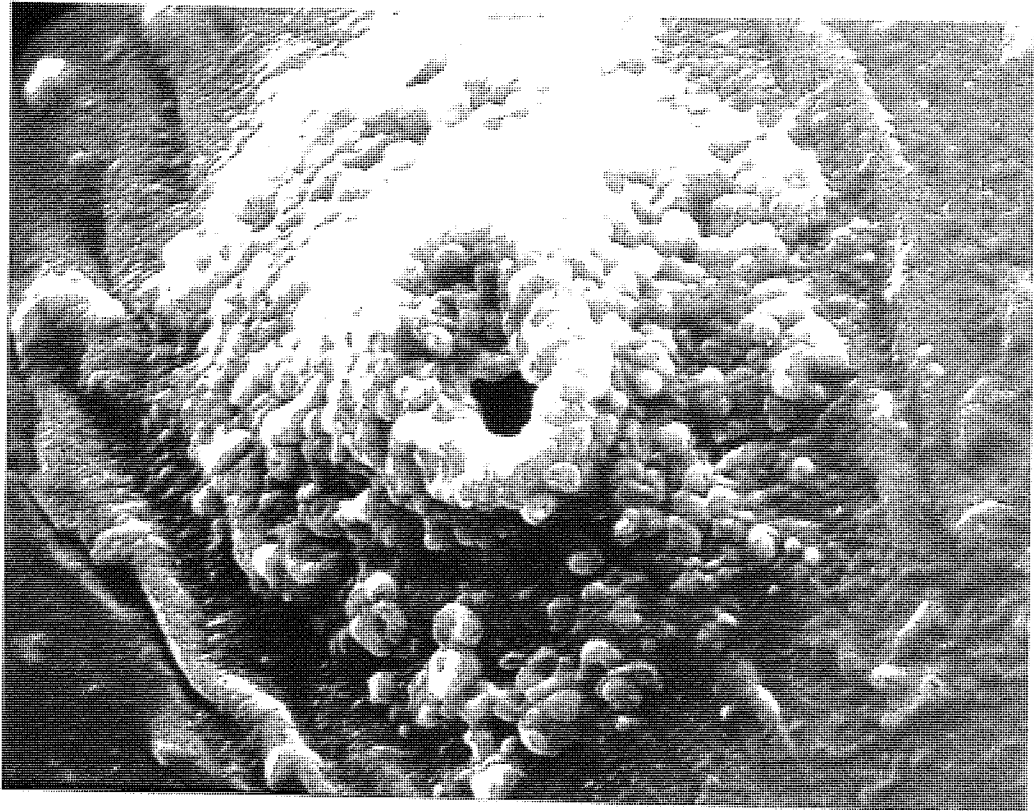
Figure 5.12 SEM of a typical white spot
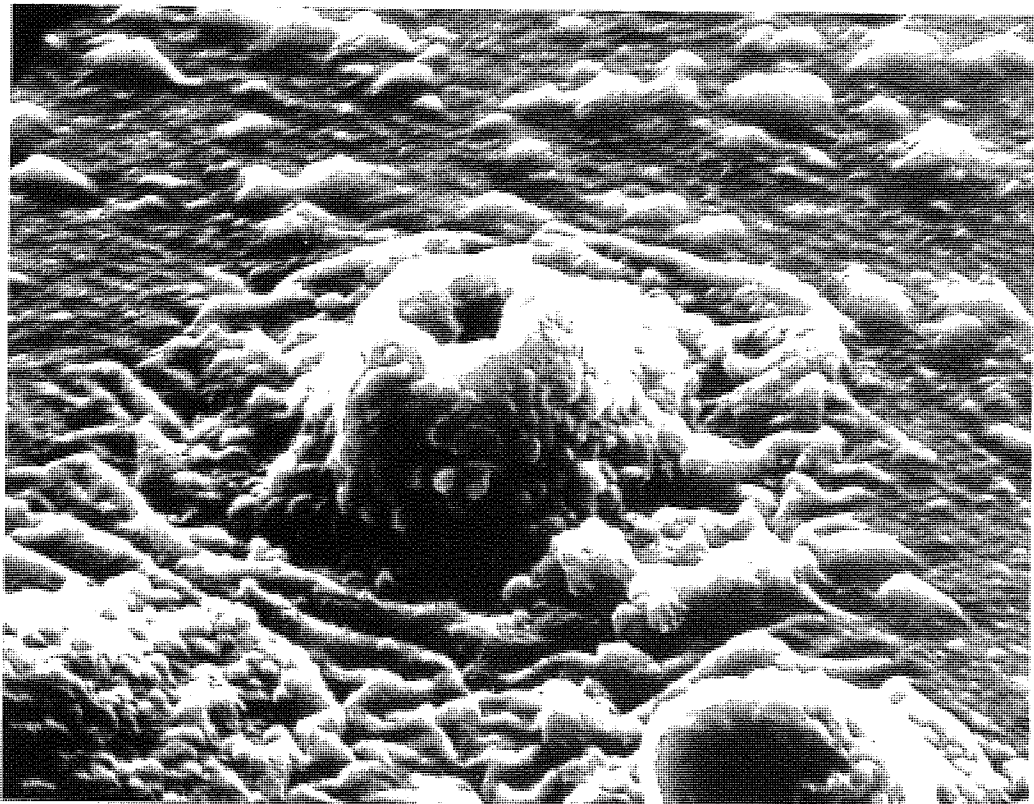
5.4 Physical forms of white spots

As described earlier, these deposits are the most amiable to physical study, these macro structures are often visible to the naked eye as pin head dots on the lens surface. These deposits have been described by many workers as discussed in Section 2.3. Much of the reported work in the literature on these deposits was by means of light microscopy with some electron microscopy. Examples of SEM and light microscopy are shown in sections 5.2 and 5.3.2.

At the outset of this project this was the state of knowledge as to the three dimensional structure of these deposits, light microscopy had shown that the deposits have a complex physical form but apart from unsuccessful studies involving sectioning, the three dimensional shape was not known. Scanning electron microscopy had given some three dimensional information, its major draw back was the harsh sample preparation required. Thus the information gained is brought into disrepute, are the features observed artifactual or are they a genuine reflection of the structures present before intervention by the investigator ?

To sum up, little unambiguous information as to the three dimensional form of these deposits was known and more importantly no details as to the foundations on which the deposit were built was known.

5.5 Confocal Microscopy

This section describes the work done on white spot deposits using confocal microscopy, this is the first time that this powerful technique has been used in this field

and offers the unrivaled advantage of being able to observe the deposits in their natural state without the need for harsh sample preparation, and to obtain detailed three dimensional information.

The three dimensional imaging of microscopic biological objects has undergone a leap forward with the introduction of the confocal scanning laser microscope (CSLM) [134]. The microscope, by means of optical techniques allows the collection of images through sections of suitable samples without the need of physically cutting the sample.

5.5.1 Theory and practice of scanning confocal microscopy

In a conventional microscope the sample is uniformly illuminated and a two dimensional image is collected from all points at once. The illumination in a confocal microscope is the same as in a conventional optical scanning microscope, a beam is focused by a conventional objective to a point, (see figure 5.13). The emergent beam is then focused to a point, at this point a photomultiplier tube is located. The beam is made to scan across the sample by means of mechanically controlled mirrors and each point is illuminated in turn, the output from the photomultiplier tube is fed to a computer, where an image can be constructed. In CSLM it is not the transmitted beam that is collected, rather it is the reflected or emitted fluorescent beam from which the image is formed.

Figure 5.13 Comparison of illumination techniques in conventional and CSLM microscopy.

Beam of illumination

Sample

Emergent beam
Conventional Microscope

Beam of illumination

Sample

Emergent beam
Confocal Microscope
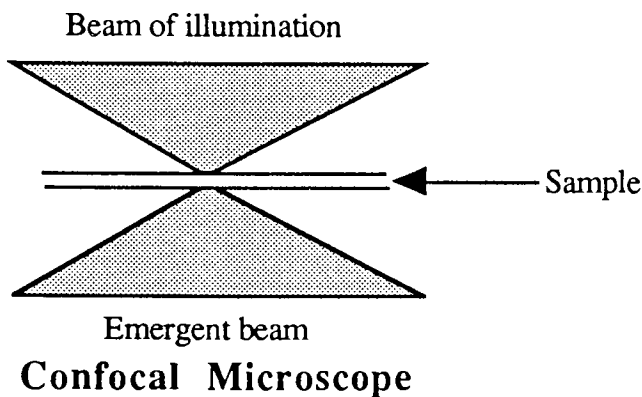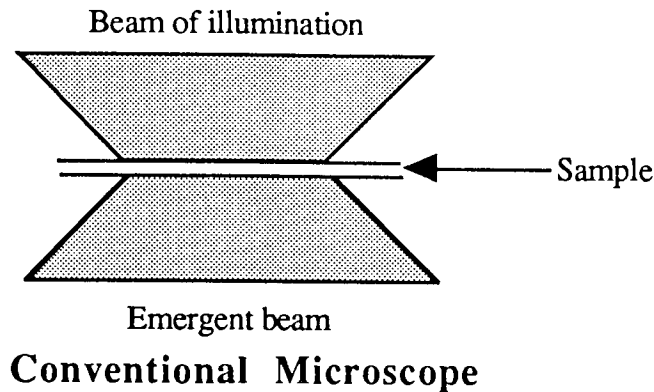
The secret of the confocal microscope is that in the placement of a carefully aligned aperture in the illuminating light beam of the microscope. A second aperture is placed in the emergent light beam at a point equivalent to the primary image. These apertures are said to be confocal with one another and with the sample. Light that is emitted by the sample in the optical plane passes through the aperture un-reduced and causes a signal in the photomultiplier tube. Light that comes from out of focus regions in the sample is defocused at the imaging aperture thus little of is passes through. The arrangement of the aperture is shown in figure 5.14, the light beam from the sample is

shown as a solid line whilst the out of focus element is shown as a broken line.

Figure 5.14 Diagram of the optics in a confocal scanning microscope.
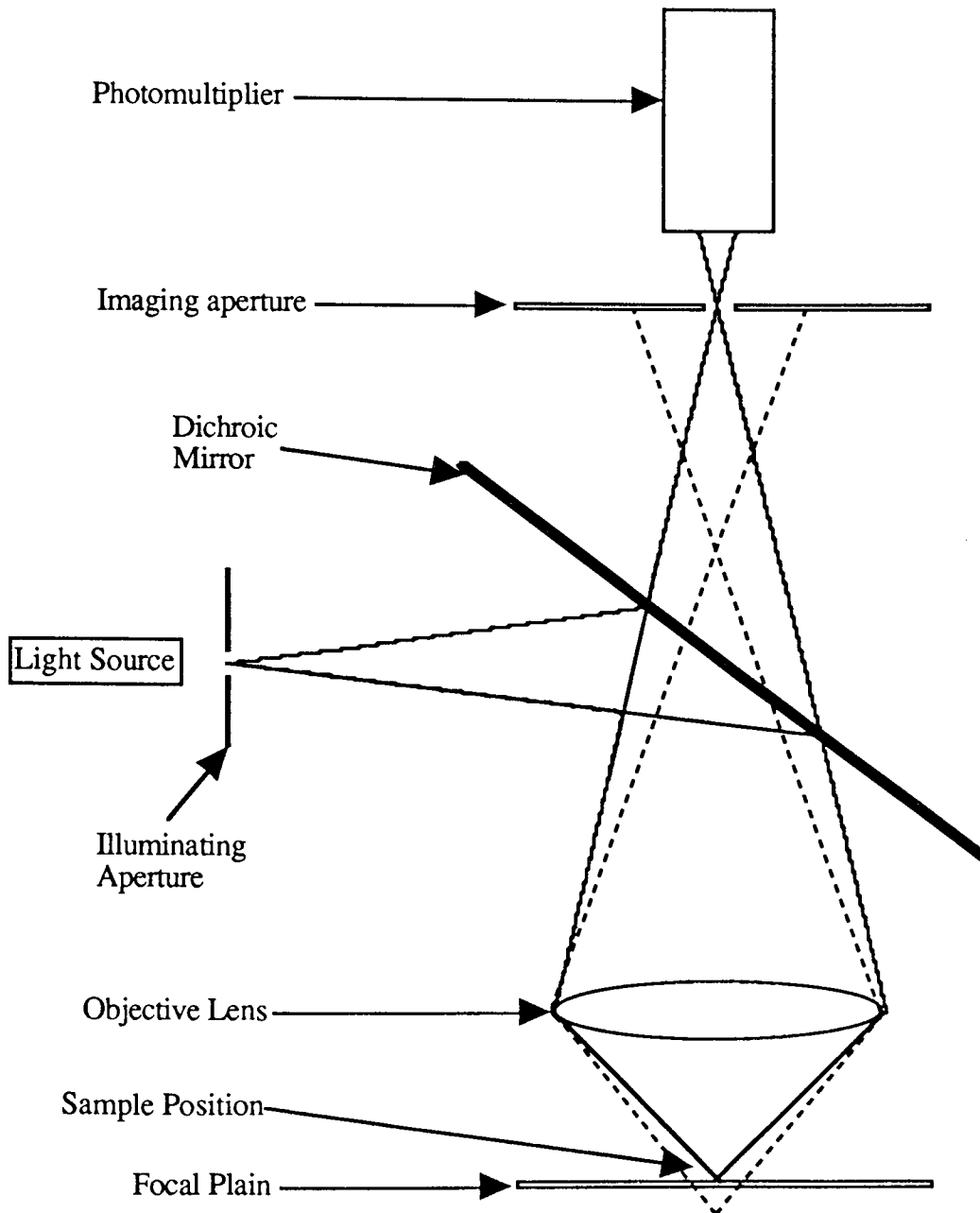
The image is sharpened in two ways, the emitted out of focus light is reduced at the imaging aperture. The second mechanism is because only the area of interest is illuminated, thus in fluorescence mode the adjacent areas of the sample are not exited by the incident illumination and therefore do not emit strongly. In reflection mode a

similar secondary sharpening occurs because of the aperture in the illuminating path. The illuminating beam used is a laser rather than a white light source, this gives higher energy throughput and optics can be corrected for a single wavelength very accurately. The result of the optics is that the microscope has, in the terms of photography, a very shallow depth of field, only objects close to its focus points are imaged. More information about the theory and practice of CSLM can be found in references 134 to 137.

The output from the photomultiplier tube is passed to a high speed microcomputer, the computer can then construct a two dimensional (X,Y image). This image can be stored in a number of ways, on computer storage medium, on video, by means of a 35mm SLR with a high resolution screen and on a colour video printer. The X Y sections can be taken at any point through the sample by means of focusing up and down through the sample. The CSLM is equipped with a motorised focus control, thus it is possible to collect automatically a large number of X Y sections through a sample. The sections are all stored on magnetic media for further manipulation. The data files so constructed often reach a size in excess of 20 Mbytes thus the use of a high speed computer is essential for their manipulation.

Typically the lenses were mounted as described in section 3.8. The images are built up a frame at a time, between each image acquisition the motorised focus control moves a preset amount. As the X Y sections through a deposit are collected each is stored on magnetic media, these files can then be manipulated. An averaged image of a structure can then be displayed on the screen, a yellow line is displayed this can be moved in any

direction by means of a mouse. The position of the required XZ section is chosen with this cursor line, the computer then manipulates the data files collected to give the desired image. The technique relies on the collection of a large number of X Y scans, as it is the number of images collected that will give the final resolution of the X Z section.

The BioRad Lasersharp MRC 500 used can also display the images in many other ways, the black and white image collected (as laser is monochromatic), can be manipulated so that a pseudo-colour image is produced, with the colours representing the height of the displayed feature, in much the same way that height is displayed on an ordinance survey map. A hard copy of these colour images can be obtained from a colour video printer.

A diagrammatic representation of the layout of the instrument and its peripherals is shown in figure 5.15.

A three dimensional image of a object can be observed by colour coding the pixel points, then offsetting certain points by a degree that represents how deep they are in the object. The result of this manipulation when viewed through the classic 3D spectacles equipped with colour filters is that the object appears to come out of the screen. The effect can be further enhanced by the use of a mirrored viewing box which makes the distance between the observers eyes greater and thus the apparent effect larger. The production of hard copy of this type of display is almost impossible.

An excellent method of display is to record each of the XY sections on video, as each

image takes a few seconds to collect, the video allows these to be played back in real

time to give an animated journey through the object under study.

Figure 5.15 Schematic diagram of a confocal microscope

Photomultiplier and beam steering

Argon Laser

Fiber optic conduit

Trinocular microscope head

Focus stepper motor

Transillumination collector

Hi-res monitor with camera

Colour graphics monitor

Control monitor

Microcomputer
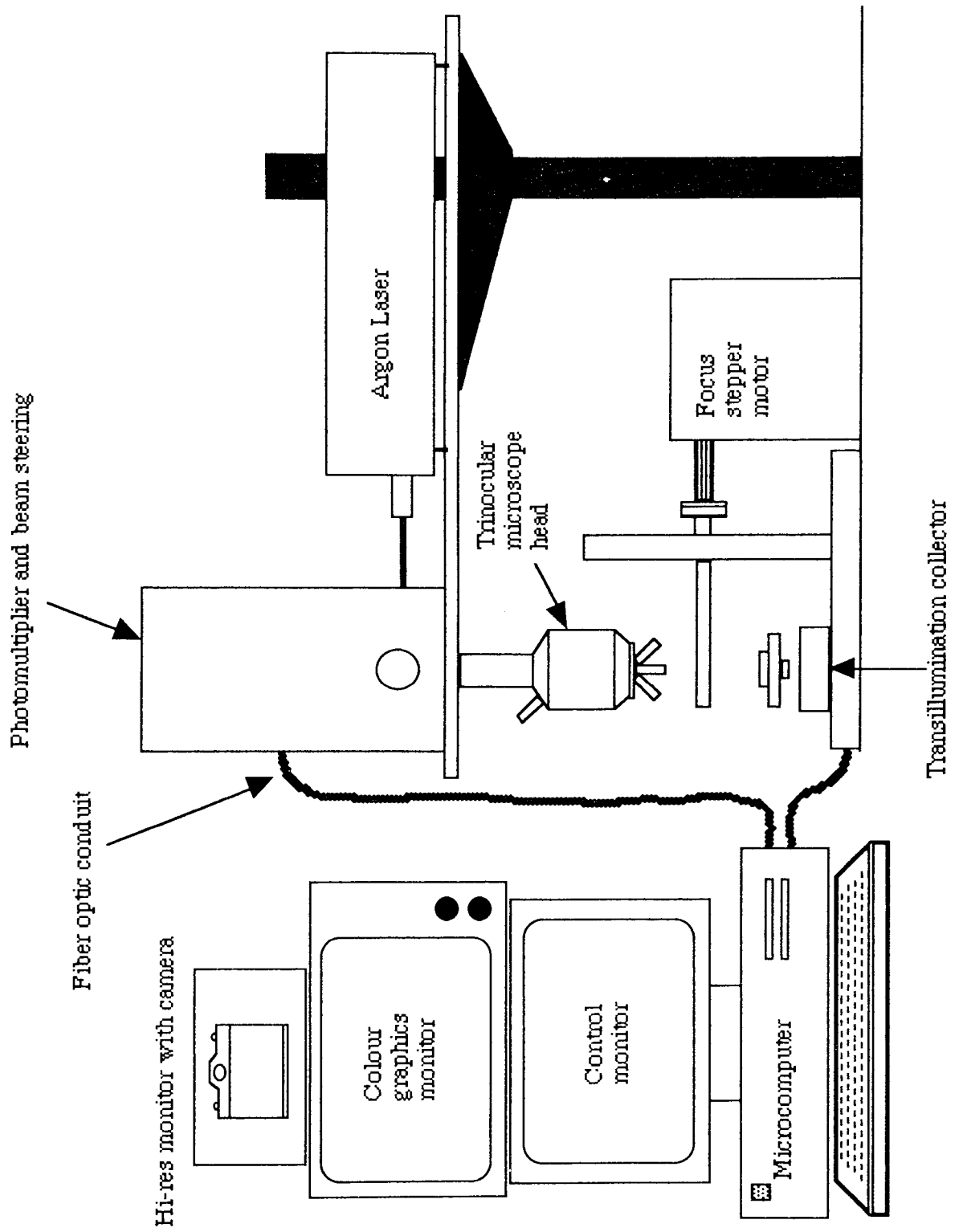
5.5.2 Confocal microscopy methodology

The sample preparation was minimal for this technique, the lens was removed from its storage vial and placed on a clean glass microscope slide. A cover slip that had been pre-treated with poly-lysine and air dried, was placed over the lens. The poly lysine coating is a commonly used method in microscopy to adhere a sample to a coverslip without using harsh treatment. This adherence is important as if the the sample is allowed to float about the advantages of the shallow depth of field produced in confocal microscopy will be diminished.

Many other mounting techniques were tried, these include the use of water immersion optics which negate the need for a cover slip, the sample was found to move as the focus was altered. Glycerol immersion objectives were also tried as glycerol has a high viscosity thus it was thought that the sample would be less likely to move during the acquisition of an image set. The samples were found to be stable but glycerol acts as a potent dehydrating agent, as the primary reason for using the confocal microscope was to obviate the need for sample dehydration (as in SEM) it was decided not to proceed with its use.

5.5.2.Results

Classic white spots proved to be very suitable subjects for study, the white spot shows intrinsic fluorescence thus it can be viewed in this mode without the need for staining. The hydrogel material that the contact lens is made from shows little or no fluorescence thus the background is negligible.

A representative selection of the images collected from white spots found on worn soft contact lenses are shown, the lenses had been obtained from optometrists. The history of the samples was not known fully, this is typical of many samples obtained in this manner. As it was the general manifestation of this deposit type that was primarily under review this does not cause a problem.

The white spots imaged have been designated A,B,C etc for ease of reference in the text.

A set of fluorescent confocal images of a white spot designated A, are shown in figure 5.16, these images scan down through the deposit, the images depicting the top and bottom of the deposit are marked for ease of understanding. An appreciation of the three dimensional shape of this structure can be gained from study of these images, but further manipulation can make this much easier. A composite averaged image through the deposit is constructed, this is shown in figure 5.17. The cursor line is positioned at the required position shown by the line. A XZ image is then computed for this point, this is shown in figure 5.18. It should be pointed out that deposit A is typical in shape but appears to have undergone some damage in that a large hole looks to have been gouged out of the bottom left of the image as displayed. This damage could be due to patient or practitioner handling. The XZ section shows clearly the overall shape of the deposit and shows well the gouged section. Figure 5.19 shows a section taken at a point that runs through the damaged section and through the central 'hole'. This volcano-like summit supports the SEM work shown in figures 5.11 and 5.12. Thus the central hole in the cone appears to be a genuine feature rather than one induced by the sample preparation required for SEM.

Figure 5.16 Set of fluorescent confocal images through a white spot A.
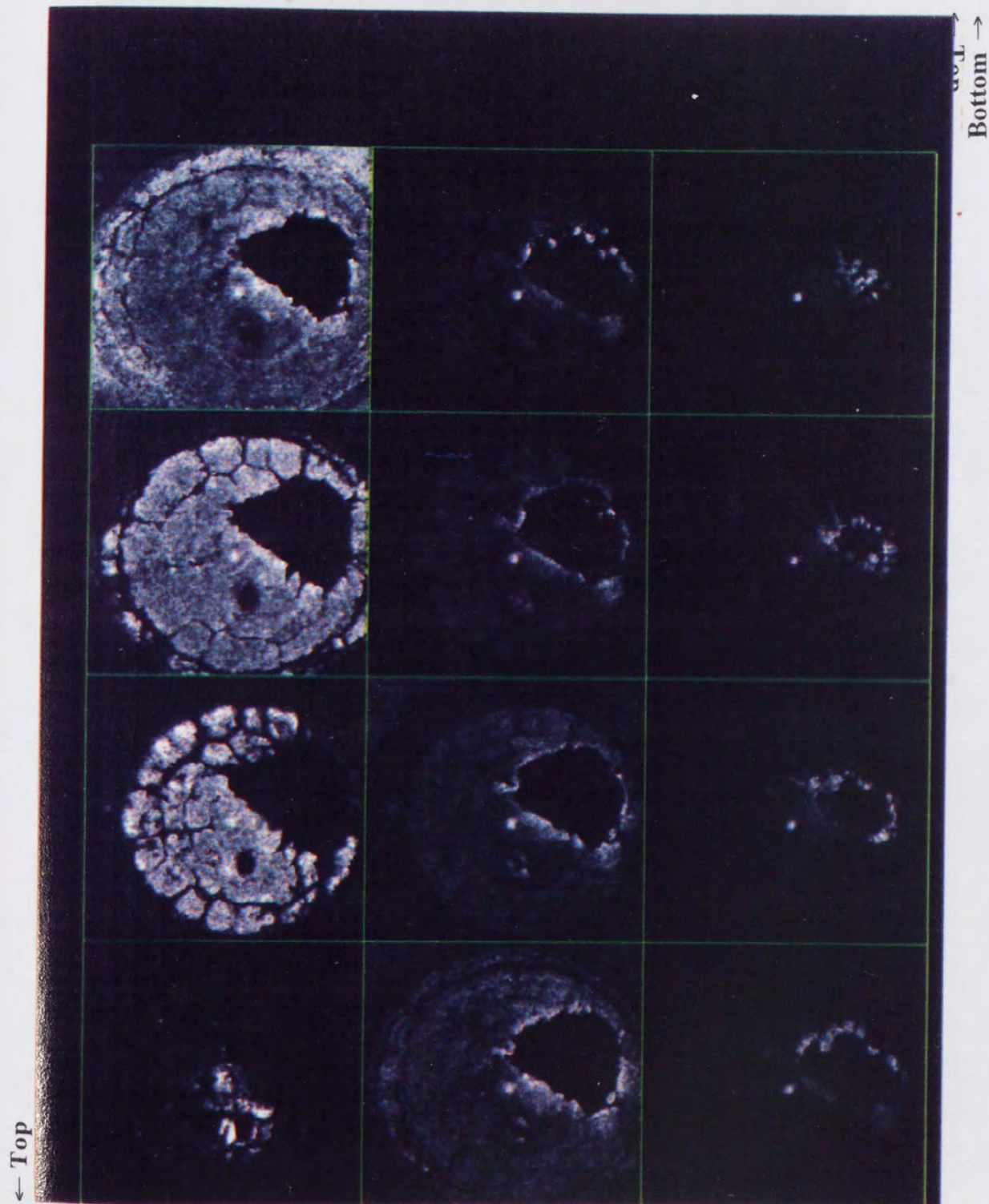
Figure 5.17 Composite fluorescent confocal image of white spot A.
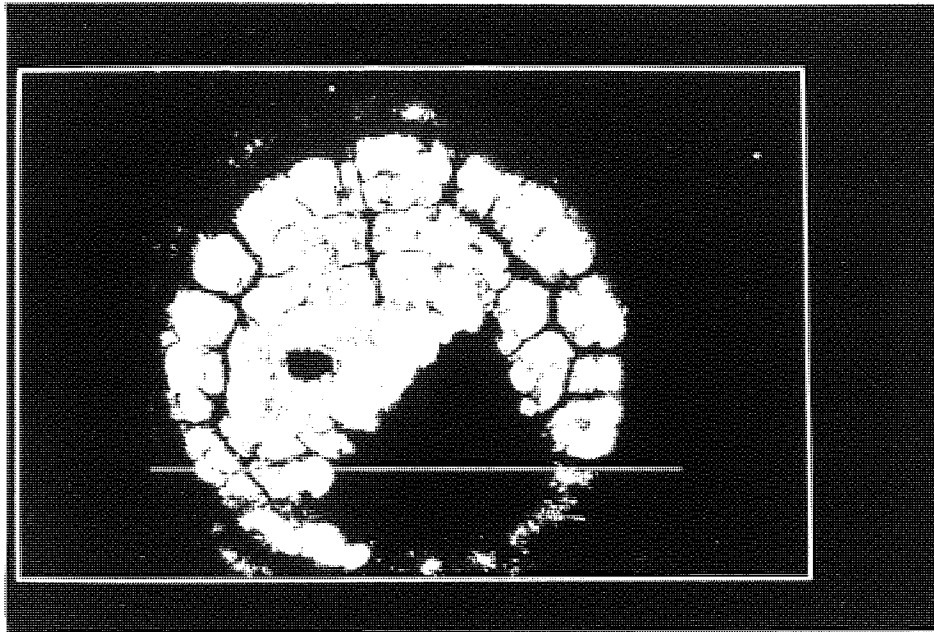
Figure 5.18 X Z section through white spot A at a point denoted by a line in figure 5.17
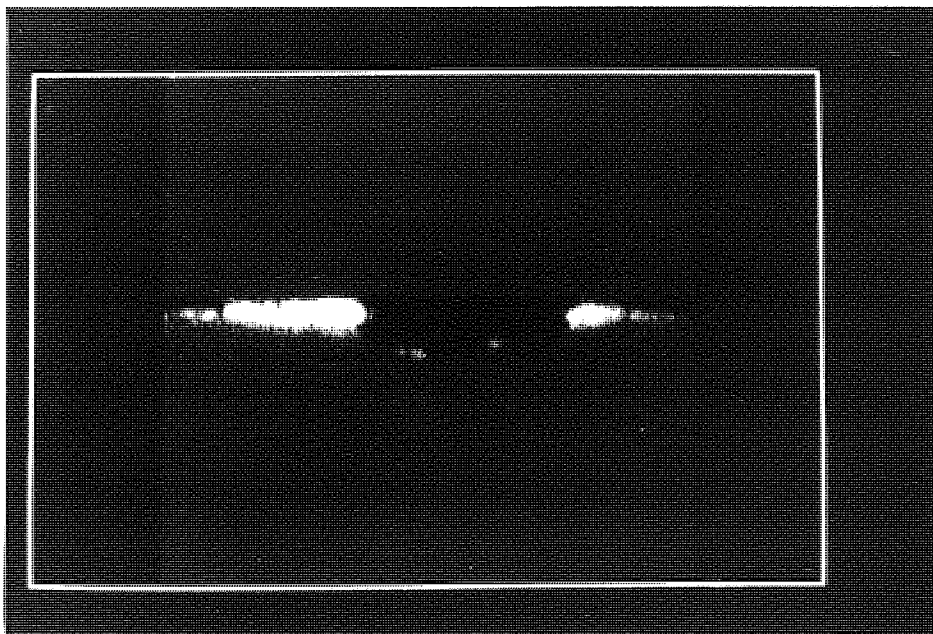
Figure 5.19 Z X section through deposit A at midpoint
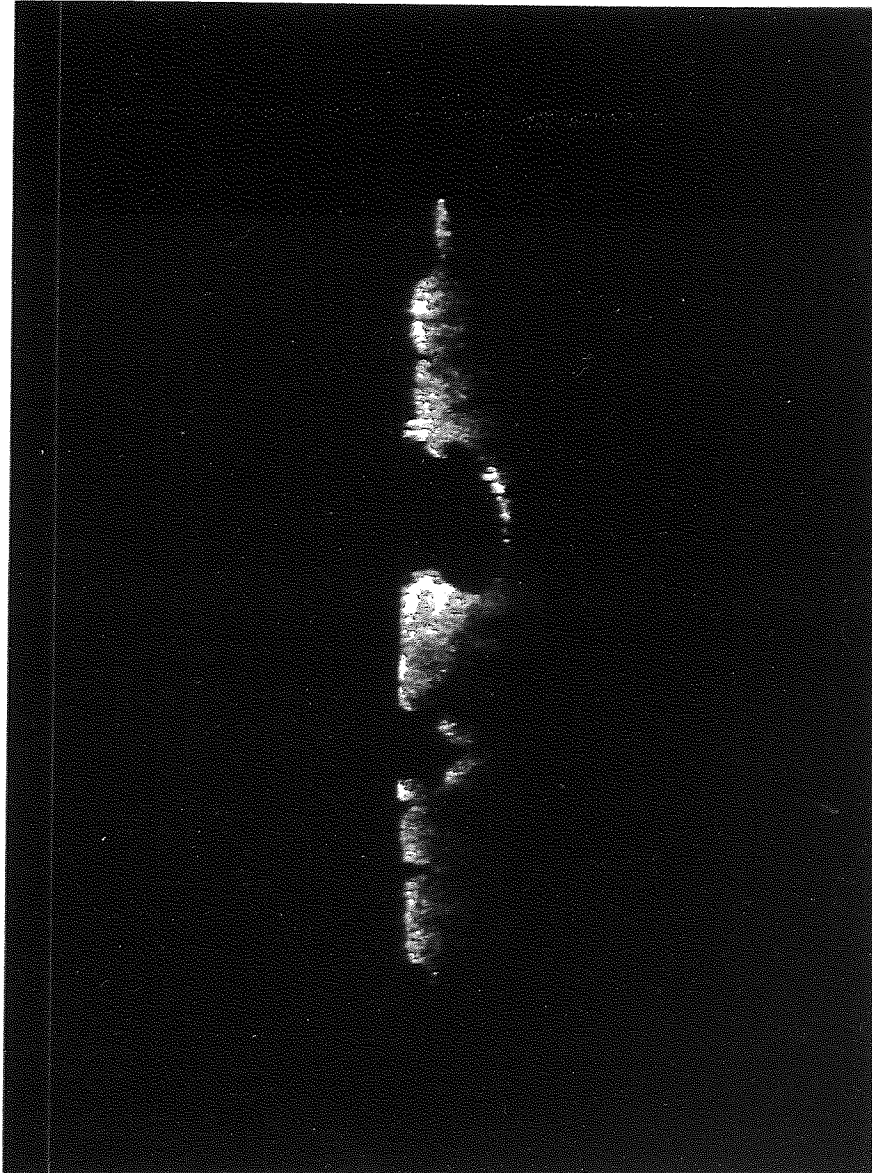
It was found that white spots were imaged well in the microscope's reflectance mode. The use of the microscope in this mode was a bonus, very few biological structures give good reflectance images, white spots proved to be an exception. The white spot itself reflects light strongly whilst the lens does not. The only artifact produced in reflectance mode is that the lens surface reflects light. This property can be used to our advantage as we can see what proportion of the white spot is above and what is below the surface of the lens.
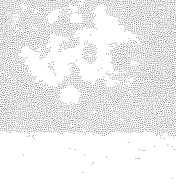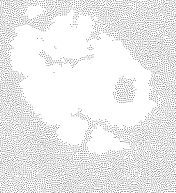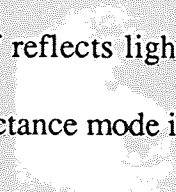
Figure 5.20 Confocal reflectance images of white spot A
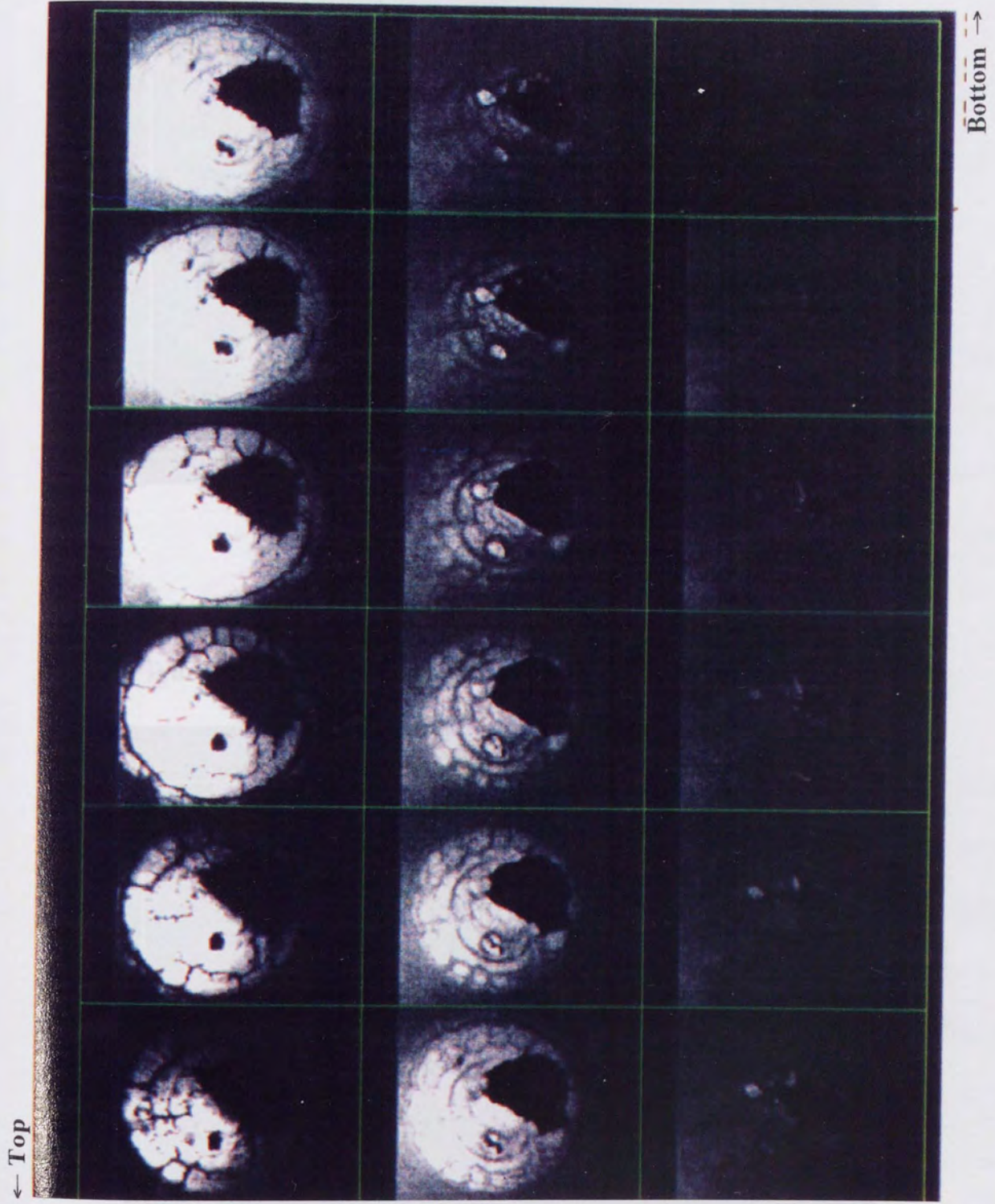
Figure 5.21 Composite reflectance image of deposit A
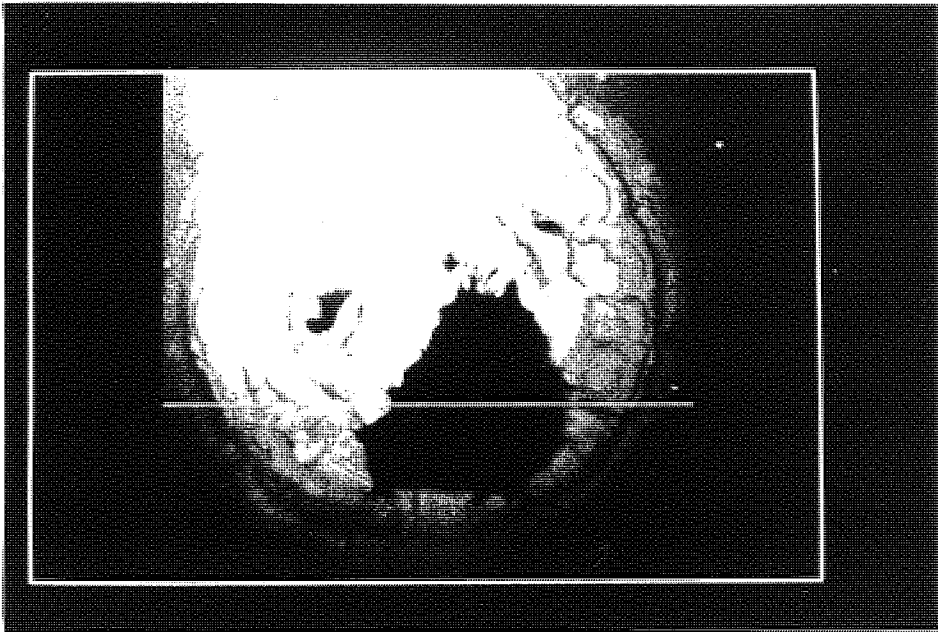
Figure 5.22 XZ section through deposit A at point shown in figure 5.21
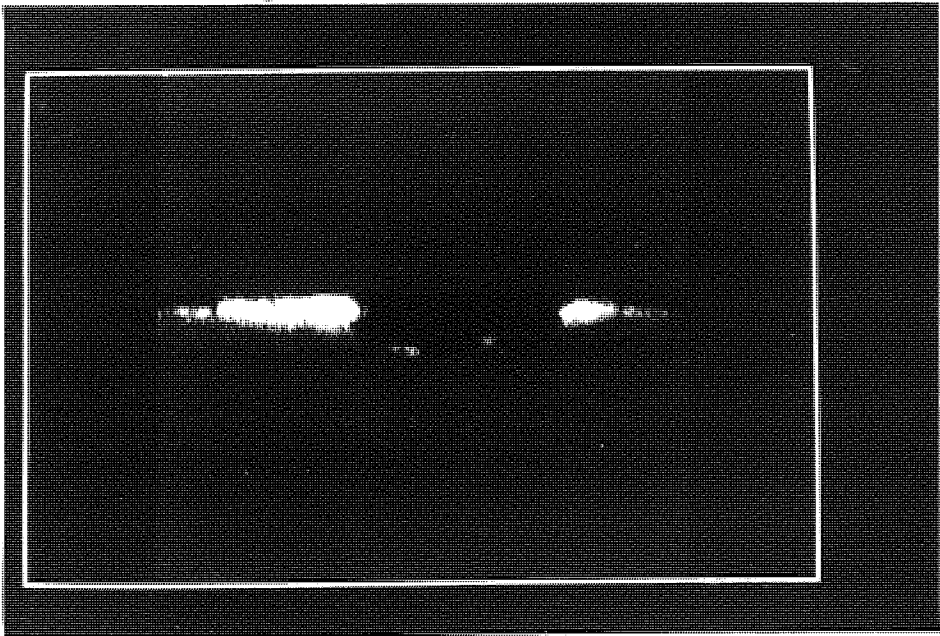
A second set of confocal images is shown in figure 5.20, these were collected in reflectance mode. Figure 5.21 shows the composite image made up from images in figure 5.20. A XZ section was taken again at the point denoted by the line in figure 5.21 it is shown in figure 5.22. This reflected image confirms the findings of the fluorescent imaging shown earlier.

The amount of the deposit above and below the surface of the lens can be deduced by looking at the images in figure 5.20, the frames showing the strong reflectance associated with the lens surface are marked. The majority of the deposit is thus below the surface of the lens, it lies within the lens matrix. The deposit could then either start to form within the matrix and grow out or the deposit could start on the lens surface and grow into and out from the initial foundation. Evidence for this second mechanism will be put forward later in this chapter.

Another set of confocal images are shown in figure 5.23 this is of another white spot designated B, an interesting feature is shown, because the lens is flattened the curvature of the lens is such that the lower surface is seen to deform, this is shown in figure 5.24 and the XZ section in figure 5.25. It was suggested that if the lenses were mounted on a curved surface this problem could be overcome. This deformation is shown diagrammatically in figure 5.26 this defect was seen more often when no coverslip was used but even with a coverslip some evidence for deformation of the lower surface of the lens was observed. As suggested earlier, a system for presenting the lenses to the microscope on a curved surface was attempted but this proved to be problematic. The space available between the objective and the stage is small, thus the device would have

to be no more than 1.5cm high. It is also a requirement that the curved surface should be able to rotate and swivel about all axes. These constraints lead to the decision that the artifact produced did not diminish the observations and that as long as it was recognised as such, posed no problem.

The deposit imaged in figure 5.23 through 5.25 is interesting in that it compares well with the doughnut like deposit seen under the white light microscope. Is the doughnut a separate deposit type to the classical white spot or is it a precursor ? One could imagine how a ring like deposit growing on all sides would ultimately lead to a volcano shaped structure as the central hole grew smaller.

Figure 5.23 Fluorescent images through deposit B
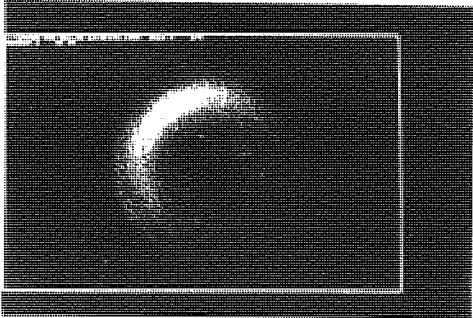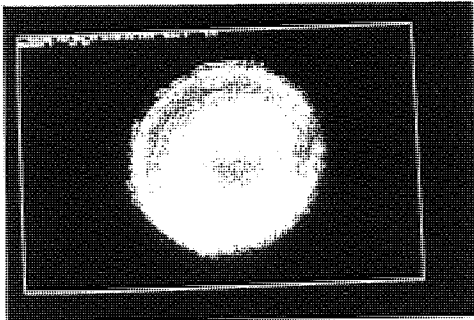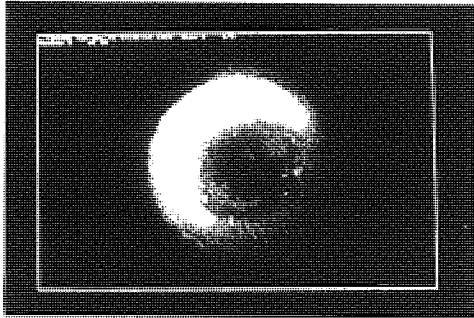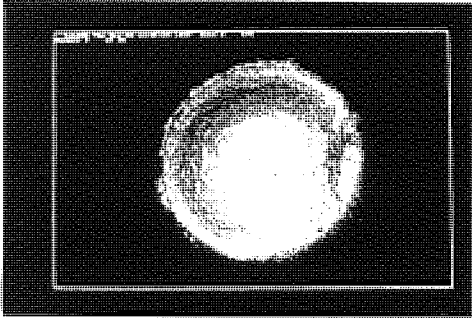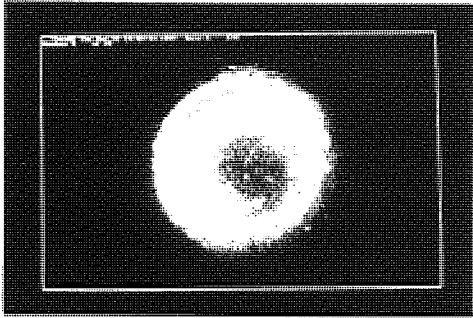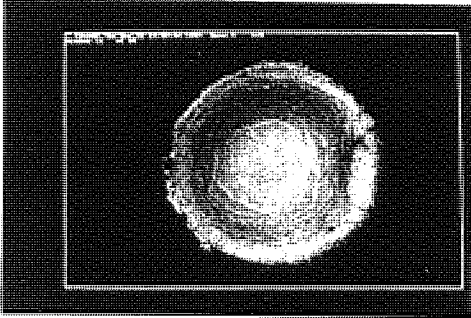
Figure 5.24 Composite image of deposit B
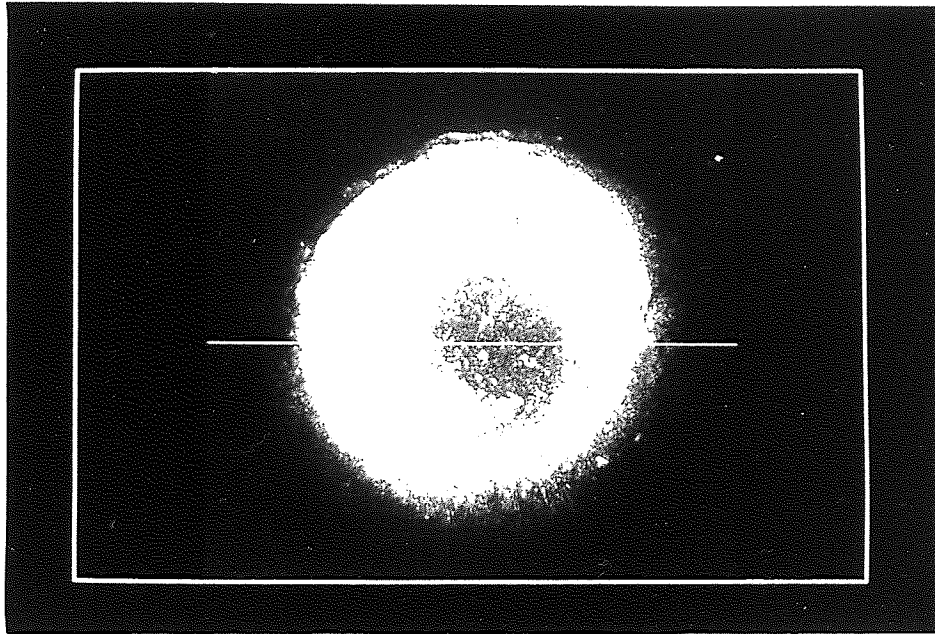
Figure 5.25 XZ section through deposit B at point shown in figure 5.24
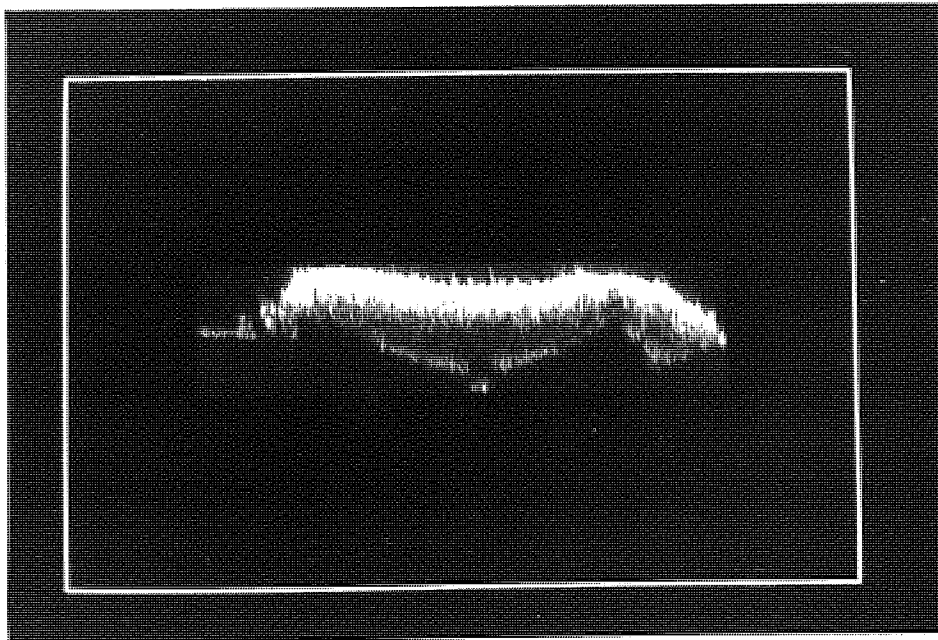
Figure 5.26 Diagrammatic representation of lens deformation as the microscope objective compresses the contact lens
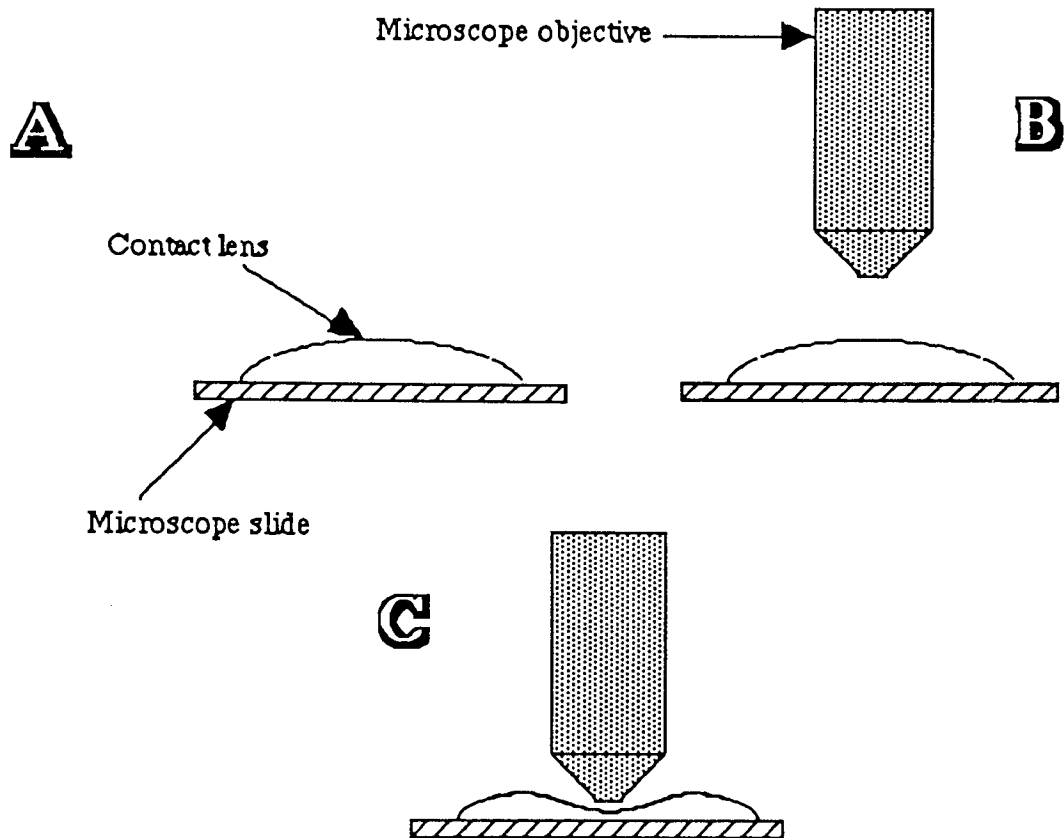
Many other deposits were imaged and many of the general trends seen in the above examples were seen. One more interesting feature was seen in a white spot deposit designated D, a set of reflectance confocal images are shown in figure 5.27, a composite image and XZ section are shown in figure 5.28 and 5.29 respectively. It was noticed that in figure 5.27 that at the base of the deposit a finger like structure could bee seen. A higher magnification study of this region was carried out, these images are shown in figure 5.29. A composite image and a section shown in figure 5.30 and figure 5.31 confirms the presence of these structures. The significance of these could be great, are we looking at the growing edge of the deposit, penetrating into

the contact lens ? Thus is the deposit like a great tree, does it grow roots down into the lens matrix as well as adding to its bulk above the lens surface. SEM work done in this laboratory by Bowers[86] showed that these finger-like structures are seen on the surface of deposits in protected crevasses. The assumption was made that the finger-like structures would be smoothed out in exposed areas by the action of the eyelids during the blink process. If the deposits are anchored by such structures this would explain the difficulty of removal encountered.

Figure 5.27 Set of reflectance confocal images of white spot D

Top→

←Bottom
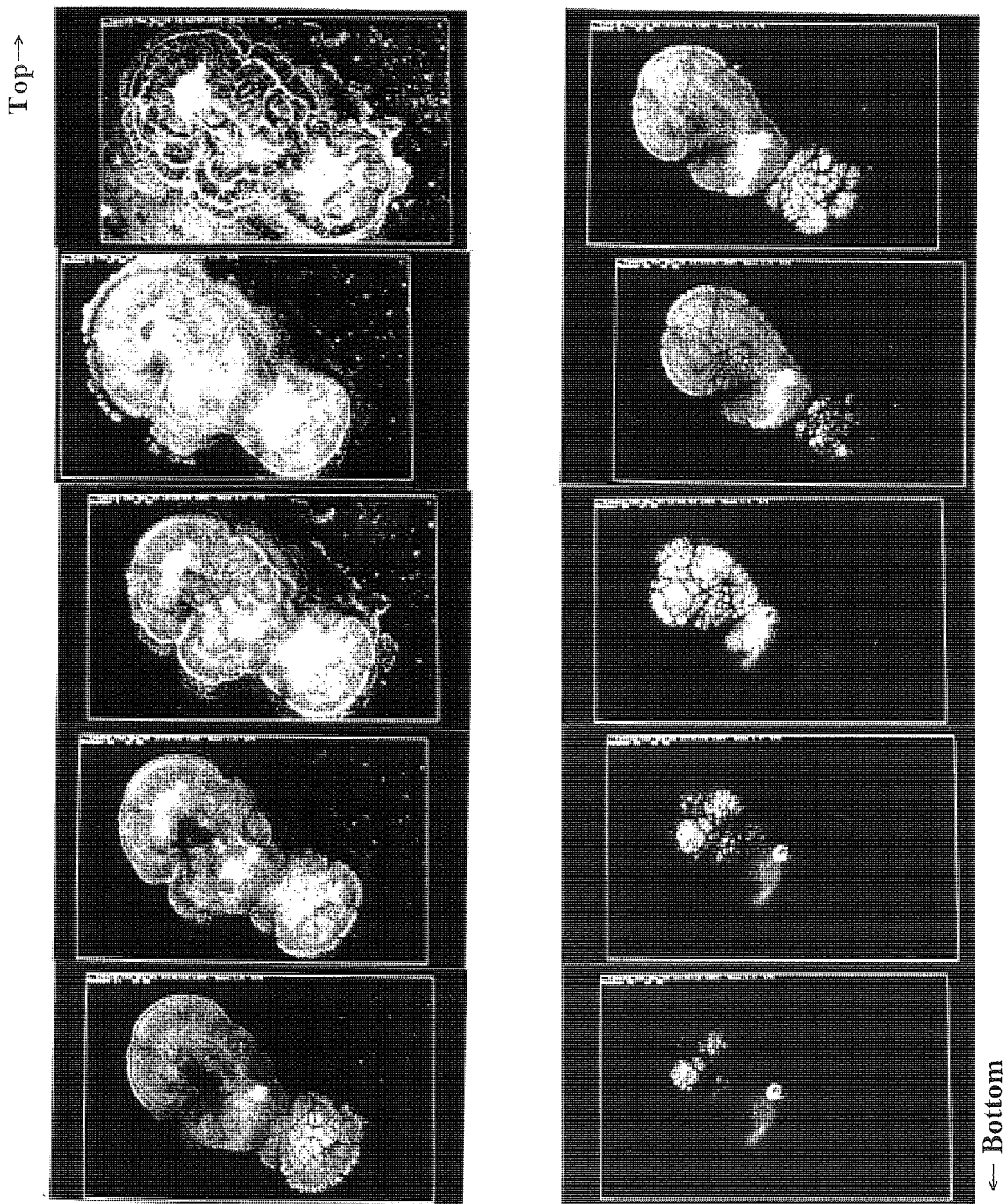
Figure 5.28 Composite reflectance image of white spot D
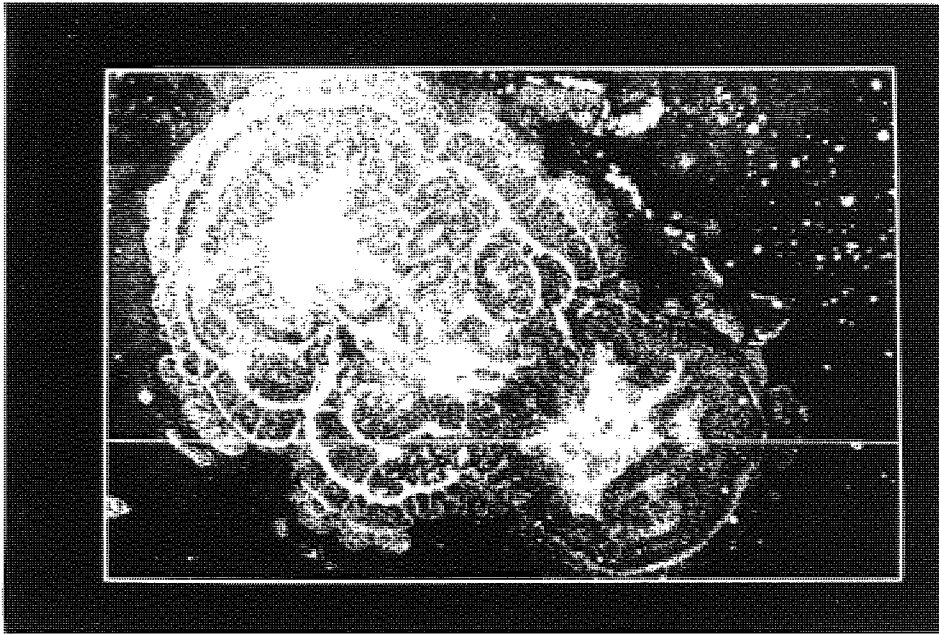
Figure 5.29 XZ section through white spot D at the position shown in figure 5.28
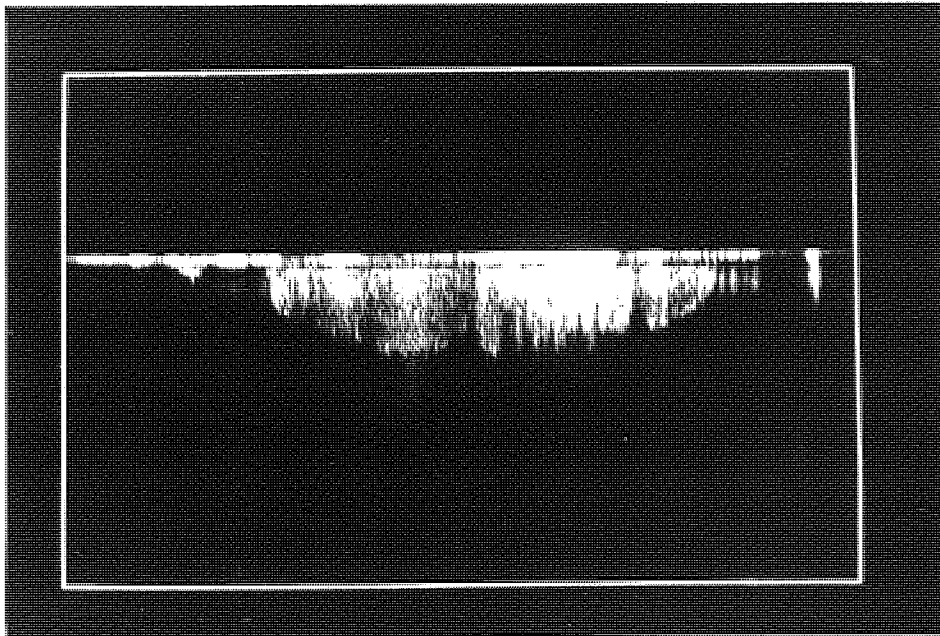
Figure 5.30 Set of confocal reflectance images at high power through white spot D
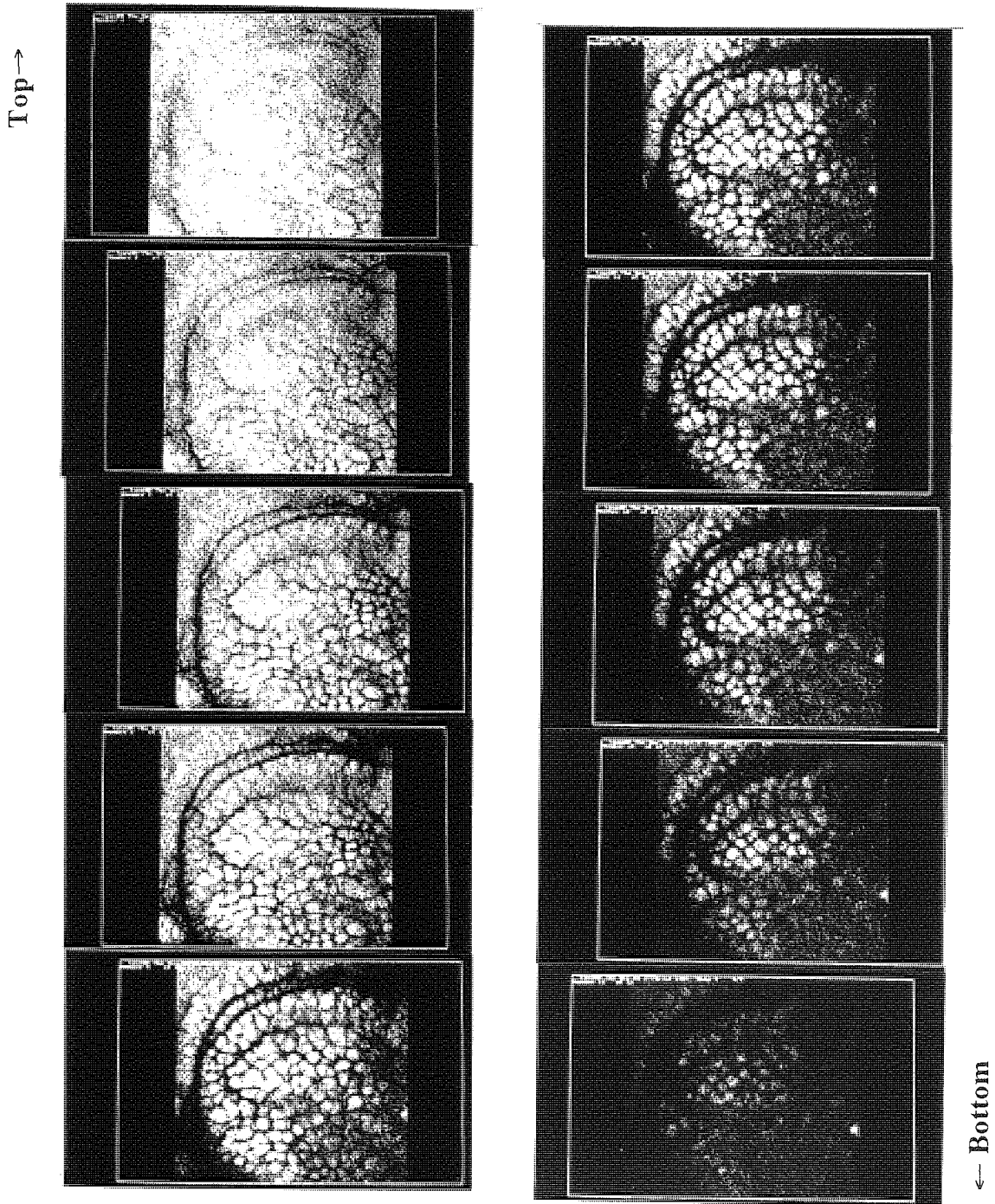
Figure 5.31 Composite reflectance through white spot D at high power
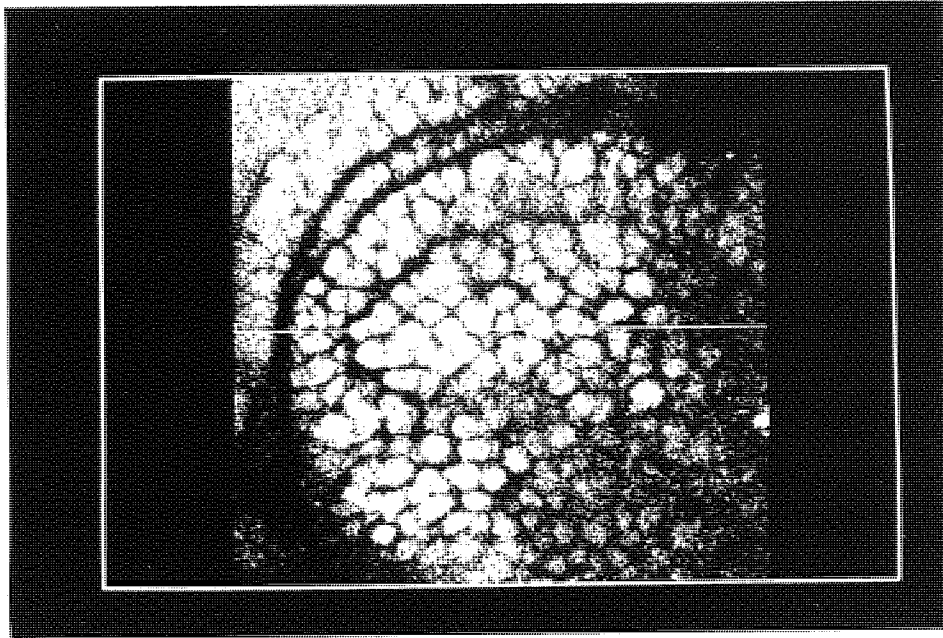
Figure 5.32 XZ section through white spot D at high power at point shown in figure 5.31
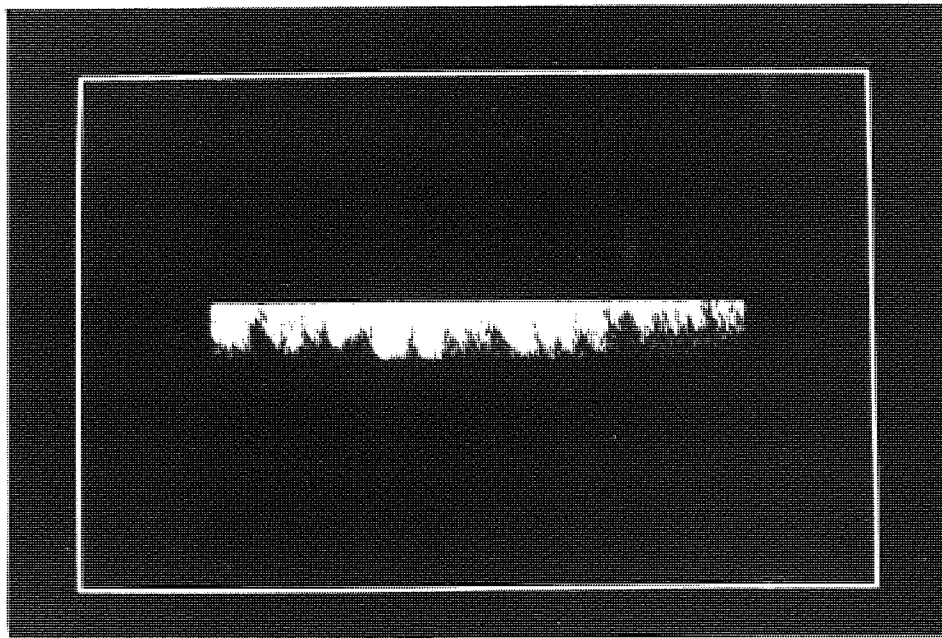
Figure 5.33 Pseudo colour image of white spot A
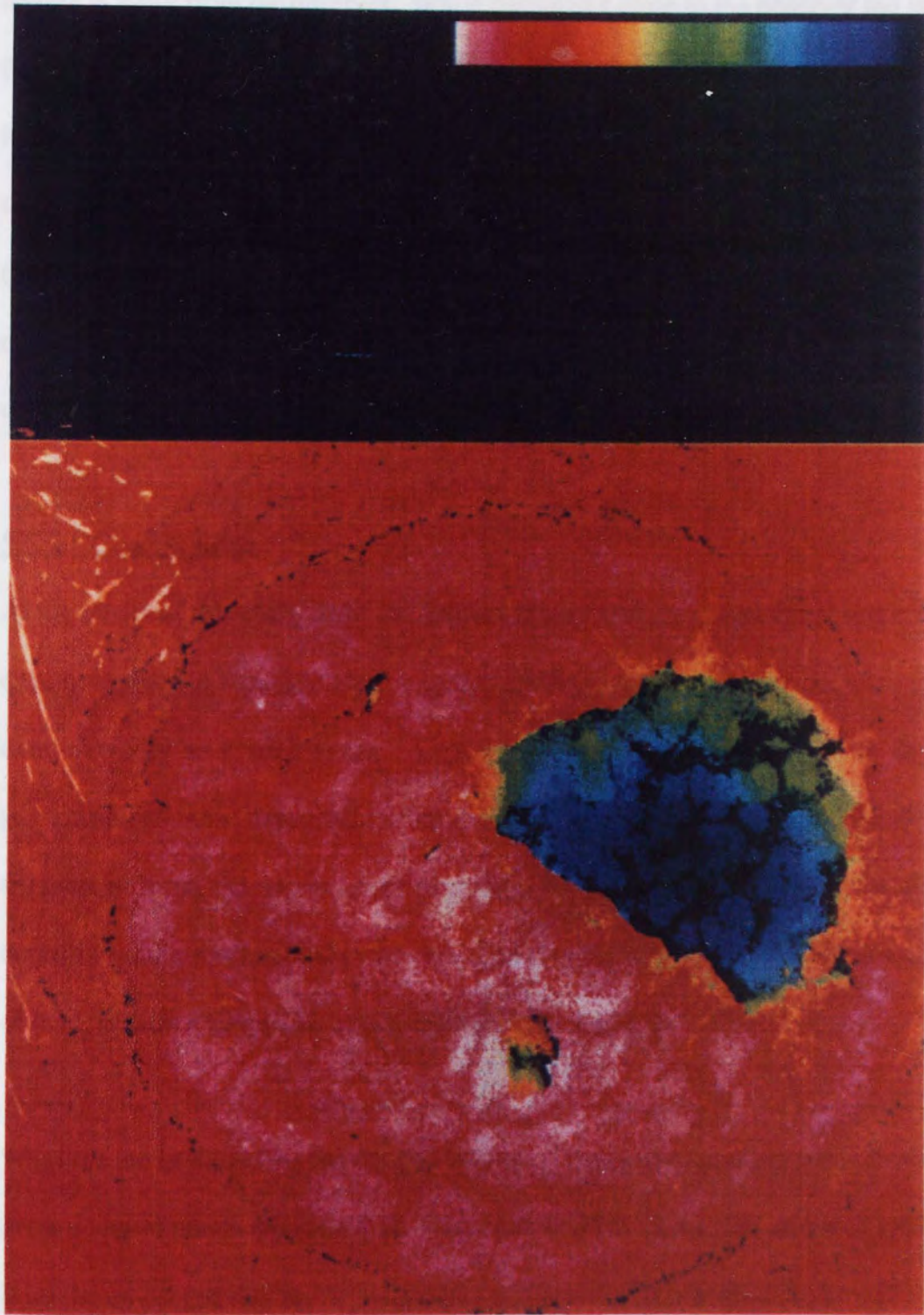
To give a better appreciation of the structure of white spots a pseudo colour image was produced, this is shown in figure 5.32. The colours represent the distance that a feature is away from the observer. White designates close to the observer red is a little further away then down through the spectrum, blue is far away with black showing the farthest features. Another way of looking at the display is that the colours are similar to those used on ordinance survey maps, white shows the snow on the top of mountains (the highest features), blue shows the depth of the oceans (the lowest features). Many of the other images could have been displayed in this manner but the power of this method of presentation is displayed well enough by this single example.

5.5.3 Conclusions

At the outset of this study little was known about the three dimensional shape of these deposit types, the structure has, by means of the confocal microscope, been deduced unequivocally as a cone shape. Little or nothing was know about the degree of penetration of these deposits into the lens matrix, this study has show, that for some deposits at least, the deposit penetrates into the gel matrix. The observation of the deposit foundations has lead to a postulation that the deposit grows into as well as out of the lens with a finger like structure at the growing edge.

What are the implications for the elucidation of the spoilation mechanism ? Are these gross physical trends mirrored at the molecular level in all manifestations of spoilation ? It can be envisaged that the deposit grows on the molecular level, into, as well as out of the lens matrix. This possibility should be borne in mind when formulating models for deposit build up.

CHAPTER 6

FTIR spectroscopy

6.1 Infrared spectroscopy:- Introduction

Infrared (IR), spectroscopy is a valuable technique for general chemical analysis, it yields information about a compounds identity and in the case of some biological molecules details of conformation.

The absorption of electromagnetic radiation in the region 0.7μm to 33μm is due to the transition between vibrational energy levels within the molecule under examination. These vibrational energy levels represent the bending and stretching as well as simple vibration of the bonds present. It is these states that are affected by the absorption of IR radiation, thus much of the chemical makeup of a molecule can be deduced from its IR spectrum.

The absorption bands found in a sample are normally given wave number units rather than the frequency or wavelength as used in other types of spectroscopy. The wave number is simply the number of waves per centimetre, and is equal to the reciprocal of the wavelength in centimetres:-

$$v = \frac{1}{\lambda}$$

equation 1

where v = wavenumber in cm^{-1} and λ=wavelength in centimetres.

Many of the bands found in the IR spectrum can be attributed to particular chemical groups present, whilst other regions containing much spectral detail cannot be ascribed to particular groups, these region are thus called fingerprint regions. If two compounds

are identical we would find that as well as the group attributable bands being identical the fingerprint region would also be equivalent. This acts as a final conformation as to the identity of an unknown compound by comparison with known standards.

6.2 Dispersive IR Spectroscopy

IR spectroscopy was until recent times carried out on dispersive instruments, in these the IR beam derived from a hot filament is split into a spectrum by means of a diffraction grating, this is then passed through the sample and the emergent beam detected by means of a thermocouple. A component in the diffraction moves so as to change the wavelength of the incident beam thus an absorption spectrum is constructed. The signal from the thermocouple is fed to a chart recorder and plotted as intensity against wavenumber. This technique proved adequate for most purposes but suffered from poor resolution, long scan times, low signal to noise ratios and a low energy throughput. The advent of the Fourier transform infrared spectrometer overcame the above shortcomings. With these spectrometers high resolution IR spectra became possible with short scan times, this was due to the intrinsic manner that the spectrum was collected, also the computer data storage inherent in the technique lead to the possibility of scan averaging and facilitated further data manipulation.

6.3 FTIR Spectroscopy

In the Fourier transform infrared spectrometer the spectrum is collected not in the conventional form but as an interferogram, it is this that is manipulated by means of a Fourier transform algorithm into a recognisable spectrum. The interferogram is formed by a simple interferometer made up of two mirrors, one moving one fixed, and a beam splitter. The Michelson interferometer below is an example of one type of

interferometer found on many commercial spectrometers.

Figure 6.1 The Michelson interferometer
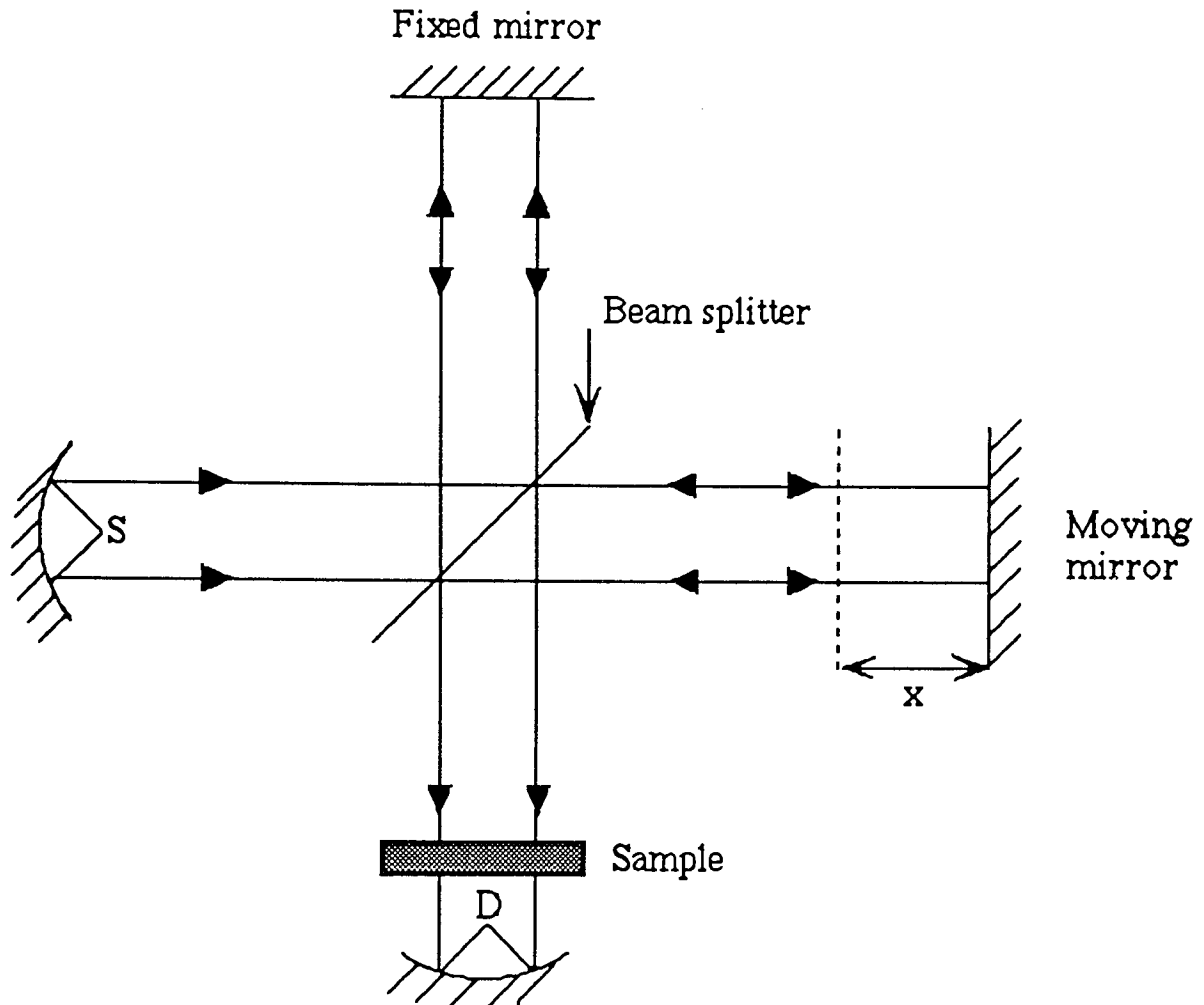
Key:- S= IR source   D=detector   x=distance the mirror travels

The IR beam from the source (S), is focused by means of a concave mirror onto a beam splitter, a portion of the beam is reflected upwards to the fixed mirror which reflects the beam back to the splitter, which allows part of this beam to pass through; this impinges on the sample and then by means of a focusing mirror reaches the detector (D). The second part of the beam passes through the beam splitter and onto a mirror that can be moved, this beam is then reflected back and a portion is directed through the sample

and reaches the detector.

The interferogram is produced by moving the mirror slowly back and forth and recording the signal produced. The interferogram is the product of the path differences that the two components of the beam have suffered. If the distance is a zero or a whole wavelength then the two beams reinforce each other, whilst is the path difference is one half wavelength the beams interfere with one another and no signal is recorded. As well as this gross effect the interferogram also contains spectral detail that can be derived by mathematical manipulation.

A Fourier transform is the means by which the spectrum of the sample is extracted from the interferogram. If the mirror displacement is x then the intensity of the beam at x is $I(x)$, if the intensity of the source as a function of frequency in cm^{-1} is $B(v)$, the equation for the signal is

$$I(v)=B(v) \cos(2\Pi xv) \qquad \text{Equation 2}$$

If a second frequency of IR is added to the above example we will see the sum of the above plus the contribution from the second source. The detector will see the sum of the two cosine waves

$$I(x)=B(v_1)\cos(2\Pi xv_1)+B(v_2)\cos(2\Pi xv_2) \qquad \text{Equation 3}$$

If all frequencies are added in up to an infinite number we get the interferogram as

collected on the instrument described by

$$I(x)=\int_{-\infty}^{+\infty} B(v) \cos(2\Pi xv).dv \qquad\qquad \text{Equation 4}$$

As we have a sample in the beams path, the absorbed frequencies will not be making a contribution to the final interferogram. The missing frequencies will make their absence felt by the presence of an inverted cosine wave across the interferogram. Equation 4 is one half of a cosine Fourier transform pair, the other half is equation 5 shown below.

$$B(v)=\int_{-\infty}^{+\infty} I(v) \cos(2\Pi xv).dx \qquad\qquad \text{Equation 5}$$

The pair of equations fully defined the relationship between the interferogram and the spectrum. The first equation describes the variation in power as a function of path-length, which is the interferogram. The second shows the variation of intensity as a function of difference in path length. The two are inter-convertible by means of the mathematical system called a Fourier transform. A number of algorithms have been devised to enable computers to perform the transform in a reasonable time period.

The FTIR spectrometer has many advantages over dispersive instrument such as improved energy throughput better resolution and signal to noise ratio. The signal to noise ratio can be further improved by virtue of the fact that the data collected is stored digitally on a computer thus many spectra can be collected and then averaged. The

noise is random and thus should average out to zero whilst the spectrum is constant and is thus unaffected by the averaging. Another advantage of digital storage of data is that it can be manipulated for a number of reasons including spectral subtraction and the quantification of a single component in a mixture.

6.4 Sampling Techniques

The IR of a sample can be gained by means of a number of accessories, the system used depends on the physical nature of the sample and on the amount of sample preparation that is desired.

The sampling techniques available during the period of research included multiple internal reflectance (MIR), IR Microscopy and photo acoustic spectroscopy (PAS), their suitability to study the system under investigation was evaluated. The next sections discuss the work done, the problems encountered and the possible solutions.

6.4.1 Multiple Internal reflectance (MIR)

MIR is a method by which the IR spectra of a surface can be acquired, this makes it attractive for the study of biomaterials in which the majority of changes take place at the solid interfaces of the implanted material. MIR is sometimes termed attenuated total reflectance (ATR), but in this discussion it will be called MIR, as it is a better descriptor of its mode of operation .

The sample should be flat and pliable enough to make a good contact with a flat surface. Hydrogel contact lenses fulfill these criteria. The sample is placed onto a crystal of an IR transparent material, two commonly used materials are germanium and

KRS-5, a mixture of thallium chloride and thallium bromide. The crystal is shaped as shown in the diagram below (figure 6.2).

Figure 6.2 The shape of a typical MIR crystal

Face on which sample is placed

Face that the IR beam enters
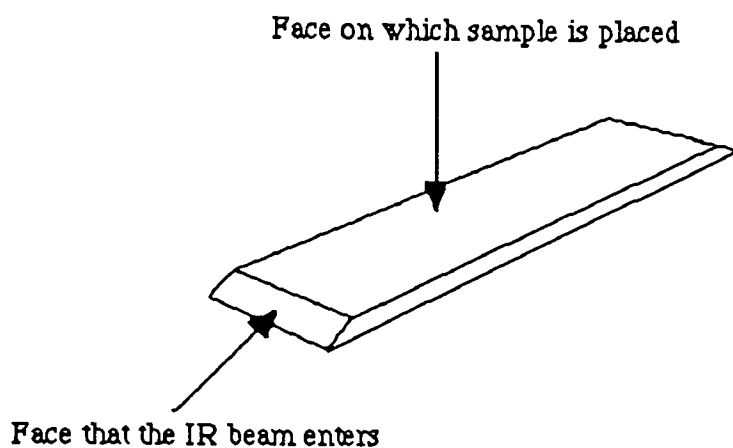
The crystal and the sample are then bolted between two steel plates (see figure 6.3), this sandwich is placed into the MIR attachment, which is a simple arrangement of mirrors to direct and focus the IR beam into the crystal and to collect the emergent beam (shown in figure 6.4).

Figure 6.3 MIR crystal bolted into place

Upper metal plate

Sample position

IR beam

Lower metal plate
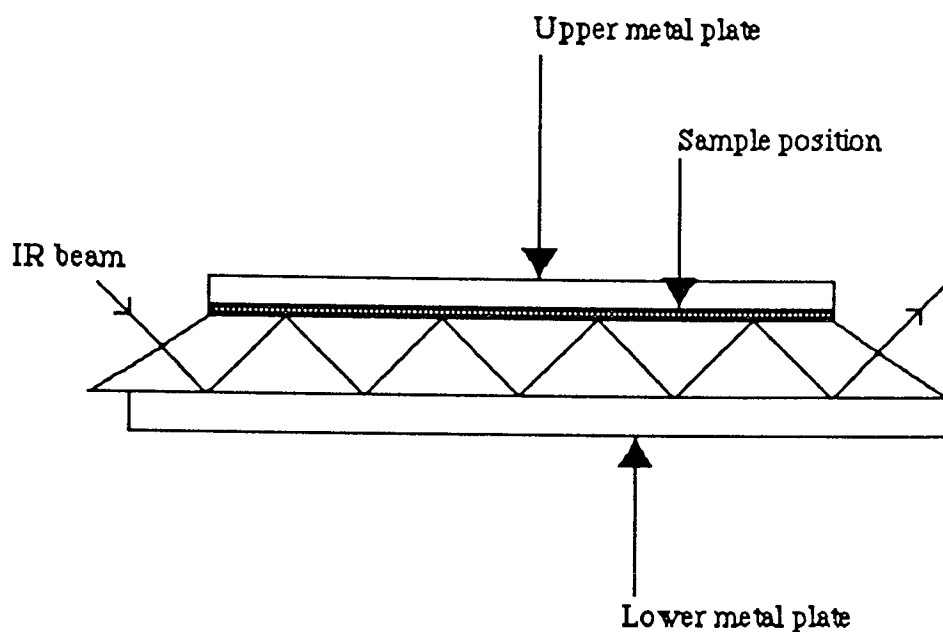
Figure 6.4 Infrared beam through the MIR attachment

Focusing mirrors

IR source

IR detector

Directing mirror

MIR crystal

Collecting mirror
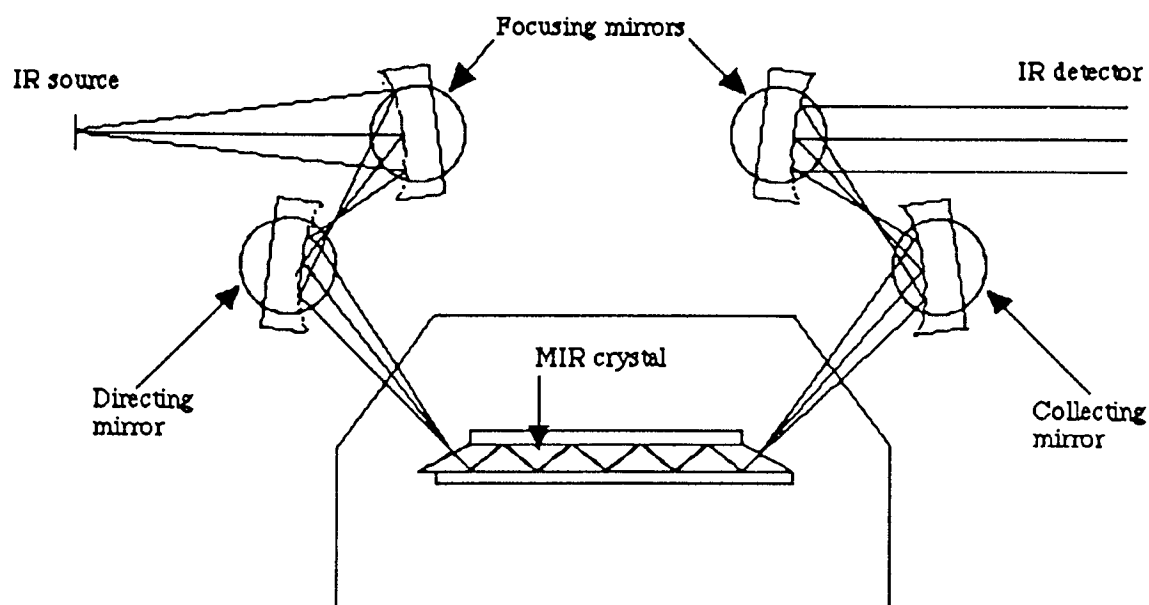
The IR beam from the spectrometer is directed by means of two mirrors onto one end

of the crystal and is reflected off the internal surfaces along the crystal. The beam

emerges from the crystal and is collected, focused and directed back into the

spectrometer again by the means of mirrors. At each successive reflection the beam is reflected internally off the inside face of the crystal, a small portion of the beam, called the evanescent wave [138], penetrates into the sample which is in close contact with the crystal. This portion of the beam is the reflected back into the crystal, it is this portion of the beam that has impinged into the sample that gives us our IR spectrum. An FTIR rather than a dispersive instrument is required because of the low energy throughput of this attachment and the necessity of successive digital manipulation of the data collected. The ability of FTIRs to collect a large number of spectra from a single sample allows the use of spectral averaging to remove the high noise levels encountered with this attachment. The energy throughput of the device is low and the noise is high due to the large number of mirrors and absorptions in the crystal, also not all the beam is accurately directed into the crystal thus considerable losses occur. It should be noted that the beam that emerges contains only a small contribution from the sample as only a low proportion of the beam has contacted the sample.

The operator has control over a number of variables in the MIR accessory, the angle of incidence of the IR beam onto the crystal face affects the depth of penetration of the beam into the sample, as the angle of incidence increases the depth of penetration decreases. The depth of penetration can also be altered by the choice of crystal material, as the refractive index increase the depth of penetration goes down. A range of materials are available to the spectroscopist but often other factors limit the choice of material such as solvent resistance or effective range in the IR. As hydrogels contain a high proportion of water the resistance of the crystal to water is of high importance, KRS-5 (thallium chloride/thallium bromide), is slowly dissolved by water thus cannot be used in the long term. KRS-5 is very toxic and thus the operator must wear gloves

at all times, and contact lenses analysed could not be placed back in the patients eye as part of an ongoing experiment. A germanium crystal was chosen because of its water resistance, negligible toxicity and high mean refractive index, thus the penetration into the sample is low. Germanium is expensive compared to KRS-5 and is very brittle and needs to be handled with extreme care.

6.4.1.1 Methodology

All the MIR infrared spectra were collected on a Perkin Elmer 1710 FTIR spectrophotometer. The data was digitally stored and manipulated on a Perkin Elmer 3600 data station. The spectra were collected at a resolution of 4cm^{-1}. Each spectra was averaged over 50 scans, this gave a scan time of ~7.6 min.

The spectrum as collected has contributions from a large number of different sources, the crystal even when new and clean has some intrinsic IR absorbance, contamination on the mirrors and steel plates also make differences to the spectra. To remove these effects the spectrum of the MIR attachment without the sample is collected and this is then subtracted digitally from the collected spectrum. The scaling of this subtraction is done by eye as no other suitable method gives better final results. The spectrum is subtracted so that the small, contamination derived-peaks, are made to disappear.

A spectrum of the clean germanium crystal was run at the beginning of each session. The lenses were placed on the germanium crystal, making sure no air bubbles were introduced. The sample was always placed on the upper face of the crystal so as to maintain the mirror alignment. The metal plates are then bolted each side of the crystal , as in figure 6.3 and 6.4.

The MIR attachment was then placed in the sample beam, a beam attenuator was placed in the reference holder and the spectrum was then collected. A sample of the saline from the vial in which the lens had been equilibrated was run on the germanium crystal in the same way as the lens.

The three spectra, crystal, sample and saline, were all transferred from the instrument computer to the data station. The spectra were then stored on disk for future reference.

It was then necessary to carry out a number of spectral subtractions, so that the biological material that had been deposited could be characterised. To do this was necessary to remove the contribution of the water (saline), and the crystal. The spectrum of the saline was first removed from the sample, the scaling of the subtraction was done by eye, i.e. disappearance of the two characteristic water peaks. This difference spectrum then had the spectrum of the crystal subtracted, again the scaling factors were judged by eye, this time on the basis of the disappearance of noise contributed by the crystal.

The spectrum obtained, is it hoped to be a true representation of the infrared spectrum of the surface, but it also includes a large contribution from the polymer matrix. The contribution from the polymer can be removed by a third subtraction, that of a unworn or unspoiled contact lens.

This multiple subtraction is very prone to errors, in some cases the spectrum from the un worn lens was simply subtracted from the worn lens spectrum. This process should remove spectral contributions from the crystal and the water in the gels as these are

constant. This method was tried but with little success, the sample size become critical if the subtraction is to be successful. The contact between the crystal and the material is another parameter that has an effect on this type of manipulation. As the water film acts as the optical couple between the sample and the crystal an change in this leads to large changes in the spectral intensity, making subtraction harder. The pressure exerted by the steel plates is the critical factor, the only way to do this would be to use some sort of mini torque wrench. The horizontal MIR described later with its spring loaded clamp can reproduce pressure on the sample and thus avoid this problem.

6.4.1.2 Results

The results from this method were very disappointing. At best a fine IR spectrum of the hydrated contact lens material was obtained. The computer being used on the Perkin Elmer 1710 optical bench is designed with routine IR analysis in mind. Using this computer it is only possible to carry out one spectral subtraction. The 1710's computer is linked to a 3600 Perkin Elmer data station, this is an obsolete device designed in the days of dispersive IR spectrometers. The data can be transferred to the 3600, this is the only method of storage of spectra on the system. The spectrum is transmitted to the second computer minus much information e.g., the wavenumber range over which it was collected is not preserved. Thus the operator must record other information if the spectrum is to be valid. Once all the spectra are in the 3600 it is possible to manipulate them. The subtraction and manipulation software is primitive, the interactive subtraction leaves much to be desired. As other workers have recorded reasonable spectra of this type it must be assumed that the spectrum collected contains the desired information, but given the manipulative tools available on this device, their

extraction is not possible.

Figure 6.5 shows the spectrum obtained from a clean Vistacon soft contact lens, figure 6.6 shows the same lens after 48 hours in foetal calf serum (FCS). The FCS treatment was used to spoil the lenses, so that the same lens could be studied before and after spoilation. If patient lenses were to be used a degree of uncertainty as to the lenses identity would ensue, also a time lag would be introduced. The FCS spoilation method has been shown in these laboratories to be a good surrogate spoilation medium. The difference spectrum was obtained from figures 6.5 and 6.6, this is shown in figure 6.7. The temptation is to sit down and start assigning the bands obtained. It was found that if the same raw data were processed on different days by the same operator the results obtained are very different. It must be assumed that the spectrum in figure 6.7 is mostly artifactual caused by over or under subtraction. The ideal situation would be a subtractive technique that did not rely on the judgement of the operator, or spectra of high quality that could be subtracted unambiguously.

Figure 6.5 IR spectrum of a clean Vistacon lens on an MIR attachment

Wavenumber(cm-1)
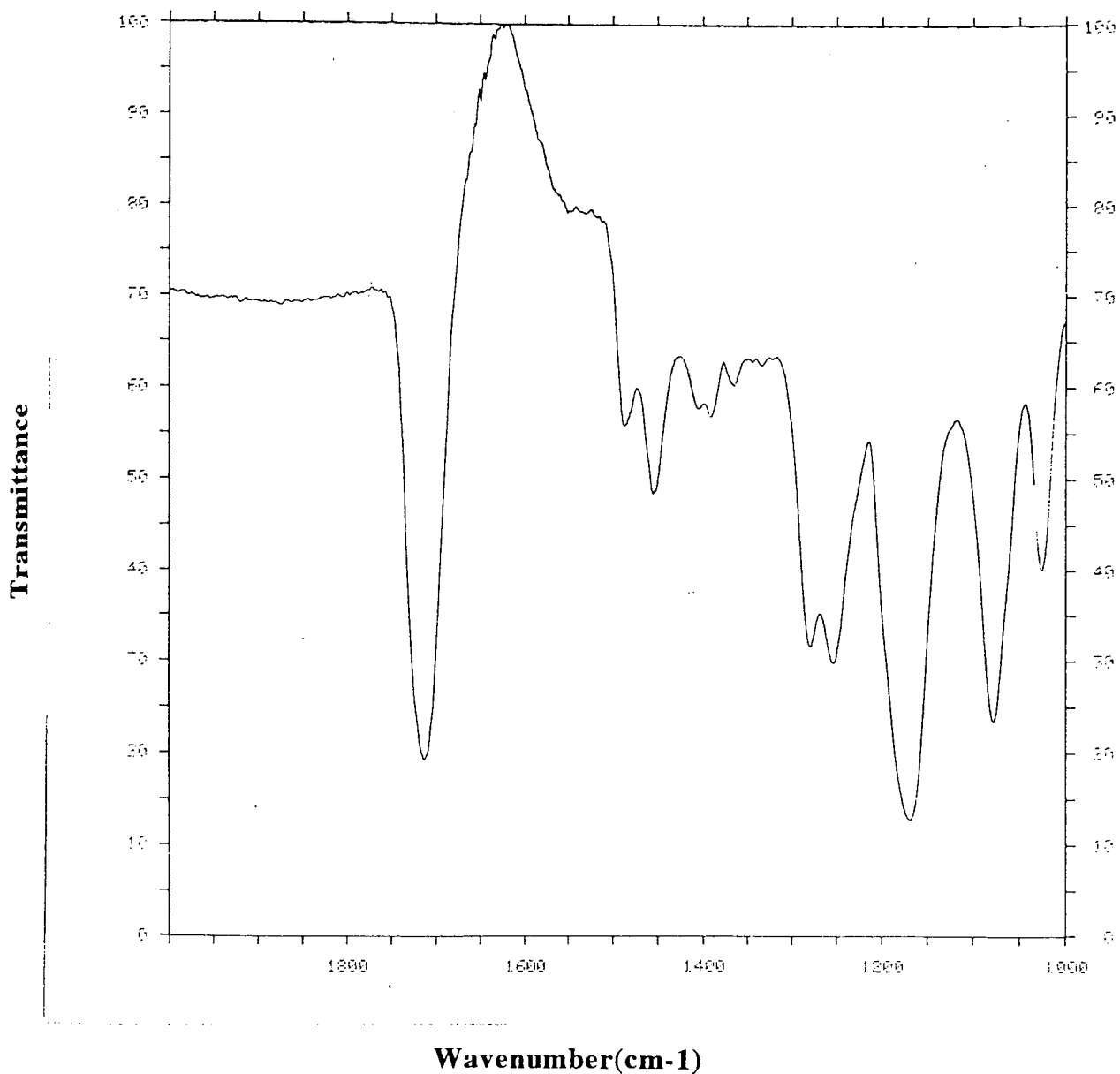
Figure 6.6 IR spectrum of a Vistacon lens after 48 hours in FCS on an MIR attachment

Wavenumber(cm-1)
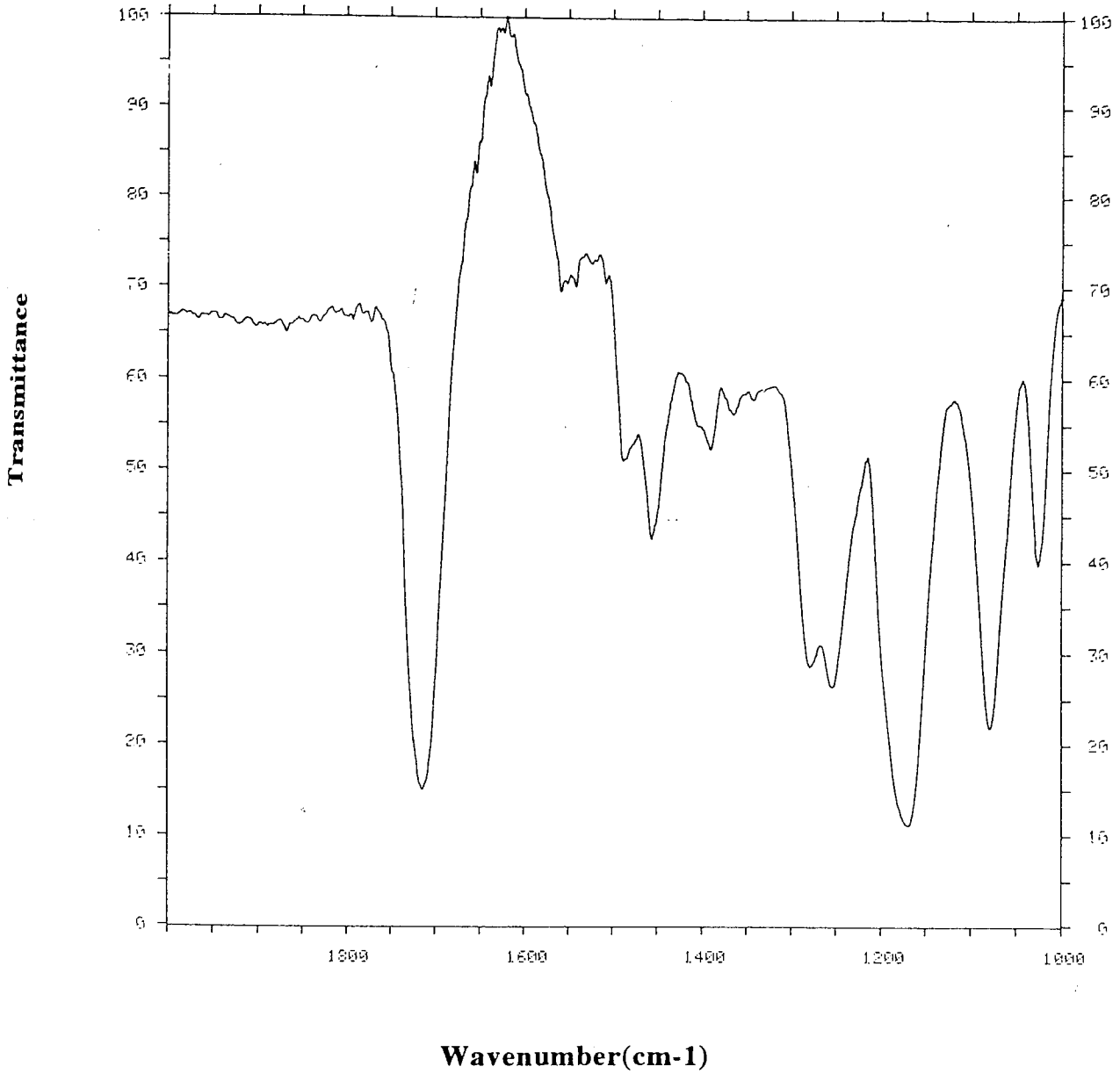
Figure 6.7 Diference IR spectrum of spectra in figure 6.5 and 6.6

Wavenumber(cm-1)
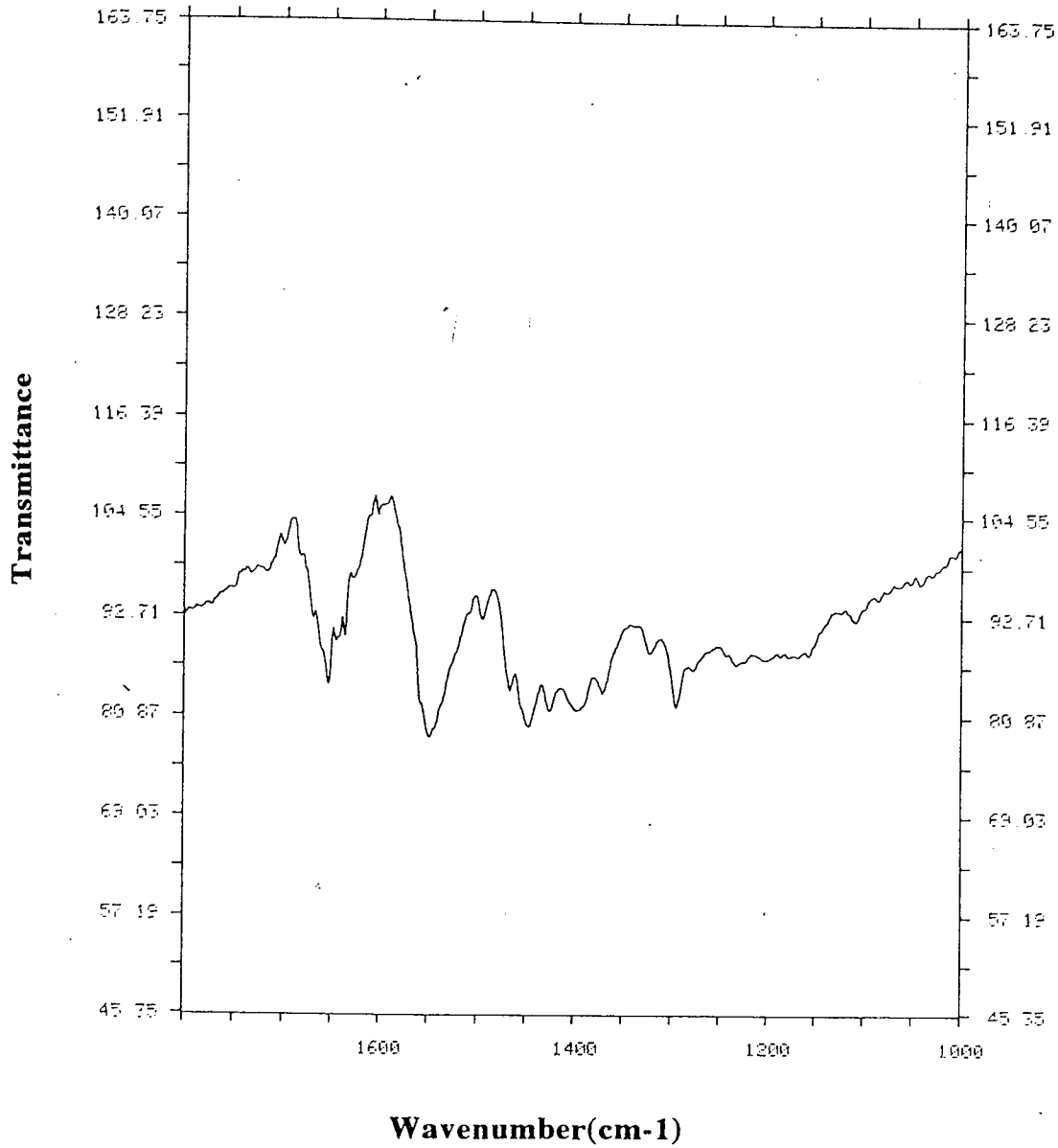
6.4.1.3 Horizontal MIR (H-MIR)

The theory and practice of H MIR (H ATR) is identical to that of vertical ATR. The only difference is the orientation of the crystal. The accessory used, a Spectra Tech Leading Edge H-ATR, is designed for ease of use and rapid sample changing. The sample is placed on the crystal face and a spring loaded clamp ensures good and reproducible contact. The spectrum is collected and a second sample can be put in its place. The throughput of samples is thus higher as no bolt removal or crystal positioning is required. This attachment was used exclusively on the Nicolet 510M bench with a Macintosh IIci computer. As will be discussed later this combination was significantly faster in spectral acquisition and the ease of spectral manipulation. The spectral information obtained once again was reserved to the bulk of polymer studied. When the spectral subtraction was carried out with care, and the protein deposition was relatively high it was possible to see some evidence of the amide bands in the spectrum. In real worn lenses no definite evidence of the presence of amide peaks from protein contamination were found.

Excellent results were obtained when the IR of a layer of cells grown on a polymer film was collected. The IR spectrum of the cell layer was easily subtracted from the polymer. This could prove to be a rapid and effective manner to estimate the cells on a materials surface. The possibility of this working is high as long as the sample size could be kept constant. The material would need to be pliable so that a good contact with the MIR crystal could be obtained. Standard curves could be constructed and unknowns estimated. The suggestion that IR could have a role in biomass estimation has appeared in the literature, Gordon et al.[139] used photoacoustic spectroscopy to

measure protein biomass.

6.4.1.4 MIR :- conclusions

The use of MIR systems to investigate soft contact lens spoilation does not appear to have a future unless software or hardware developments make the acquisition of spectral information from a low level contaminant in a bulk polymer possible. The technique certainly has a general use, it is a rapid way of obtaining a good IR spectrum from a solid film without the need for any sample preparation. Liquids can also be analysed by the use of a special crystal and holder, the solution can be recovered with only minimal loss.

6.4.2 Infrared Microscopy

The term infrared microscopy is a little misleading in that it suggests that an IR image of an object is collected, such an instrument does exist. The preferred but little used term is IR microspectrometry. In IR microscopy an infrared spectrum of a very small region of a sample is collected. The microscope achieves this by acting as a beam condenser. The IR beam passes out of the optical bench and through a series of reflecting lenses termed Cassegrains. The beam is then directed onto the sample, the transmitted or reflected IR beam is then redirected back into the spectrometer and ultimately to its detector. The selectivity of the technique is further enhanced by the use of an aperture in the pathway of the emergent beam, thus an area of interest can be masked off from the surrounding area and only IR features from this region are collected. In the microscopes manufactured by Spectra Tech, a second aperture is placed in the illuminating beam and is used to mask the sample identically to the first aperture. This system is termed redundant apertureing, the result is that better selectivity of region is

obtained, this is due to the fact that adjacent areas are not illuminated by the IR beam and thus scattered radiation from these regions make less of a contribution to the final spectrum. The redundant apertureing system is a patented by Spectra Tech and thus is not found on other microscopes. A schematic diagram of an IR microscope is shown in figure 6.8, this shows the microscope being used in transmission mode. The beam is shown being diverted back into the spectrometer to be detected, on the Analytical grade microscope used in this study a detector is built into the microscope. The dedicated detector used is a liquid nitrogen cooled MCT detector manufactured by Nicolet Instruments.

Figure 6.8 Schematic diagram of an IR microscope.
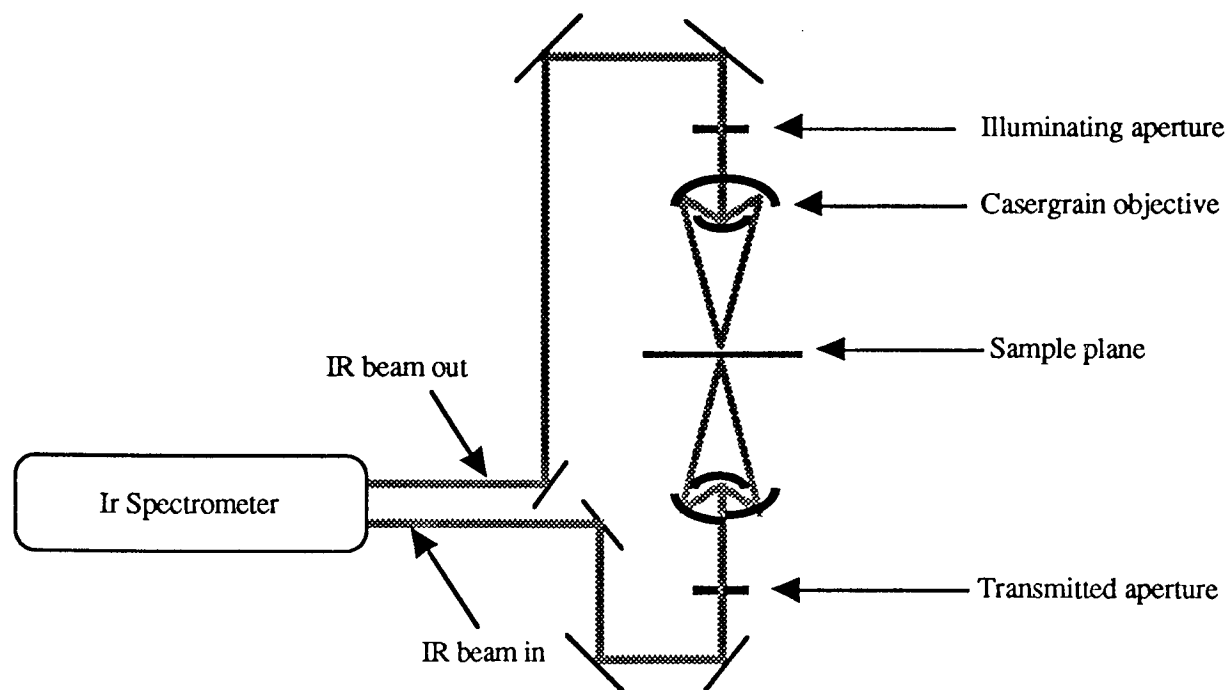
The IR microscope used is built up upon a Olympus white light microscope. All of the glass lenses and objectives are removed, these are replaced with all reflecting Cassegrain lenses. The quality of Cassegrain lenses are very high, as a result it is possible to view the subject under study through the reflecting objective, without the need for white light objectives.

Figure 6.9 Electron micrograph of a section cut through a soft
contact lens
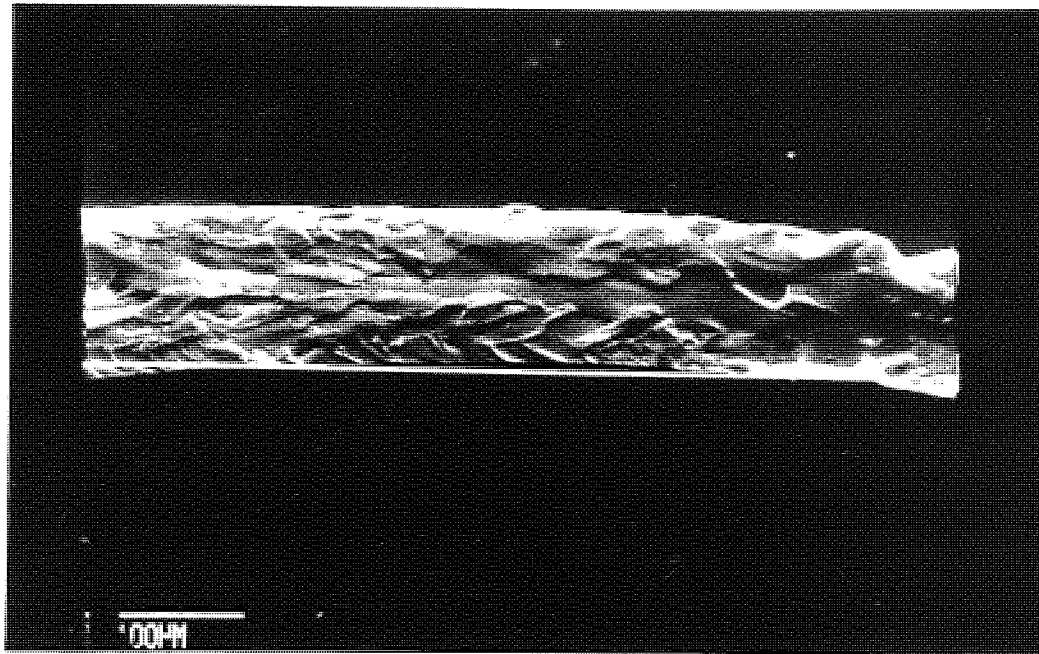
Figure 6.10 High power electron micrograph through a section
through a soft contact lens
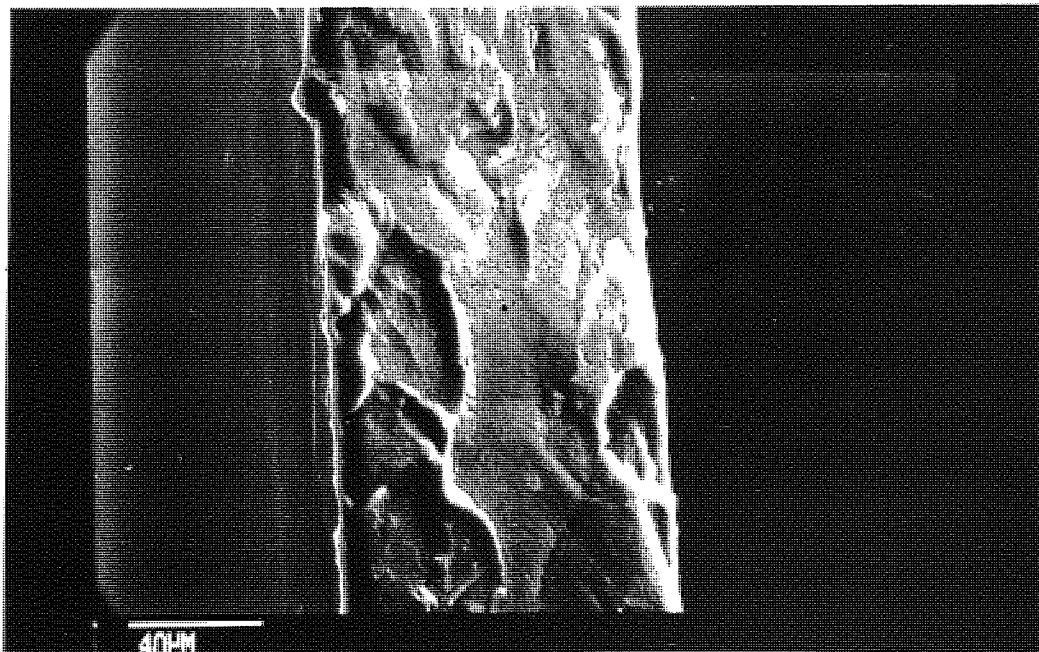
Figure 6.11 Photographs through the IR microscope of a fibre with progressive apertureing
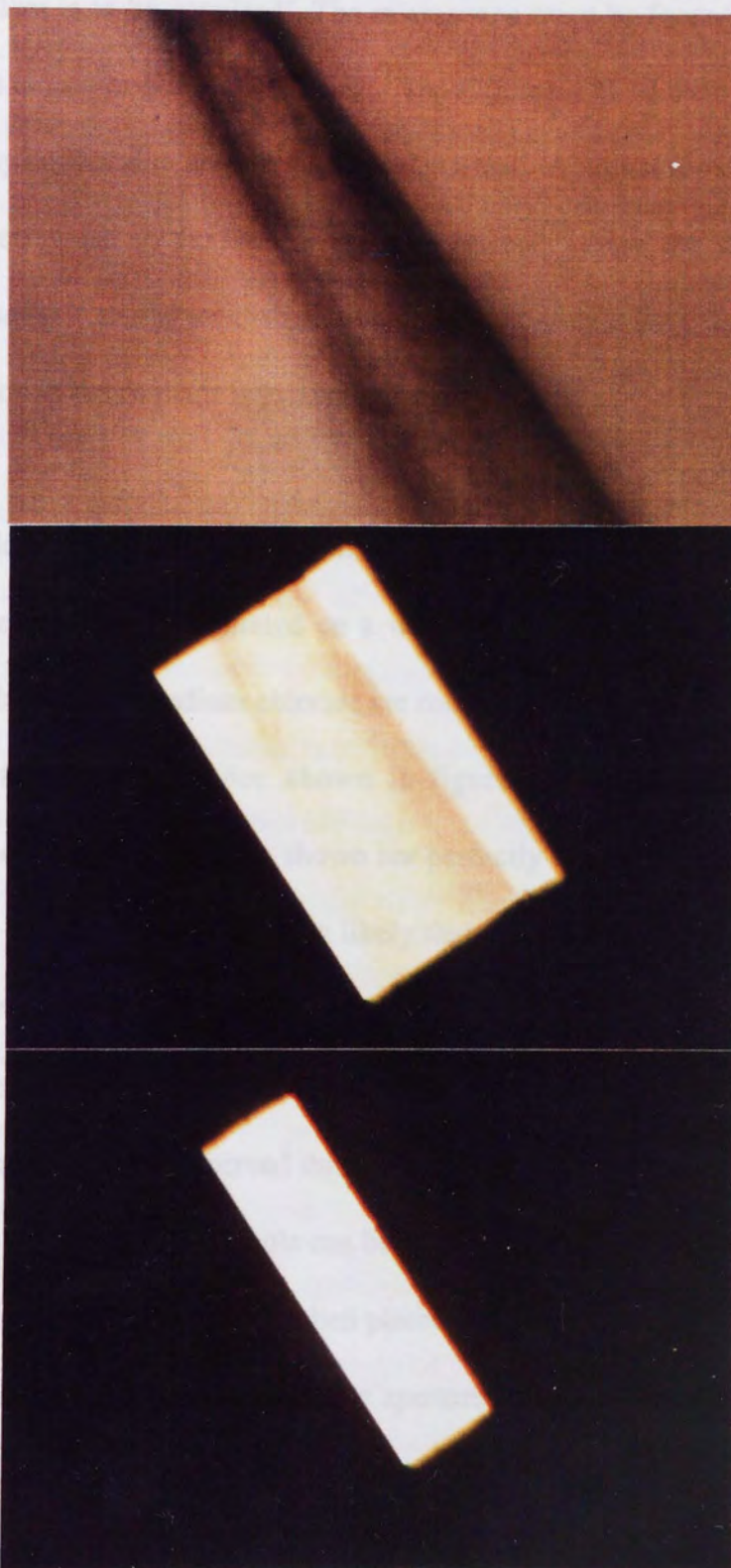
This ensures co-linearity between white light observation and alignment with the IR spectrum that is to be acquired. The microscope must be focused and adjusted on a daily basis to ensure good performance. The alignment of all the optical components is checked by performing an energy throughput test. A signal to noise ratio test should also be performed to ensure correct performance. Also the signal to noise ratio measurements if stored on disk can be monitored over a long period of time so that deterioration in the detector is picked up rapidly.

The sample after suitable preparation, in the case of soft contact lenses sectioning on a freezing microtome, is mounted on a window made of an IR transparent material. Calcium fluoride and sodium chloride are commonly used. The microtome produces a sample with the appearance shown in figure 6.9 and 6.10 under the electron microscope. The surface is as shown not perfectly smooth, this is ideal as if the cut was clean and the surface flat it is likely that a good deal of the IR beam would be reflected from the sample rather than passing into and through it. The sample should be made to lie flat on the window so that it is all contained within a narrow field of focus. The sample can then be observed through the reflecting objective, in our case a times 15 cassegrain was used. The sample can be examined and the region of interest observed. A set of knife blade apertures are then placed in the lower part of the microscope in the beam emerging from the sample. The apertures are at the same point of focus as the sample and can clearly observed. Figure 6.11 part 1, shows a typical fibre sample under the microscope, in part 2 the knife blades can be seen moving in on the sample. The blades are then advanced so that no stray unabsorbed portion of the IR is allowed through, this is shown in figure 6.11 part 3. The second aperture is then placed in the

illuminating beam so that it coincides with the first aperture, this aperture is also at the same focal point as the sample and can be easily seen by the operator.

The white light illumination is then switched off and the IR beam is sent along the same path. A series of safety interlocks are incorporated so that the observer cannot expose himself to the laser beam that comes along with the IR beam out of the spectrometer.

The IR spectrum can then be collected, the energy throughput is much lower in the microscope than in conventional KBr disk transmission. The MCT detector used is very sensitive and makes up for this deficiency, in fact the detector is so sensitive that it is very easy to saturate the detector leading to invalid spectra. As the spectrum is being collected the size of the interferogram displayed should be monitored to ensure it is no more that 10 volts. If the signal is too large it is a simple matter to reduce the size of the area from which the spectrum is being collected. The data was averaged over at least 50 scans at a resolution of $4cm^{-1}$, if time was a consideration, a resolution of $8cm^{-1}$ makes data collection much faster. The optical bench has a maximum resolution of $1cm^{-1}$, if the very fine detail of spectra are to be analysed this would give the optimum results, the time involved with the concomitant fourier transform would make data acquisition a lengthy process. The process can be divided, the computer can collect the raw data as an interferogram, the spectrum can then be constructed 'off line', when time is available. The Macintosh IIci computer can be made to run much faster than its published clock speed of 25MHz, by the addition of a fast ram cache card or a dedicated accelerator card. The expense of such an addition would be minimal compared with the initial expenditure on the microscope and optical bench.

6.4.2.1 IR microscopy results

The initial work with the microscope involved the development of a method suitable for obtaining the spectrum of a soft contact lens. It was thought desirable if we could analyse the lens non-destructively. Whole lens analysis proved impossible, the lens thickness means that totally absorbing bands are obtained. This proved true for both the hydrated and the dehydrated lenses. Sectioning of contact lenses had been carried out in these laboratories for some time. The method developed involved mounting the contact lens in water on the stage of a freezing microtome. The action of compressed CO_2 on this water causes it to freeze. A CO_2 cooled blade was used to cut the ice and the lens embedded in it. These sections were collected in a drop of water off the blade. The sections can be placed under the microscope in the hydrated state, it was found that the lenses start to dehydrate rapidly during sample acquisition. The gradual dehydration causes noise in the final spectrum because the sample did not remain constant. To improve this situation the lens fragments were dehydrated in a vacuum oven at reduced pressure at 40°C. The lenses were then placed under the microscope on a CaF_2 window. This proved to be a far more satisfactory arrangement, the lenses are hygroscopic and water peaks are found in the IR spectrum even after dehydration, but the lens rapidly reaches equilibrium with the atmosphere and the spectrum is stable with respect to time.

Figure 6.12 shows the IR spectrum of a section through a soft contact lens the typical collection area for these studies was $300\mu m^2$. The whole spectrum from 4000 to $400cm^{-1}$ is shown the contribution from water is clearly visible as a broad band at 3100 to $3600cm^{-1}$ and a narrower band at $1700cm^{-1}$. The contributions from the components of the hydrogel are visible. It would not be a worthwhile exercise to

disseminate all these bands as it is not lens composition that we are interested. A worn lens of the same material as that in figure 6.12 is shown in figure 6.13. At first glance the two spectra appear the same but if we consider the likely position of bands caused by adsorbed protein we see more. Figure 6.14 shows a magnified section of the spectra shown in figures 6.12 and 6.13. The spectra clearly show the presence of the amide peak due to the adsorbed protein as a shoulder at about 1755. The lens under study had only been worn for 2 weeks as part of a clinical trial and thus could not be described as heavily deposited, thus the power of detection exhibited is impressive.

It would be worthwhile at this point to discuss the IR spectra of proteins and other biologically important molecules. IR spectroscopy was until recently little used in biochemical investigations of large molecules. The resolution of the available spectrometers and the difficulty of spectral manipulation made the unravelling of the complex IR spectra impossible. Recent advances in IR spectroscopy, computational manipulation and the reduction in the cost of FTIR has lead to a revaluation of IR in this field. A body of literature has appeared that has started to equate the IR spectra of proteins with the tertiary structure of the protein. In appendix A2.1 a number of tables appear these equate the position of the bands in the IR spectra of a wide range of proteins with the presence of α-helices, β-pleated sheets and random structures within these proteins. The body of work presented in appendix A2.1 is drawn from a large number of papers as detailed in the appendix [140-153]. The spectra in question have been collected by a wide range of sampling techniques and using a range of instruments and solvents. The result of this is that although useful, the data contained should be treated with care. The data is presented in the appendix A2 as a spread sheet, this allows the solvent and sampling technique to be ascertained if doubt exists as to the

validity of any particular data. The information in the available literature points to the wavenumbers of interest being those around 1650 and 1545 which represent the amide 1 and amide 2 bands respectively.

The conformation of proteins has been attributed to bands appearing in the IR spectrum, these bands are often not visible in the raw spectrum, manipulation and enhancement being required. Much of the work has centred on the bands that go towards making up the amide 1 and 2 bands, small shifts in the values of the many bands that contribute to the overall shape of these bands. The overlapping bands have been enhanced by the use of a Fourier self deconvolution (FSD)[142,143,150], this method allows the overlapping bands that make up the IR spectrum of proteins to be resolved into its constituent bands. A second method[140,145,146], is that when two bands overlap and one band appears as a shoulder the value for the peak centre can be ascertained by taking the second derivative. The fourth derivative can also be taken, this has the advantage of having the peaks of interest pointing upwards. The values obtained by these methods can be seen in table A 2.2. There is little general agreement apart from on a few points. Much of this variation is due to the vast array of sampling techniques, physical form of samples and solvent used.

Figure 6.12 IR spectrum of an unworn soft contact lens

Wavenumber (cm-1)

N1c/IR
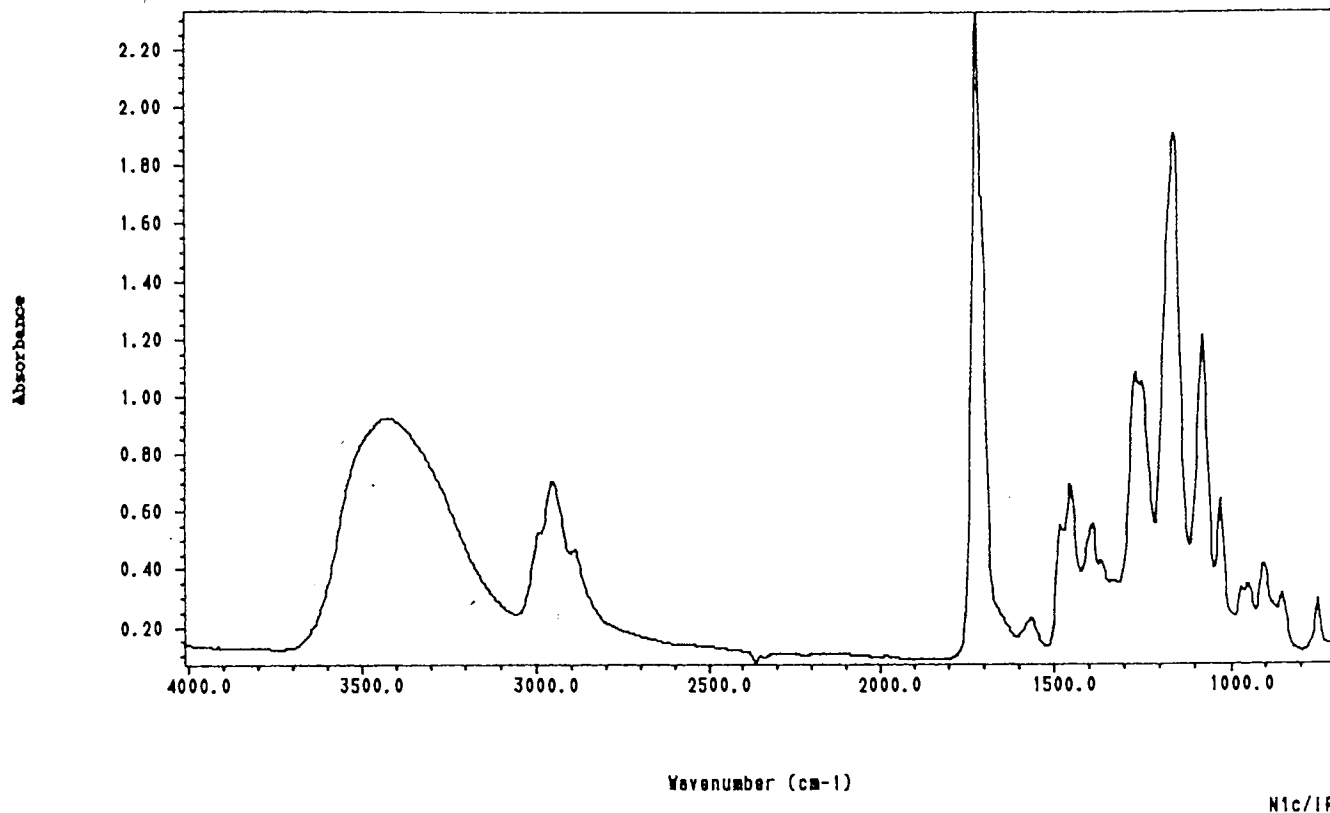
Figure 6.13 IR spectrum of a worn contact lens (same material as that in figure 6.12)

Wavenumber (cm-1)

N1c/IR
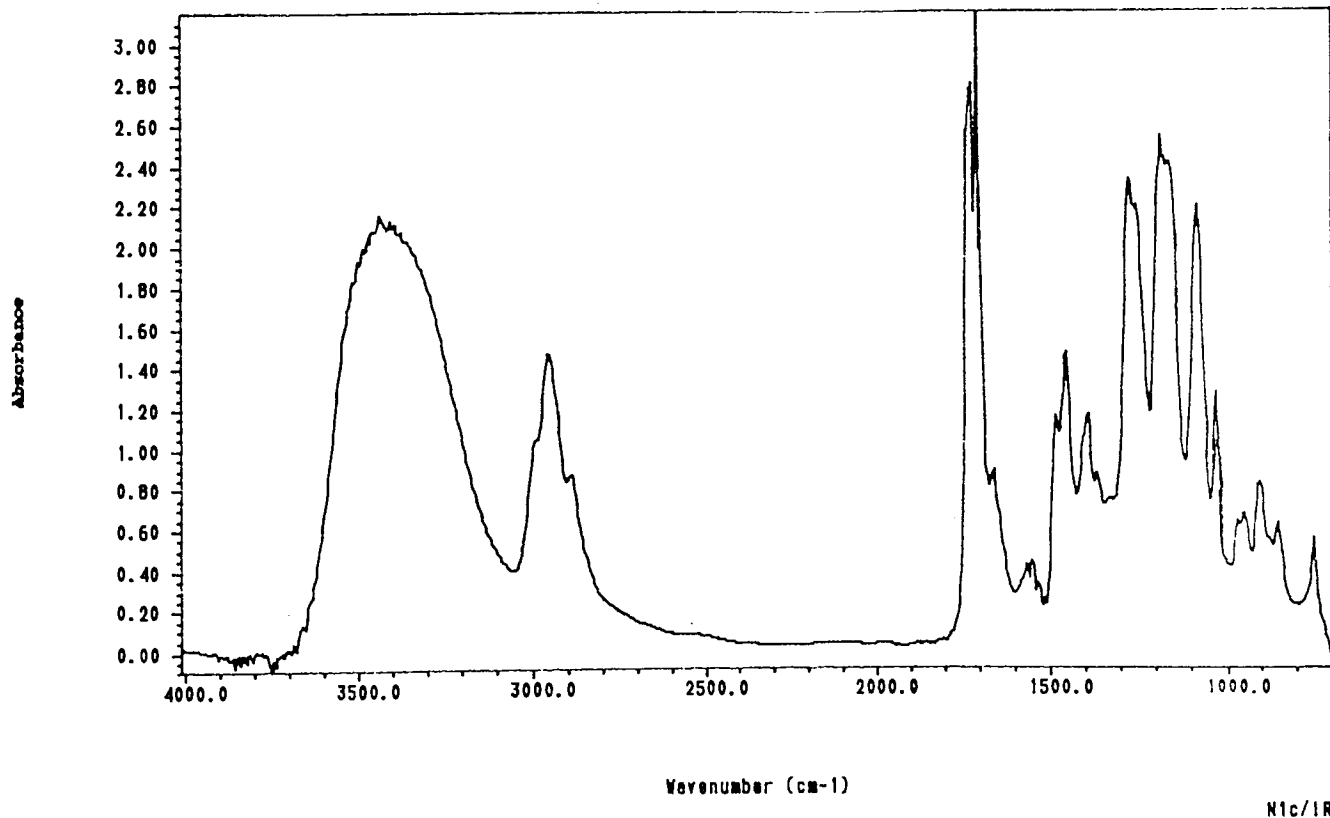
Figure 6.14 Spectra of worn and unworn soft contact lens

Wavenumber (cm-1)

N1c/IR
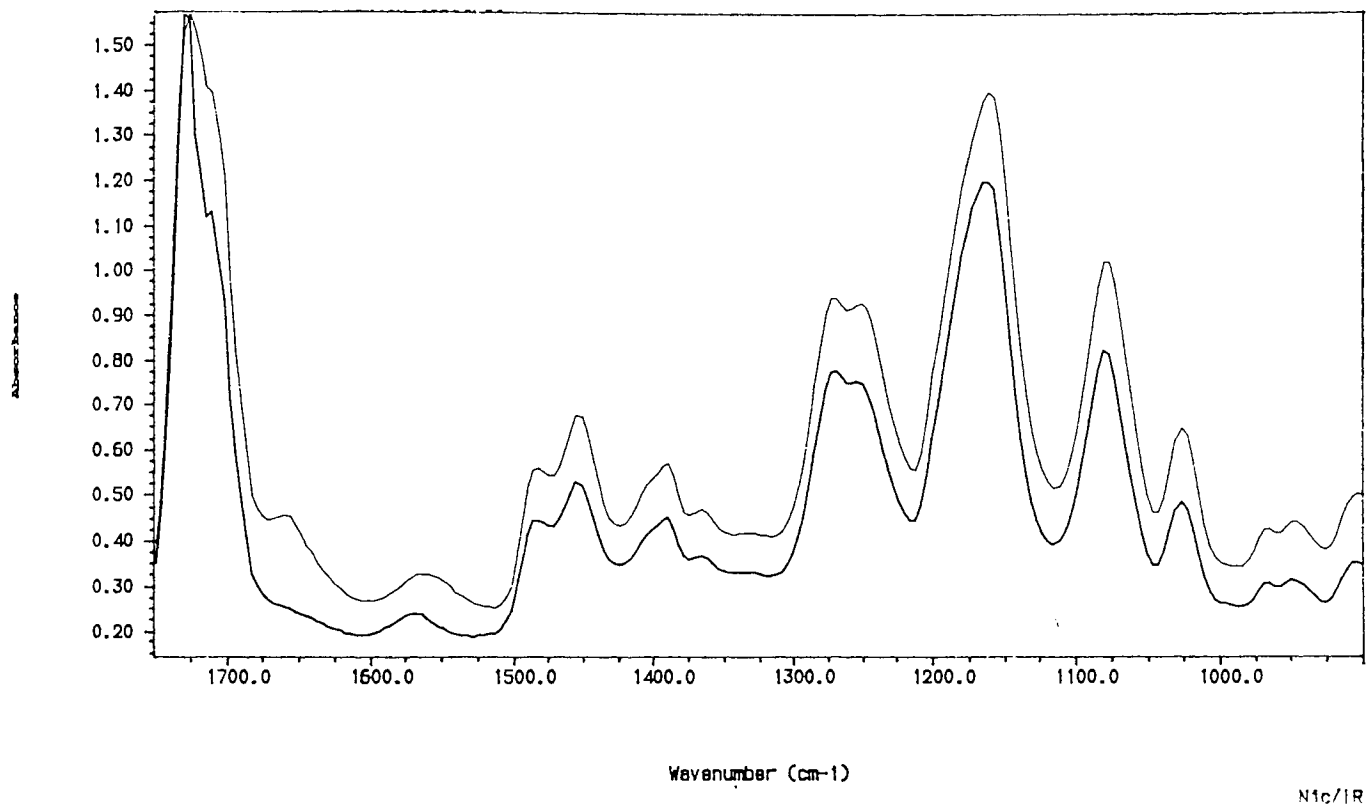
(upper spectrum is worn lens, lower spectrum is unworn lens)

In these present studies the spectra often contain information as shoulders, thus rather than performing a subtraction it might be possible to analyse the raw spectra and from it accurately find the position of the amide bands. The Macintosh Nicolet IR software is not able to carry out FSD, it is possible to transfer the data to an IBM compatible computer running the Nicolet IR software. The IBM version does allow this type of manipulation. The cost of purchasing a second computer is unacceptable, Nicolet claim that later versions of the operating software will have the facility for FSD.

All was not lost, 2nd and 4th derivative analysis is possible, this will give the peak positions of shoulders. We are therefore not able to deconvolve the spectrum into its constituents but we are able to see the true position of important peaks. It is necessary to take the fourth derivative because the software will not perform a peak pick on the negative peaks seen in the second derivative. It should be pointed out at this time what derivative manipulation is. The first derivative is a measure of the rate of change seen across a spectrum rather than the absolute values. A second derivative is the result of carrying a derivative analysis on the first derivative, it gives the rate of the rate of change. Shoulders on the original spectrum appear as negative peaks on the second derivative The fourth derivative is identical to a second derivative but the peaks are positive.

The 4th derivative analysis was carried out on data collected off a worn contact lenses, an example of this is shown in figure 6.15, along with the raw data. The shoulder of the amide peak is shown to have the absolute position of $1652 cm^{-1}$ in this case.

The significance of this measurement in *in vivo* lenses is unclear, we have a complex system under analysis. The system comprises, the contact lens material, water, and the biological material that has built up on the lens. As the lens has been worn in the eye the adsorbed material could be one or more of up to sixty different tear components. The complexity cannot be therefore over-stressed, complete understanding from the IR is unrealistic. If these limitations are taken in to consideration the method has allowed protein to be detected *in situ* in the lens matrix, this is something that very few other methods have allowed. The possibility of quantifying the adsorbed protein is a possibility, the internal standard could be one of the known peaks caused by the lens material, thus an allowance for section thickness would made.

Figure 6.15 IR spectrum of a worn contact lens, along with 4th derivative analysis of the same spectrum
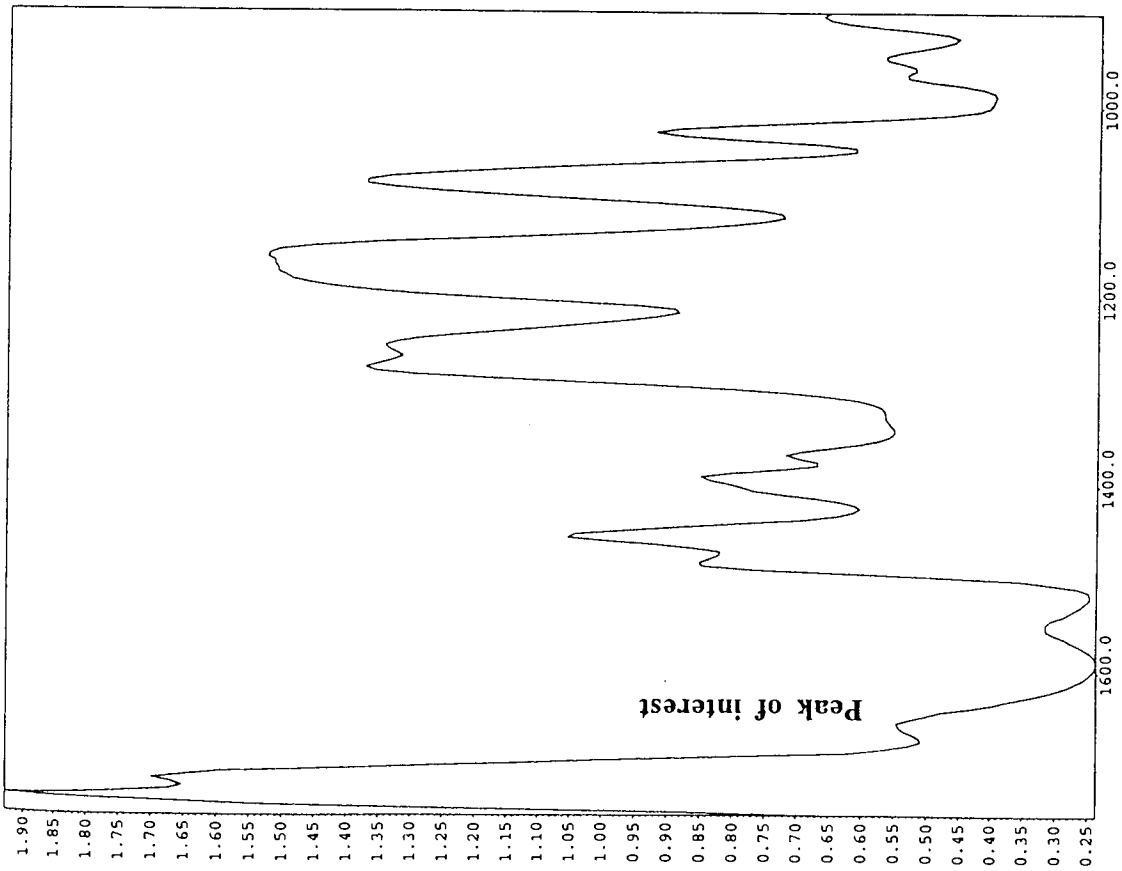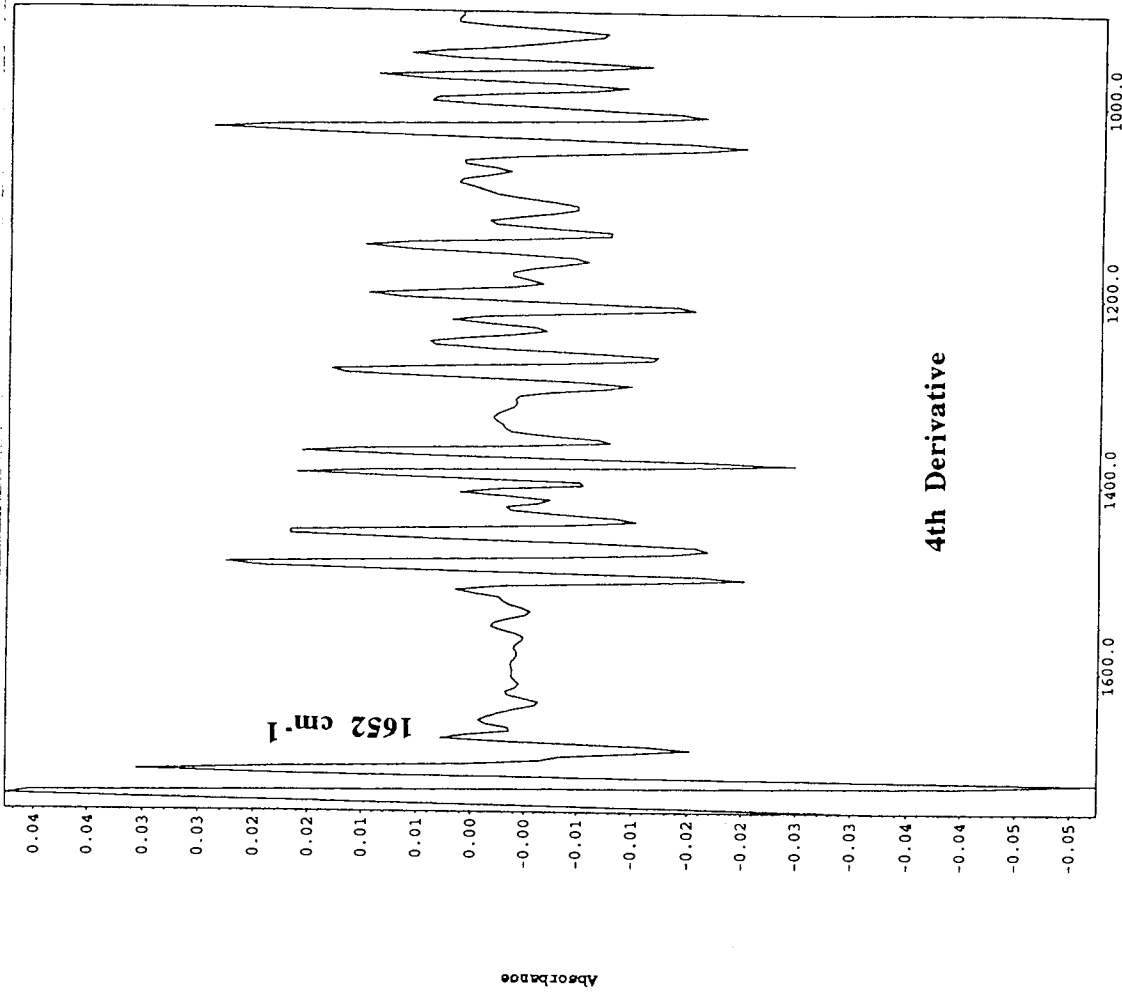
It was decided to collect the IR spectra of some proteins known to occur in tears. The acquisition of this data proved to be more difficult than first thought. Most proteins are supplied by the manufacturers as freeze dried powders. As IR microscopy's principal role is in the analysis of white powders suspected of being illegal drugs, it was thought that the protein powder analysis would be routine. The spectra obtained from some proteins such as lactoferrin were of an excellent quality. Lysozyme crystals proved to be very difficult to analyse, the crystals are large and needle like. The spectra obtained had totally absorbing bands due to the thickness of the crystals. Crystals were then chosen that were of a more suitable thickness. A second problem was encountered, the spectra had spikey noise like peaks. This was believed to be due to specular effects off the shiney faces of the crystal. Crystals were chosen for their surface characteristics as well as their thickness, the problem is clearly illustrated in figure 6.16 which shows two spectra of lysozyme collected from two very different crystals.. This method lead to the collection of the IR spectra of a number of proteins of interest. A typical spectrum of this type can be seen in figure 6.17 along with its 4th derivative. Many more of these spectra and the 4th derivatives can be seen in the second part of appendix A2 of this thesis.

Figure6.16 Spectra of lysozyme crystal good and bad

Lysozyme crystal Transmission Microscopy
Lysozyme Transmission microscopy single cryst

Wavenumber (cm-1)

N1c/IR

Figure 6.17 Spectra of a typical protein along with its 4th derivative
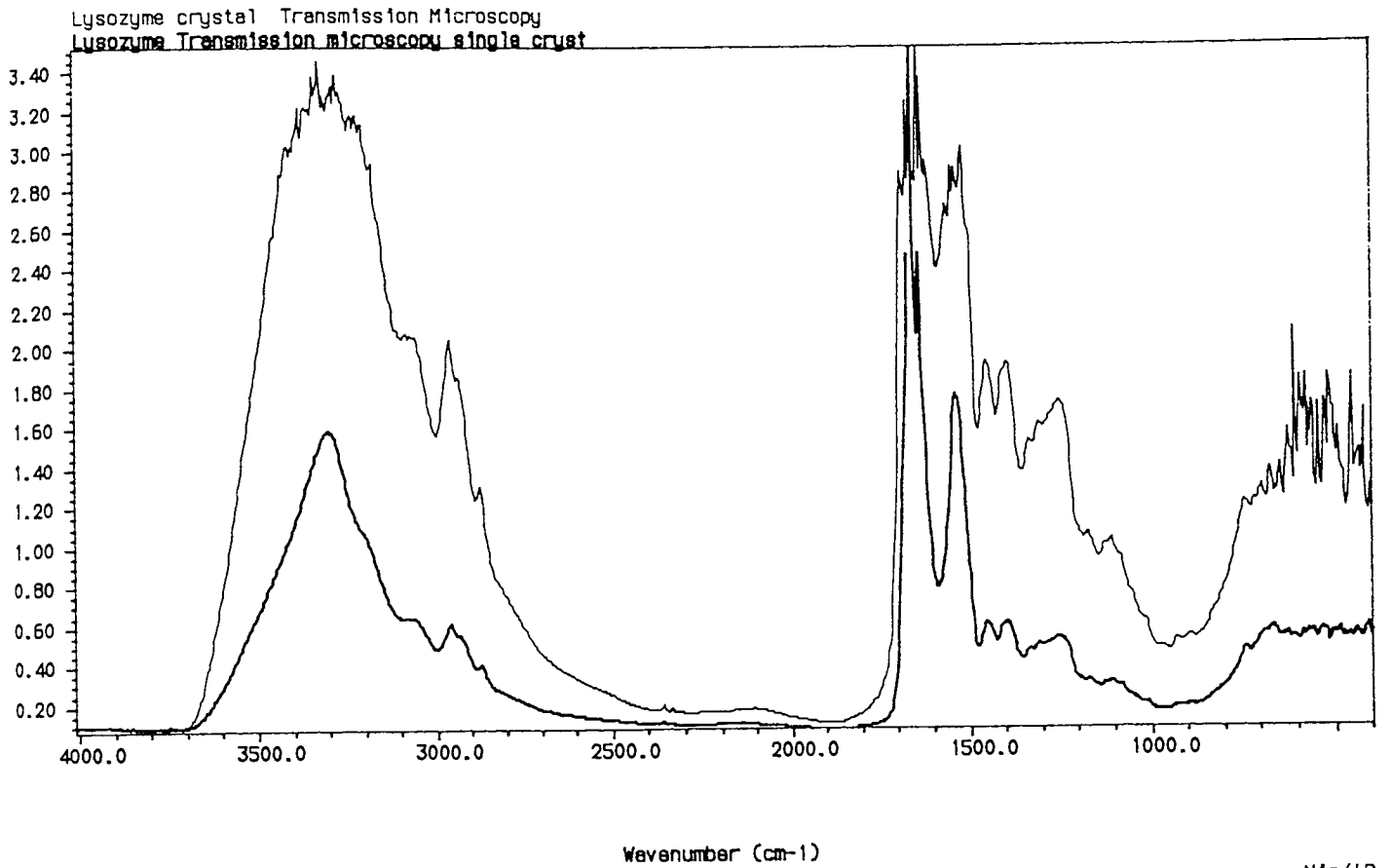
IR spectrum of Lactalbumin and its 4th derivative
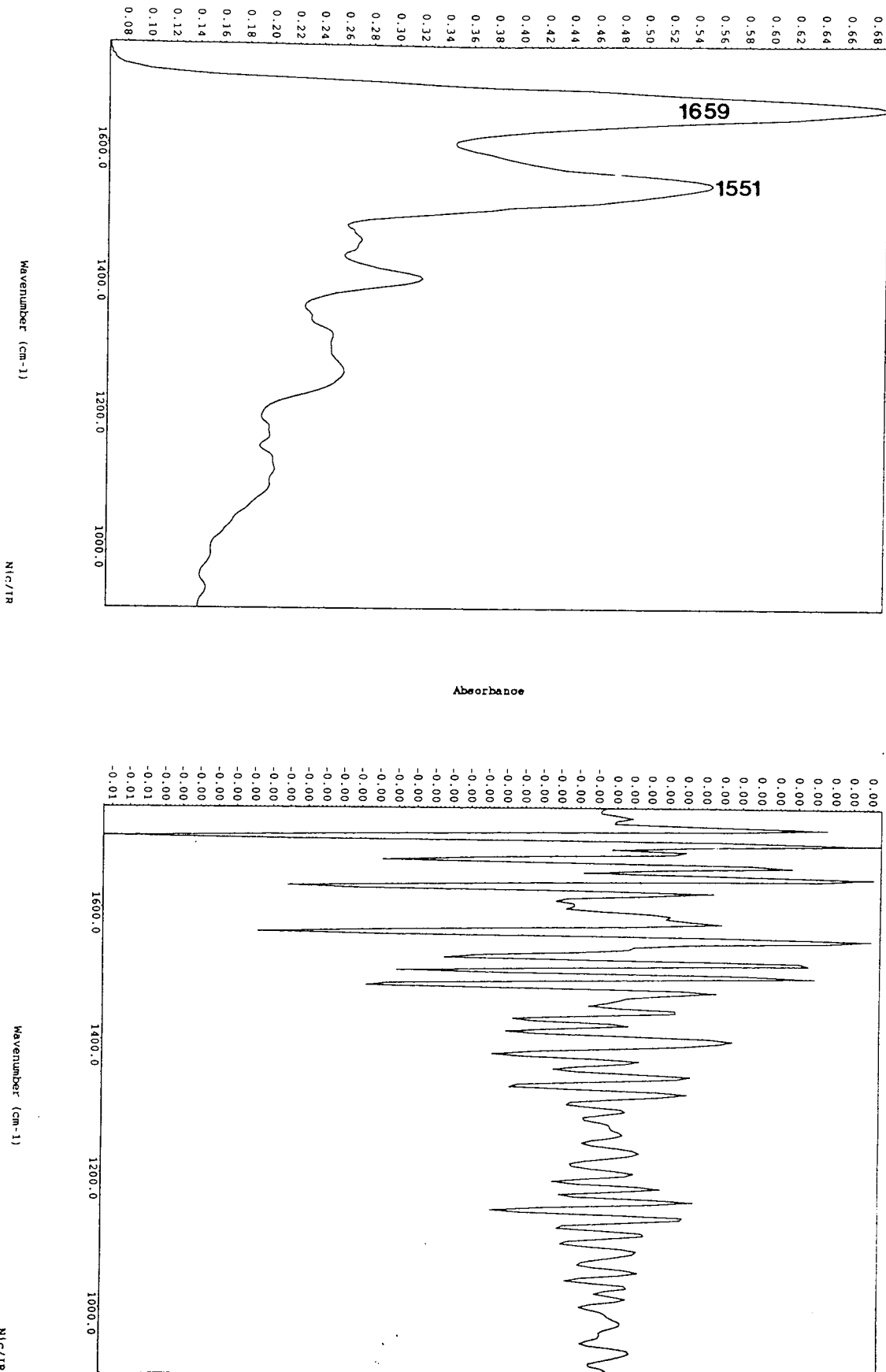
Figure 6.18 IR of a white spot deposit
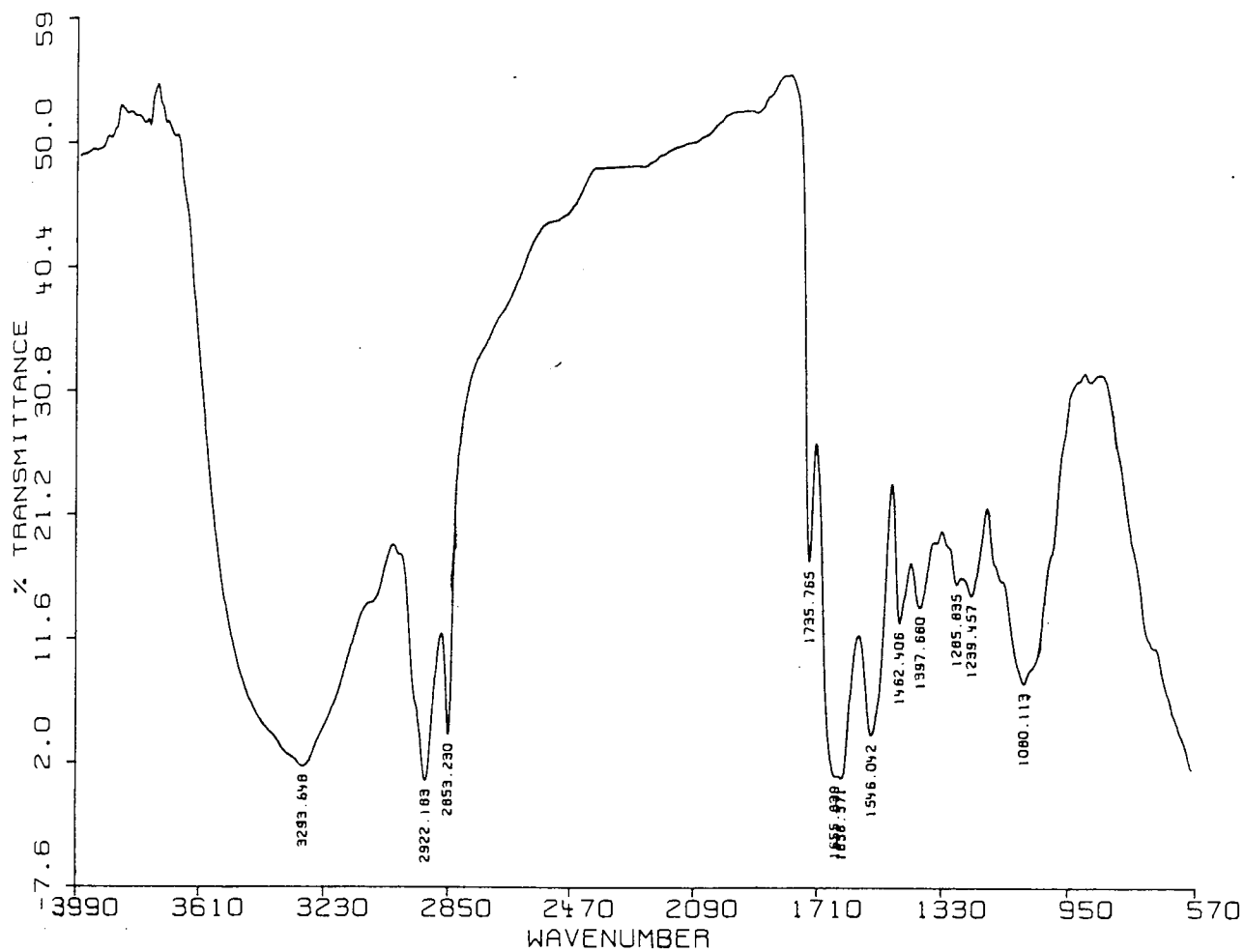
The significance of these spectra is open to debate, the proteins are not in their natural state when dry in a crystal form. The most suitable manner of examination would be to study proteins in solution. This is technically possible, it requires the use of a specially made cell with a short path length (so the water band is not totally absorbing), and equipped with IR transparent windows. The spectra obtained would of course have a large contribution from the water present, this could be removed by manipulation of the data, with some obvious loss of information. The alternative is to dissolve the protein in D_2O making the water subtraction unnecessary. Both of these methods have been used by other workers, both have their disadvantages. Electronic subtraction leads to loss of spectral information, whilst using D_2O as a solvent assumes that the proteins conformation in D_2O is the same as that in water. The cells required for this study were not available at the time so the arguments as to the best method for analysis became rather academic.

It had been hoped that other manifestations of a lack of biocompatibility such as the white spot deposits could be studied using IR microscopy. The analysis of white spots proved difficult, the thickness of the deposit and lens made transmission IR an impossibility both of the deposit *in situ* and also of the excised white spot. The only partial success was achieved by removing the white spot from the lens with a platinum needle. The white spot was then rolled flat using a tiny stainless steel roller against a flat steel plate. The thin film obtained was mounted on a CaF_2 window under the microscope. The spectrum obtained is shown in figure 6.18 it was recorded in transmission rather than absorbance. The amide band at 1655 and 1650 is clearly visible. As the white spot deposits are likely to contain a wide range of species it is

unlikely that the IR spectrum obtained will be fully interpretable. If FSD had been available it is likely that more information could be obtained from this spectrum.

6.4.2.2 Conclusions and suggestions about IR microscopy

The power of being able to discover the conformation of a protein on a contact lens could have important implications. It is unlikely that the study of *in vivo* spoiled lenses by this technique will divulge all of their secrets. We are dealing with a complex situation, we have a complex multi-component mixture, the IR spectrum is consequently complex. The likely advances using this system will be the study of single proteins interacting with contact lenses. It should be possible to follow the fate of the protein that binds to a lens as a function of time. It is likely that the more irreversible the interaction of the protein with the material, the more denatured it will become. Thus the disappearance of the α and β substructure would equate with a significant interaction with the contact lens.

The construction of a library of IR spectra of all the important species believed to be involved in soft contact lens spoilation would be a useful exercise. It would be possible to ascertain how many of the IR bands from these compounds would overlap and how much unambiguous identification would be possible. In the case of the proteins it is likely, as figure 6.18 shows, that the IR spectra are so similar that spectral enhancement and deconvolution will be necessary. The facility to do this at Aston is not available at present but with the increase in the sales of the Macintosh II controlled Nicolet benches it is reasonable to expect the manufacturers to include this feature on future software releases.

On a practical level the performance and ease of use of this analysis technique could be improved by the use of a diamond anvil, this device allows a shard of material to be flattened between two diamond windows. The sample is made very flat and excellent IR spectra can be obtained. The sectioning of lenses would become unnecessary improving the throughput of samples significantly. The diamond anvil is as its name suggests an expensive piece of equipment. The use of this device would also make it necessary to purchase new Cassegrain objectives that could correct for two windows rather than the preset one window type currently used. Because of these considerations it would be inadvisable to use this type of sample preparation.

To conclude, the method as developed does have flaws but it is an advance on what has been done in the past. The route to improvement is clear, deconvolution software is essential, as is the collection of protein spectra from solutions rather than dry crystals. The first of these would allow the reevaluation of the data presented in this thesis, so that more information could be extracted from it. The second improvement would make the spectra of known proteins more significant.

6.4.4 Photoacoustic spectroscopy

Photoacoustic spectroscopy is a novel way to aquire an IR spectrum of sorts. The IR beam of a conventional FTIR spectrometer is directed into an enclosed sample holder with an IR transparent window. The chamber is sealed from the outside environment, the IR beam is directed onto the sample, which is normally presented as a powder. The IR beam interacts with the sample causing a heating effect. The heating causes a small acoustic signal, this is picked up by a sensitive microphone mounted in the chamber. the chamber is pre filled with helium, this eliminates any water vapour and also the

signal caused by atmospheric carbon dioxide. The helium also acts as an excellent acoustic coupling gas, improving the signal transference. The output from the microphone is passed to the optical bench and computer, it is converted by means of a Fourier transform into a recognisable IR spectrum. The PAS cell used was an MTec 200 and was linked to a Nicolet 510M bench and Macintosh IIci computer as discussed before. The possibility of collecting the IR spectrum of a single contact lens using this device was investigated. The lens was placed in a hydrated state into the cell, the IR beam caused the lens to dehydrate. This caused two problems, the lens curled up during the acquisition of the spectrum, and as the lens dehydrated the water vapour saturated the interior of the cell. The repeated use of wet sample in the cell would damage the microphone and its electronics. The spectrum obtained was as a result of these problems unusable. The possibility of running dehydrated soft contact lenses was investigated. The lenses were found to have too glossy a surface, the IR beam was reflected off the surface with little or no acoustic signal being created.

The analysis of proteins in their dehydrated state was attempted with the PAS cell as it would be a quick and simple method to obtain an IR without any sample preparation and allowing total sample recovery. The proteins gave poor PAS signals, it was hypothesised that as the proteins are freeze-dried the crystals formed are flat faced and highly reflective. The IR beam would, as with the dehydrated contact lens, reflect off the surface causing no thermal effects and thus no acoustic signal.

It is possible that this method could give IR spectra of both lenses and proteins if the samples were prepared correctly. The drawback is that the spectra obtained tend to be more noisy than the corresponding spectra obtained using the IR microscope and if

spectral manipulations such as deconvolution and enhancement is to be carried out, this

becomes an even greater drawback.

CHAPTER 7

Miscellaneous techniques

7.1 Miscellaneous techniques:- General

The analytical techniques discussed thus far in this thesis are only a selection of the methods that were attempted. Before a technique is adopted, a large number of criteria need to be assessed; these include sensitivity, usefulness of the results the ease of interpretation of results and the time required for a typical analysis. Even when the scientific criteria above are satisfied we must look at the more mundane matters of cost and availability. At the outset of the project the systems of analysis available were surveyed, a number of candidates were selected and investigated further. There comes a point when it is necessary to stop speculating and to 'suck it and see', to test out the applicability of a method.

7.2 HPLC of mucins

It was decided that the quantification of mucins on a single contact lens would be a useful marker for possible biocompatibility studies. Mucin is a complex multi-molecular gel, its analysis is very difficult, unlike proteins, a single mucus species is not of a homogenous molecular weight. The backbone of the mucus molecule is a protein and thus is manufactured in the cell using a molecular template. Sugar moieties are added to the mucin molecule by enzymes, the specificity of the enzymes determines the similarity between like molecules. As a rule of thumb, the longer the sugar chain the more the chance of variability. As a result of this the molecular weight of an individual mucin species is variable. This fact means that many analytical systems cannot be used. An alternative method of quantification is to digest the mucin molecule in to its constituent amino acids and saccharides, as amino

-224-

acids are found in all proteins their estimation would not be useful. The saccharides present in mucus glucoproteins include [70-72] fucose, mannose, galactose, glucose, N-acetyl galactosamine, N-acetyl glucosamine and the sialic acid N-acetylneuraminic acid. Of these the hexosamines N-acetyl glucosamine/galactosamine are the most suitable for quantification.

A suitable HPLC system for this analysis has been published [154]. It makes use of a highly crosslinked cation exchange resin for the separation. The detection of saccharides, especially at low concentrations, is very problematic because of their lack of chromophoric activity. The detection system used by Honda and Suzuki [154] involved post-column derivatisation with 2-cyano acetamide. The resulting flow was monitored at 280 nm, reproducible linear results were obtained in the range 0.1 to 80 nmol of sample.

Post-column derivatisers are very expensive pieces of equipment and as one was not available another route to this separation and detection was attempted. The equipment available consisted of an isocratic low pulse HPLC pump, a Rheodyne injection valve, a 18 ODS (octa decyl silane) column, a refractive index (RI) detector and a data collection computer. The RI detector at first sight looked a good bet, RI is a commonly used detection system in saccharide analysis in the food industry. In this field sample size is seldom a problem, also the saccharides analysed tend to be sucrose, glucose, mannose and saccharine. The analysis of the amino sugars is not such a simple procedure. The RI detector proved to be in some respects too sensitive, the peak caused by the arrival of the injected solvent peak was so large as to mask peaks arriving soon after. The computer integrator had extremely

inflexible operating software, thus the integrator would scale all the peak sizes to the size of the solvent peak. In many other respects the computer proved to be more of a hinderance than a help. It had only limited random access memory (RAM) and as a result often ran out of memory on long runs. The computer had no means of storage on magnetic media and thus the only record of a separation was the paper hard copy. The computer also suffered from badly written software, the menus to change operational variable were often nested within many other menus, this made it labyrinth-like. The alteration of a single variable could often prove to be a long process. A chart paper recorder was used for a short period of time but no integration for peak height and area determination was available. A solvent system of variable ratios of acetonitrile and water was tried to optimise the separation of model mixtures. The isocratic pump was unable to deliver a gradient elution of the column, this is the preferred method. The pump proved to be very prone to bubbles of dissolved gas in the solvent, various degassing method were attempted the most successful was constant degassing of the solvent with helium. This introduced another problem, to reduce the size of the solvent peak the samples were being dissolved in the eluting solvent. The act of bubbling helium through the solvent caused the more volatile acetonitrile to evaporate, thus the solvent is depleted by a tiny amount as the run proceeds. The upshot of this is that the base line of the detector output tends to drift and the solvent peak becomes a problem again. The separations that were being obtained were not good, the unambiguous identification of the hexosamines required was not proving possible. This was due to two reasons; the RI detector is not discriminative in that it cannot be made to ignore some compounds as in a wavelength selective UV detector. The second reason was that the retention times of the

compounds were not reproducible. It was decided at this point that this analysis was not going to be possible given the time and equipment available.

7.3 GC MS

It has been reported in the literature that mucus can be analysed by gas chromatography followed by mass spectrometry (MS). This method does not quantify the mucus but gives much information about the mucus' structure. In MS several of different ionisation techniques can be used, it has been found that to analyse mucus fragments it is necessary to use a 'soft' ionisation system such as fast atom bombardment (FAB). Mucus samples must be first partly digested, then the saccharide chains that will be characterised must be derivatised. The derivatisation is necessary to make the molecules volatile, the mixture can then be separated by the GC. The output from the GC column is passed to the MS where much structural information can be gained about the mucin side chains. The combination of GC MS with FAB is rare. SERC has a facility at University College Swansea with such a service. The possibility of samples being processed there was investigated. The SERC facility insists on a number of conditions before analysis will be carried out. The sample must be accompanied by a capillary GC trace, no non-volatile components may be present and no particulate matter may be present.

In our case these conditions are difficult to satisfy, the samples are tiny to begin with, if a capillary GC is carried out we would be left with an even smaller sample for the GC MS.

The condition that no non volatile material should be present is difficult to satisfy, because these are clinical samples often other compounds are present. If some pre treatment is required then the sample size would be reduced. The problem of particulates is similar if pre filtration is carried out the available sample size is once again reduced.

Because of these problems and the small number of samples that the SERC facility were willing to analyse, the time involved was deemed to be too lengthy.

7.4 NMR

Nuclear magnetic resonance spectroscopy is possibly the single most powerful analytical technique available to the chemist. A huge number of variations on simple proton NMR have been developed, these can give information about identity, stereo chemistry and association between molecules. NMR is rapidly becoming important in biochemistry, for small molecules it has always been possible, but the latest advances have allowed the analysis of larger molecules such as proteins peptides and nucleic acids. Because of the size of these molecules the NMR spectra are complex. Modern computing techniques have allowed the assignment of many of the signals seen in the NMR of small proteins. The improvement in the field strength of the magnets used has led to more fine detail in these complex spectra.

As a result of this success it was decided to run some high field two dimensional NMR spectra of proteins found in tears. It was hoped to run the spectra of the proteins in their natural state and again after harsh heat treatment. The problems encountered were manyfold.

The proton signal from the water used as a solvent was huge, this was reduced by signal pre-saturation. This reduced the water peak but information near to this position was also lost. The next step was to increase the size of the protein signal by dissolving as much as possible of the protein in water. The solution must not become viscous as band broadening will occur.

A two dimensional NMR of lysozyme in its natural state is shown in figure 7.1. The information it contains is enormous. When the solution of the heat denatured protein was prepared it was found that the viscosity of the solution was very high, it had become a thick gel at 100mg/ml. The high viscosity was a parallel to what happens when we boil an egg, the egg white becomes a solid as the protein is denatured. This problem could have be overcome but what direction the research would take was unknown.

If we want to study proteins adsorbed to a hydrogel we must get an NMR of a solid. This required the use of a technique called 'magic angle spinning', in which the NMR tube is spun at very high speed at a preset angle to the magnetic field. Spinning a gel at these high speeds causes the gel to escape from the tube. If the physical problems are overcome we have a problem of the added complexity that the hydrogel contributes to the NMR spectrum. It was decide to proceed no further with this study, the time involved would be large. The results obtained would also require expert interpretation or a great deal of study on the part of the investigator. The study of this system by NMR is not impossible and if any one technique could be chosen as the most likely to yield useful information, this would be it.

Figure 7.1 Two dimensional NMR of Lysozyme in its natural state

PPM

PPM

PPM
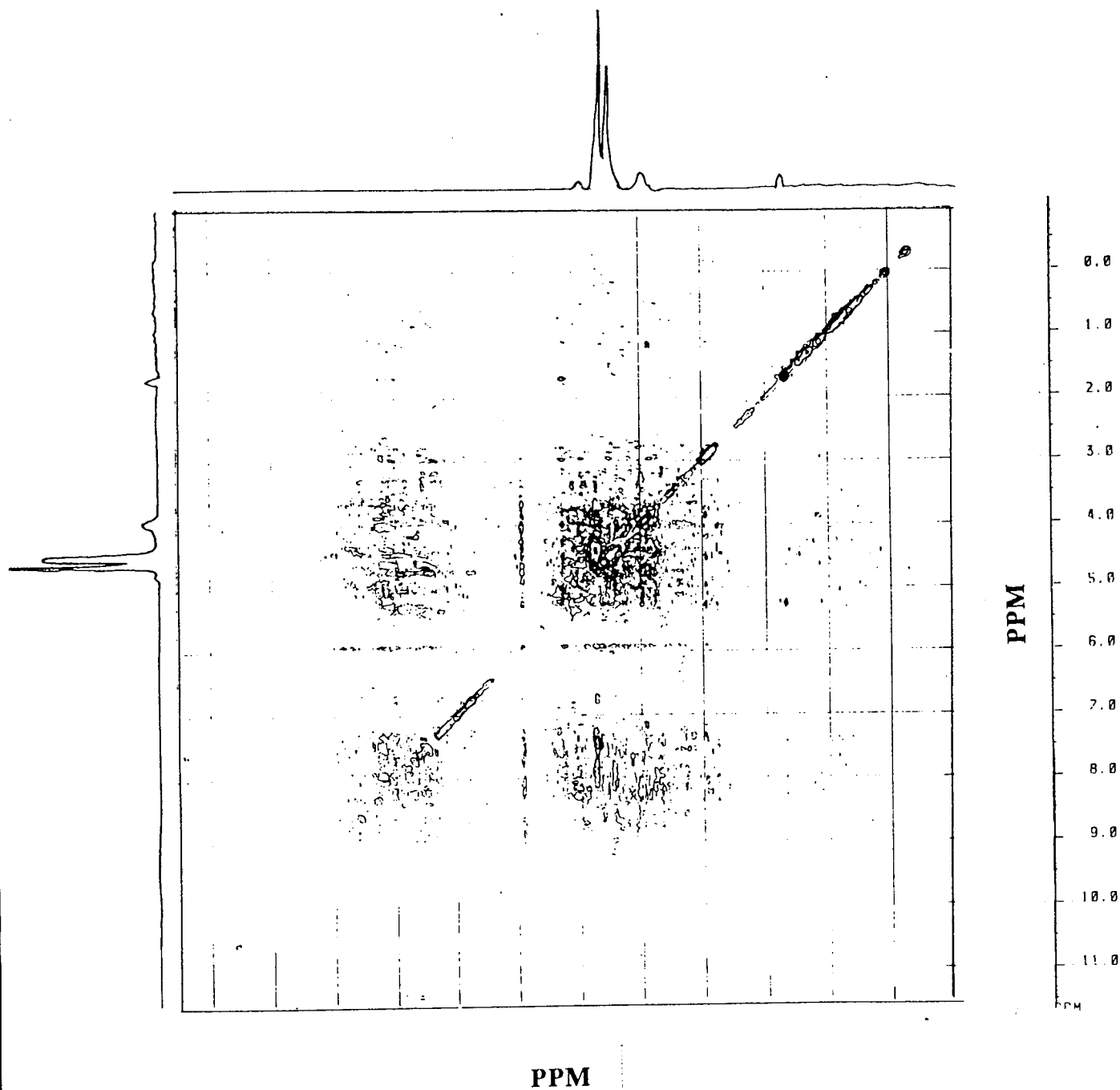
7.5 Fluorescence spectroscopy

Work in these laboratories using fluorescence spectroscopy has lead to a practical method of assessing the amount of protein and lipid that accumulates on and in soft contact lenses. The lenses are mounted in water in a cuvette, and the fluorescence spectra collected. It has been found in these laboratories that if lenses are excited with light of wavelength of 280nm two emission peaks are obtained. A peak at 360nm attributed to proteins and a second peak at 480nm from the lipids present. If the lenses are excited at 360nm the peak at 480nm from the lipids is stronger.

Because of the success of this work and of the availability of equipment for solid sampling fluorescence spectroscopy (often termed dispersive fluorescence). The solid sampling attachment is a block that can be mounted in the excitatory beam of the spectrometer. The detector is mounted at about 90° to the excitatory beam as shown in figure 7.2. The orientation should be such that no directly reflected beam is directed onto the detector.

Figure 7.2 Arrangement of the solid sampling fluorescence attachment.
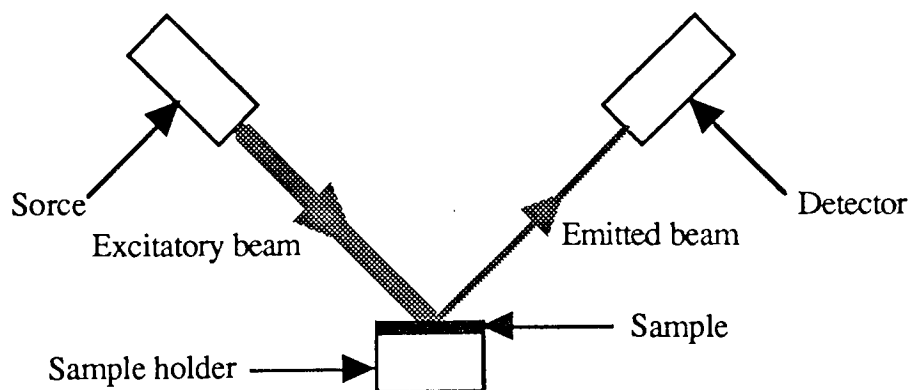
The sample holder has a quartz window that holds the sample in place. The contact lens was placed behind the window and a total photon count emmision spectrum was run. Fluorescence was obtained when the sample was excited at 280nm, 360nm and at 420nm. An emision spectrum was collected at each of these wavelenghths. It was found that the fluorescent peak was located very close to the excitatory wavelenghth. The size of the peak caused by the excitatory beam was very large due to the reflectance off the surface of the contact lens. The Perkin Elmer spectrometer used did not have a very discriminatory monochromator system. The spectrometer always gave a peak at two times the wavelength of excitation, this was due to the nature of the diffraction grating used in the monochromator. The upshot of this was that the emitted spectrum had two regions in which no information could be obtained. As the emission near to the excitatory wavelength is important, the information from solid sampling fluorescence is limited. The lens was mounted without the quartz window to try and reduce the size of the reflected beam. It was found that the lens rapidly dehydrated during the spectral aquisition, as the lens dehydrated it also curled up causing the reflected signal to change in magnitude over time.

As the fluorescence technique in wet conditions was being pursued with vigor within the group the solid sampling method was seen as unnecesary and problematic.

CHAPTER 8

Conclusions and the way forward

8.1 Conclusions:- General Comments

This thesis has detailed the research into the investigation of the manifestation of the build-up of biological material on soft contact lenses. The problem is a difficult one to study. The sample sizes are very small and the material of interest is bound to a solid substratum. Many analytical techniques used commonly in biological research do not have the necessary sensitivity. In most fields of research, if the sample proves hard to detect the researcher can increase initial sample size. This option is not open in the study of contact lens spoilation. Early work on spoilation and on patient tear composition has shown variations between patients and in a single patient over a period of time. Thus the possibility of increasing sample size by pooling of samples would decreases the relevance of the results obtained. If sample size is fixed we must look to the possibility of increasing the sensitivity of available techniques or using intrinsically sensitive systems. The second problem is that the material of interest is bound to the contact lens material. When the material is solubulised we do not know how thorough this process has been. Allthough it is difficult to monitor the degree of removal, one method developed recently in these laboratories [155] is to mount the contact lens in a fluorescence spectrometer. The intrinsic fluorescence of the adsorbed material is utilised to monitor the residual material. This technique is open to criticism, as it can tell us if any material is left on or in the lens, but not how much material remains. As this is the only method of detecting the level of spoilation and thus effectiveness of material removal, it must be used.

The material removed from the contact lens by whatever method must then be analysed. Of the methods in current use the most widely utilised is electrophoresis. The work

published using this method has tended to be naive. Often workers have used primitive 'routine' electrophoresis equipment that have little flexibility as to gel type or buffer systems. The gels obtained have not exhibited optimal separation because of the nature of the systems used. When reasonable separation has occurred the assumptions made as to the identity of the species present has relied often on little more than relative mobility. The use of unambiguous stains such as antibody probes has not been common. The idea that electrophoresis is a panacea for the analysis of spoilation is mistaken. It must be assumed that the authors of such work are unaware or not self-critical enough to appreciate the pitfalls and shortcomings of the technique.

The removal of material and its analysis will at best give us only part of the information we require. The ideal manner of analysis would be to study the material *in situ*. IR microscopy is the first technique to achieve analysis of the material on and in the lens matrix without the need for solubulisation. The spectra obtained give partial information as to the identity of the adsorbed proteins and have the potential for giving a clue as to the conformational integrity. The conformational integrity data will prove very valuable in the search for a material that encounters as little protein adsorption in the eye. It is likely that large conformation changes indicate that the protein is tightly bound to the lens material. If the protein has undergone significant changes in its secondary and tertiary structure it is likely that this protein will elicit immunological reaction in the eye. The study of a large range of commercial and novel materials should be undertaken. The response of these materials to a range of typical tear proteins , monitored by FTIR microscopy, would prove, I believe most useful.

The *in situ* examination of the physical form of the deposits that build up on soft

contact lenses has the same advantage as *in situ* chemical analysis. The deposits studied by confocal laser microscopy were seen in their natural milieu. The system was not disrupted, making the structures seen more significant. The white spot deposits gave up some of their secrets under this device. The finger-like structures seen are undoubtedly responsible for the resistance to removal of these objects from the lens. The discovery that the deposit had grown into, as well as out of the lens material is important. It was only by means of this exciting new piece of equipment and its optical sectioning abilities that these discoveries were made.

The eye is an excellent body site to study, samples are readily available and the lessons learnt here will have implications for other biomaterials. The commercial importance of soft contact lenses is also an impetus for research. The study of this interfacial system is also of great scientific interest. A great driving force is the need to develop new improved materials, the resulting improvements in wear and care properties would lead to large financial rewards. All of these reasons mean that this is a topic that must be pursued.

The complexity of the study of the spoilation mechanism means that no one analysis system will ever be able to elucidate all the species responsible. We must understand the mechanism that underlies this phenomenon. The evidence we have to go on is limited in that we can only study the remnants that are laid down, rather than monitoring the system in its dynamic state. The evidence is further reduced by our inability to full analyse the materials on the lens.

The way forward to a full understanding of the problem should involve the techniques

that this project has developed. Of the other methods that are available, fast protein liquid chromatography (FPLC), a variation on HPLC has promise. It is reportedly highly sensitive and capable of separating proteins effectively. The expense involved is great, the initial capital outlay is substantial the columns used are costly and have a limited lifetime. It would be advisable, as this would be a speculative evaluation to try and obtain time on a system elsewhere.

The use of immunological probes to study the location of species on the lens surface has great possibilities. If these probes were coupled with confocal microscopy the result would have the potential to be significant. It was hoped to do experiments of this type during this project but as always time became the limiting factor.

Capillary electrophoresis is a new technique and thus is in its infancy, it has the advantage over conventional electrophoresis, of improved separations and sensitivity. The results are produced in an on-line form improving sample throughput. Though this method holds great promise it would be wise to leave well alone until it matures into a routine piece of equipment.

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Appendix 1

A 1.1 Solutions for electrophoresis

The stock acrylamide solution was made up as follows, note the total acrylamide concentration is 30% this is the sum total of acrylamide plus the bis acrylamide. The percentage of bis acrylamide the crosslinking agent is expressed as a percentage of the total monomers present.

1 Stock monomer solution (30% w/v total acrylamide 2.7% of which is bis acrylamide)

acrylamide	58.4g
bis acrylamide	1.6g
H_2O	to 200ml

(this solution should be stored at 4°C in the dark, it has a shelf life of 4-6 weeks)

The buffers added to the monomer solutions before polymerisation are set out below, note that all references to tris refer to the free base of tris (hydroxymethyl) aminomethane ($C_4H_{11}NO_3$) Mr 121.1 . This is sold by Sigma chemicals as Sigma 7-9 buffer. These solutions should be stored at 4°C for an optimum shelf life of 2-3 months.

2 Running gel buffer (4x concentrate) (1.5M tris pH 8.8)

Tris	72.6g
H_2O	to 400ml

pH should be adjusted with HCl to pH 8.8

3 Stacking gel buffer (4x concentrate) (0.5M tris pH 6.8)

tris   12.0g

H_2O to 200ml

pH should be adjusted with HCl to pH 6.8

The other solutions needed to produce the gel slab are shown below.

4 10% SDS solution (10% w/v sodium dodecyl sulphate)

SDS   50g

H_2O to 500ml

5 Initiator solution (10% ammonium persulphate)

ammonium persulphate   0.5g

H_2O to 5.0ml

The initiator solution should be made up fresh each day (to ensure successful polymerisation)

If the separating gel is cast and some time is to elapse before the stacking gel is to be added the running gel overlay solution below should be used to protect the gel from adverse osmotic effects.

6 Running gel overlay (0.375M tris pH 8.8 0.1% SDS)

tris   25ml of solution **2**

SDS   1.0ml of solution **4**

The electrophoresis is carried out in the following buffer, this can be made up in bulk to

save time in the future, up to 30 litres can be made up at a time. It has a relatively good shelf life and can be stored at room temperature. The buffer in the upper tank should be discarded after every run, but the buffer in the lower tank may be used up to 4 times before discarding.

7 Tank buffer (0.025M tris pH 8.3 0.192 M glycine 0.1% SDS)

tris	30g
glycine	144g
SDS	100ml of solution **4**
H_2O	to 10.0 litres

All protein solutions before electrophoresis need to be suitable, treated to ensure dissociation and that they are completely reacted with SDS for SDS page. This is achieved by adding an equal volume of the following x2 concentrate treatment buffer to the solution. The glycerol is included to increase the density and viscosity of the solutions so that they do not diffuse out of the sample wells prior to electrophoresis. 2-mercaptoethanol agent breaks disulphide bridges in proteins, it is also a potent stench agent and a potent toxin it should therefore be handled with gloves and in a fume hood. Its role as a stench agent ensures that workers who use it often always get a seat on the bus, on the way home from the lab.

8 Treatment buffer (0.125 M tris pH 6.8   4% SDS 20% glycerol 10 % 2-mercaptoethanol)

tris	2.5ml solution 3
SDS	4.0ml solution 4

glycerol	2.0ml
2-mercaptoethanol	1.0ml
H_2O	to 10.0ml

The buffer should be divided into suitable aliquots and frozen.

A 1 2 Regime for gel casting

The gels were cast using the Hoefer gel casting stand. The components were all washed in Decon 90 and then rinsed in distilled water and then air dried. The stand was assembled according to the manufactures instructions. Gloves were worn throughout, to make sure no protein was transferred from the hands to the equipment and to also protect the worker from the acrylamide that had been in contact with these parts. It cannot be over-stressed as to the importance of protection from this compound, as it attacks, in men, the testicles and can cause damage to the future offspring of workers.

10% separating gel and 4% stacking gel

To make a gel of the above composition for two gels the following volumes a re required

	Separating gel	Stacking gel
Acrylamide solution 1	20.00m	2.66 ml
Buffer 2	15.00ml	
Buffer 3		5.00 ml
H_2O	24.10 ml	12.20 ml
Ammonium persulphate solution 5	300μl	100μl
10% SDS Solution 4	600μl	200μl

TEMED	20μl	10μl

The TEMED acts as a catalyst for the polymerisation and was used as supplied.

More details as to the procedures for gel casting can be found in section 4.3.1 of this thesis.

A 1.3 PAGE blue staining

PAGE blue stain is known under different name according to the suppliers, Sigma Chemicals call it Brilliant blue G stain, ICI term it Coomassie blue whilst BDH sell it as PAGE blue. The product used during these studies was supplied by BDH thus the name PAGE blue will be used. This was the simplest stain used during this study. The gels were fixed and stained in a single solution of methanol : water : glacial acetic acid in the ratio 5 : 5 : 1. containing 5%(w/v) page blue.

Page stain / fixative solution

methanol	228 ml
glacial acetic	45 ml
H_2O	228 ml
Page blue	2.5 g

The Page blue was supplied by (BDH Chemicals). The gels were placed in this solution for 1 hour. The gels were constantly agitated on a Vibrax shaker (Northern Media). The stain/fixative can be used many times before it need be discarded.

The gels were destained with a solution made up as follows

Page destain solution

Methanol	400ml
Glacial acetic acid	100ml
H_2O	1500ml

A 1.4 Silver staining

The silver staining protocol was based on that of Heukeshoven and Dernick[132] as modified in the Sigma Chemical Company, silver stain kit AG-25.

Fixing

The gels were fixed in 30% ethanol 10% glacial acetic acid. The solution was changed 3 times, 300ml was used each time and the solution was changed every 20 min.

The gels were then washed three times with 300ml of distilled water, each wash lasted 10 min. The details of the following solution are not given as they were used as supplied with the kit. No information as to the exact composition of the solutions is not given by Sigma Chemicals for commercial reasons. the procedure is described to give some idea as to the time and complexity involved in this procedure.

The gels were brought to equilibrium with a silver solution for 30 min. The gels were then rapidly washed for 10-20 seconds. 150ml of the developer solution was added to the gels and then discarded after 5-8 min. The remaining 150ml was then added, the gel should be monitored closely so as to give the darkest bands possible with minimal

background staining. When the gel is sufficiently dark the developer solution is poured off and a stop solution added for 5 min. The gels are then washed three times with 300ml of distilled water. The gels are then treated with a reducer solution for 10-30 seconds and are then rinsed under running tap water. It should be remembered that the gels are fragile and that if the water hits the gels directly they are likely to be broken into many pieces. The gels can be recycled through the procedure for reportedly higher sensitivity. The total procedure takes in the region of 3 hours and 30 min, as no step takes more than 30 min it is difficult to carry out whilst another procedure in in progress.

A 1.5 Colloidal Brilliant blue G Stain

This is a product manufactured by Sigma Chemicals and sold as Brilliant blue G colloidal concentrate (product number B-2025). The reported sensitivity of this stain is ten times greater than coomassie blue, and is comparable to that of silver staining kits.

The gels are fixed with the following solution, which can be purchased from Sigma as Fixing Solution (product number F-7264), or can be made as follows.

5 x concentrate of fixer Solution for colloidal Brilliant blue G stain

(Trichloroacetic acid 60% (w/v) 5-sulphosalicylic acid 17.5% (w/v))

30g	Trichloroacetic acid
8.75g	5-sulphosalicylic acid

The solution should be stored in the dark at room temperature. Before use the solution should be diluted to its working strength with distilled water. The working solution is 12% (w/v) trichloroacetic acid and 3.5% (w/v) 5-sulphosalicylic acid. These solutions

are capable of causing severe burns to exposed skin thus gloves should be worn when handling them.

The gels should be fixed in 200ml of the fixer solution for 1 hour with gentle agitation. The stain should be prepared as per the supplied instruction. This involves adding 800ml to the Brilliant blue G colloidal concentrate. This should be mixed by inversion only. Immediately before use 4 parts of the stain solution should be mixed by vortex for 30 seconds with 1 part methanol. The solution formed is stable for 4 hours only. Typically 100ml of this solution was made up. The gel is placed in it for 1 to 2 hours with gentle agitation. The gels were destained with 10% acetic acid in 25% methanol for 10 to 30 seconds. The gel should be washed then with 25% aqueous methanol this should be discarded and the gels should be destained in 25% aqueous methanol for up to 24 hours. The gels can be stored for a few months in distilled water in the dark. The gels can be scanned at 600nm if required.

A 1.6 Electro Blotting

The apparatus was assembled as described in chapter 4. The blot was carried out in the following buffer. The proteins are transferred from the gel to the nitrocellulose membrane by the action of a potential difference place across the two. The proteins are tightly bound to the membrane and can now be visualized by two principal methods, labeled antibodies and a biotin/avidin linked reaction.

Electroblotting buffer (tris 20mM glycine 150mM and 20% v/v methanol)

tris   12.11g

glycine   56.3g

methanol	1000ml
H_2O	make up to 3.5 litres

A 1.7 Biotin Avidin Blot

A biotin avidin blot kit supplied by Bio-Rad (Cat. No. 170-6512) was used. The reagents can be purchased individually but for general ease and higher reproducibility this commercial kit was utilised. The blot is dependant on the high affinity that biotin has for avidin ($K_D \approx 10^{-15}M$)[133].

The following solutions were made up

Borate-Tween solution (BT) (0.05M sodium borate 10-hydrate, 0.2% Tween 20 pH 9.3)

$Na_2B_4O_7.10H_2O$	34.14g
Tween 20	4ml
H_2O	to 2 litres

Tris buffered saline (TBS) (0.02M tris 0.05M NaCl)

tris	4.84g
NaCl	58.44g
H_2O	to 2 litres

adjust pH to 7.5 with HCl

Tris Tween solution (TTBS) (0.02M tris 0.05M NaCl 0.2% Tween)

Tween	2ml
TBS	1 litre

Avidin horseradish peroxidase conjugate solution (A-HRP)

Avidin HRP conjugate	100µl (of stock solution supplied)
TTBS	to 1 litre

Phosphate buffered saline (PBS) (0.01M phosphate buffer)

sodium monobasic phosphate	0.105g
sodium dibasic phosphate heptahydrate	0.600g
NaCl	2.550g
H_2O	to 300ml

adjust to pH 7.2 with HCl

NHS Biotin solution

contains 75mM N-hydrosuccinimide biotinate in dimethyl formamide, was used as supplied.

HRP colour development solution (HRP-C)

4 chloro 1 naphthol	60mg
methanol (ice cold)	20ml

just before use add

H_2O_2 (30%)	60µl
TBS (at room temp)	100ml

use immediately

Procedure for biotin avidin blot

1 The membrane is washed in 100ml BT for 10 min this is repeated 3 times

2 Discard the wash solution and replace with 100ml BT

3 Agitate the membrane in the solution and add 200µl of the NHS biotin

4 Incubate for 15 min

5 Wash the membrane in 100ml of BT for 5 min repeat

6 Wash the membrane in 100ml of TTBS for 5 min repeat

7 Prepare the Avidin -HRP solution as described above

8 Incubate the membrane in the above solution for 1 hour

9 Wash the membrane in 100ml of TTBS for 5 min repeat

10 Wash the membrane in 100ml of TBS for 5 min repeat

11 Prepare the HRP-C solution as described above

12 Incubate the membrane in the HRP-C solution until the bands become visible, normally about 15-30 min

13 Stop the development when the bands are visible and the background staining is not excessive by the washing repeatedly in distilled water.

The membranes can be dried between two sheets of blotting paper, the membrane can be stored for a long period of time without fading if it is kept in the dark.

Antibody staining of blots

The use of highly sensitive and selective antibodies on nitrocellulose membranes allows the detection and identification of tiny amounts of specific compounds. Two antibodies are used, a primary antibody to the moiety of interest and a enzyme linked secondary

antibody. The second antibody binds to the primary antibody and causes amplification in the reaction.

The solutions required for this procedure are detailed below

1   **TBS** (20mM tris, 150mM sodium chloride)

tris   2.42g

sodium chloride   8.77g

pH to 7 with HCl, distilled water to 1 litre

2   **TBST** (20mM tris,150mM sodium chloride, 0.1% (v/v) Tween 20)

TBS   100ml

Tween 20   0.1ml

3   Blocking buffer (**B**)(50mM tris, 500mM sodium chloride, 0.5% Tween 20)

tris   6.06g

sodium chloride   29.22g

Tween 20   5ml

pH to 8 with HCl, distilled water to 1 litre

4   Ponceau red stain (**P**)(0.2% Ponceau red in 100mM sodium acetate pH 5)

Ponceau red stain   0.2g

Sodium acetate   0.6g

to 100ml water adjust to pH 5.

5   **NBT** (Nitro blue tetrazolium 50mg/ml in methanol)

Nitro blue tetrazolium   100mg

methanol   to 2ml

store at -20°C in darkness

6   **BCIP** (5-bromo 4-chloro 3-indolyl phophate 50mg/ml in dimethyl formamide)

BCIP   100mg

dimethyl formamide   to 2ml

store at -20°C in darkness

7   Stop solution (20mM tris, 5mM EDTA pH 8.0).

tris   2.42g

EDTA   1.37g

adjust to pH 8.0 with HCl

distilled water   to 1 litre

These solutions can be made up in bulk and stored at 4°C, they should be allowed to come up to room temperature where necessary. Excessively long storage should be avoided as one batch of these reagents were found to be infected with a fungal infestation. The solutions should thus be checked before use.

The nitrocellulose membrane is pre-stained with Ponceau red, a non specific protein stain. this has low sensitivity but major protein bands will show up, this facilitates the cutting of the gel into separate lanes for different antibodies.

The procedure for antibody staining used was as follows.

1   Remove the nitrocellulose sheet from the sandwich, NB do not allow to dry out during these procedures.

2   Soak membrane for 5min in TBS at room temperature with gentle agitation.

3   Stain with Ponceau red for 5-10min or until some bands become visible.

4   Wash extensively in distilled water, the bands should become clearer.

5   The membrane can be cut into the necessary pieces for separate analysis.

6   Block the unused binding sites on the membrane with 4% w/v milk powder in buffer B for 15-30 min or overnight at 4°C. If lactoferrin is being analysed use 1% (w/v) bovine serum albumin (BSA) as the block protein.

7   Incubate with primary antibody in TBST(1:4000), for 2 hours at room temperature.

8   Wash with TBST three times for 10min each

9   Incubate with secondary antibody in TBST(1:7500) for 30min at room temperature.

10 Wash with TBST six times for 10min each.

11 Blot on dry filter paper

12 Make up colour development substrate solution, to make 5ml use 33µl NBT solution, 5ml AP buffer, 16.5µl BCIP substrate. This solution is stable for 1hour put should be protected from light.

13 Incubate membrane with colour development solution until bands appear.

14 Stop the reaction with stop solution S

The membranes can then be dried between sheets of filter paper. The blots should be stored in darkness to ensure that the bands do not fade over time.

Appendix 2

A 2.1 Infrared wavenumber tables

This appendix contains the wavenumbers of a number of chemical groups and some biologically important structures found in proteins.

Table A 2.1 Infrared wavenumbers of some biologically important structures (in order of wavenumber)

Wave number(cm^{-1})	Conformation
785	CH3 rocking
937	a helix
970	phosphatidylcholine
970	choline
1090	sulphur containing groups
1105	sulphur containing groups
1118	tyrosine
1135	sulphur containing groups
1240	amide 3
1250	P=0 stretch whole sperm
1287	a helix
1317	CH2 CH2
1400	carbonyl
1455	CH2 CH3
1458	phenylalanine
1476	phenylalanine
1515	tyrosine
1516	tyrosine
1518	tyrosine
1530	amide 2
1542	amide 2
1548	amide 2
1550	amide 1
1550	amide 2
1583	COOH protein
1659	random
1615	C-C stretch,no structural information
1620	b
1624	border of b sheet
1624	b sheet
1627	b sheet
1630	unknown
1630	b sheet

1630	antiparallel .b sheet
1630	C=0 amide I
1630	b
1630	b
1632	b sheet extended chain
1633	b
1635	b sheet
1635	Amide I in sperm tails
1635	choline
1638	b sheet
1638	b sheet
1639	b
1642	random coil and b sheet
1642	b sheet
1643	random
1644	b
1646	random
1648	amide 1
1649	a helix and random
1650	unordered
1650	amide 1
1651	a
1652	amide1
1654	a
1654	a-helix and random coil
1654	a-helix
1654	a helix only
1655	turns-general
1655	a and random
1655	C=0 amide I
1656	a
1656	b turns or b antiparallel
1656	a helix
1657	a
1660	distorted helices
1660	disordered
1660	Amide I
1660	Amide I in Lysozyme native
1663	a
1664	turns
1664	b turns
1666	turn
1668	turn

1670	turn-specific proteins only
1670	Amide I Lysozyme denatured
1672	antiparallel-b sheet
1672	b turns
1672	turn
1673	b turns
1675	b-spec
1675	a
1680	turn
1683	turn-spec
1684	antiparallel b
1685	b sheet-turn
1687	b turns and sheet
1687	antiparallel b sheet
1687	antiparallel b sheet
1688	turn-spec
1688	turn
1690	antiprallel chains
1694	turn-spec
1715	free carboxyl groups
1718	carbonyl stretch
1721	carbonyl,H bond
1730	ester hydroxyl
1740	carbonyl,Hbond
1740	phospholipid ester carbonyl
2554	a cysteine SH stretch
2566	b cysteine SH stretch
2592	b cysteine SH stretch
2854	acyl-lipid symmetrical
2930	acyl-lipid asymmetrical
3015	unsaturated lipid

Wavenubers given over a range

820-850	carbohydrates sulphate groups
300-2800	acyl-lipid
1080-1060	P-0-P stretch
1200-1040	C-0-C stretch
1365-1300	OH in sugars
1543-1530	amide2
1620-1640	b
1622-1639	b
1625-1639	b
1626-1624	b strands exposed

1630-1640	b
1635-1675	b
1636-1680	b sheet antiparallel
1640-1648	r
1650-1658	a
1650-1660	a
1652-1659	amide 1
1654-1668	a
1655-1660	a and random coil
1663-1680	turns
1731-1741	carbonyl-lipid
1760-1700	phospholipid
2995-2825	C-H stretch

Key to table A 2.1

a=α helix
b=β sheet
r= random

Table A 2.2 Infrared wavenumbers of some biologically important structures (shown for each feature).

Wavenumber (cm-1)	details of conformation
A helix	
1650-1658	a
1650-1660	a
1663	a
1675	a
1654-1668	a
1655-1660	a/random coil
1657	a
1649	a helix and random
1287	a helix
1651	a
1656	a
1654	a
1654	a-helix and random coil
1654	a-helix
1654	a helix only
937	a helix
1656	a helix
1660	distorted helices

B sheet

1620-1640	b
1620-or below	b
1675	b-spec
1639	b
1630-1640	b
1684	antiparallel b
1625/1639	b
1622/1639	b
1635-1675	b
1685	b sheet-turn
1630	b sheet
1630	antiparallel.b sheet
1644	b
1636-1680	b sheet antiparallel
1626/1624	b strands exposed
1638	b sheet
1687	b turns and sheet
1630	b
1635	b sheet
1687	antiparallel-b sheet
1672	antiparallel-b sheet
1624	border of b sheet
1656	b turns or b antiparallel
1642	random coil and B sheet
1633	b
1673	b turns
1672	b turns
1664	b turns
1687	antiparallel vibrations
1624	b sheet
1627	b sheet
1632	b sheet extended chain
1638	b sheet
1642	b sheet

Random

1640-1648	r
1646	random
1643	random
1660	disordered
1659	random
1650	unordered

Amide I

1652	amide1
1652-1659	amide 1
1660	amide I
1660	amide I Lysozyme native
1670	amide I Lysozyme denatured
1650	amide 1
1550	amide 1
1648	amide 1

Amide II

1550	amide II
1548	amide II
1543-1530	amide II
1530	amideII
1542	amide II

Amide III

1240	amide 3

Substructures

1740	carbonyl,H bond
1721	carbonyl,H bond
1583	COOH protein
1317	CH2 CH2
1400	carbonyl
1455	CH2 CH3
785	CH3 rocking
1718	carbonyl stretch
2995-2825	C-H stretch

Turns

1670	turn-spec
1683	turn-spec
1688	turn-spec
1694	turn-spec
1655	turns-general
1663/1680	turns
1668	turns
1664	turns
1666	turns
1672	turns
1680	turns
1688	turns

Lipids

1760-1700	phospholipid
300-2800	acyl-lipid
2854	acyl-lipid symm
2930	acyl-lipid asym
1230	asym phosphate
1088	sym phosphate
1731-1741	carbonyl-lipid
1740	phospholipestercarbonyl

Amino Acids in proteins

1515	tyrosine
1516	tyrosine
1476	Phenylalanine
1518	Tyrosine
2554	a cysteine SH stretch
2566	b cysteine SH stretch
2592	b cysteine SH stretch
1458	phenylalanine
1118	tyrosine

937	a helix
1287	a helix
1649	a helix and random
1651	a
1654	a
1654	a-helix and random coil
1654	a-helix
1654	a helix only
1656	a
1656	a helix
1657	a
1660	distorted helices
1663	a
1675	a
1650-1658	a
1650-1660	a
1654-1668	a
1655-1660	a/random coil

B sheet

1624	border of b sheet
1624	b sheet
1627	b sheet
1630	b sheet
1630	antiparallel.b sheet
1630	b
1632	b sheet extended chain
1633	b
1635	b sheet
1638	b sheet
1638	b sheet
1639	b
1642	random coil and B sheet
1642	b sheet
1644	b
1656	b turns or b antiparallel
1664	b turns
1672	antiparallel-b sheet
1672	b turns
1673	b turns
1675	b-spec
1684	antiparallel b
1685	b sheet-turn

1687	b turns and sheet
1687	antiparallel-b sheet
1687	antiparallel vibrations
1620-1640	b
1620-or below	b
1622/1639	b
1625/1639	b
1626/1624	b strands exposed
1630-1640	b
1635-1675	b
1636-1680	b sheet antiparallel

Random

1643	random
1646	random
1650	unordered
1659	random
1660	disordered
1640-1648	r

Amide I

1550	amide 1
1648	amide 1
1650	amide 1
1652	amide1
1660	amide I
1660	amide I Lysozyme native
1670	amide I Lysozyme denatured
1652-1659	amide 1

Amide II

1530	AmideII
1542	amide II
1548	amide II
1550	amide II
1543-1530	amide II

Amide III

1240	amide 3

Substructures

785	CH3 rocking
1317	CH2 CH2
1400	carbonyl

1455	CH2 CH3
1583	COOH protein
1718	carbonyl stretch
1721	carbonyl,H bond
1740	carbonyl,H bond
2995-2825	C-H stretch

Turns

1655	turns-general
1664	turns
1666	turns
1668	turns
1670	turn-spec
1672	turns
1680	turns
1683	turn-spec
1688	turn-spec
1688	turns
1694	turn-spec
1663/1680	turns

Lipids

1088	sym phosphate
1230	asym phosphate
1740	phospholipestercarbonyl
2854	acyl-lipid symm
2930	acyl-lipid asym
1731-1741	carbonyl-lipid
1760-1700	phospholipid
300-2800	acyl-lipid

Amino Acids in proteins

1118	tyrosine
1458	phenylalanine
1476	Phenylalanine
1515	tyrosine
1516	tyrosine
1518	tyrosine
2554	a cysteine SH stretch
2566	b cysteine SH stretch
2592	b cysteine SH stretch

Name	ref	Year	Instrument	Method	deconvolution	protein	system	solvent	cm-1	conformation
Surewicz	140	1988	various	various	FSD/2nd	various	various	D2O	1650-1658	a
								D2O	1640-1648	r
								H2O	1650-1660	a
									1620-1640	b
									1620-or below	b
									1630	unknown
									1670	turn-spec prot
									1683	turn-spec
									1688	turn-spec
									1694	turn-spec
									1675	b-spec
									1655	turns-general
					least-squares	Bacteriorhodopsin	dry film		1663	a
					least-squares	Bacteriorhodopsin			1639	b
					nth derivative	Bacteriorhodopsin	solution	D2O or H2O	1630-1640	b
					nth derivative	Bacteriorhodopsin			1684	antiparallel b
					nth derivative	Bacteriorhodopsin			1660	distorted helices
					FSD or derivative	rhodopsin			1675	a
						rhodopsin			1625/1639	b
					fsd	myelin			1622/1639	b
					resolution enhanced	myelin		D2O	1646	random
						myelin			1663/1680	turns
						myelin			none	a or b
						myelin+lipid			1635-1675	b
						myelin+lipid			1654-1668	a
Areas	141	1989	J-Ash raman	capillary	Lippert method	phospholipase	soln and solid film	2H2O and H2O		
									1655-1660	a random coil
			PE 1750	absorption	2nd and FSD				1685	b sheet-turn
									1630	b sheet
									1515	tyrosine
									1546	not structural
Arrondo	142	1987	Digilab FTS 15	absorption	FSD and 2nd der	Ca-ATPase	solution	H2O	1652	amide1
				CaF window				D2O	1646	
								H2O	1550	amide2
								D2O	1460	
								?	1760-1700	phospholipid

Name	ref	Year	Instrument	Method	deconvolution	protein	system	solvent	cm-1	conformation
Arrondo	142	1987							1657	a
									1630	antipar. Bsheet
									1682	antipar. Bsheet
									1668	turn
									1692	turn
									1643	random
									1516	tyrosine
									1731/1741	carbonyl-lipid
									300-2800	acyl-lipid
Bhushan	143	1990	PE 1750 BaF2	absorption	FSD	lipid protein in acetylcholine receptor	solution	D2O	2854	acyl-lipid symm
									2930	acyl-lipid asym
									1740	carbonyl,Hbond
									1721	carbonyl,Hbond
									1230	asym PO2
									1088	symPO2
Carpenter	144	1989	PE1700	absorption solid-aqueus	primary spectra	carbohydrate and dried protein	solid and aqeous	D2O	1365-1300	OHin sugars
									1652-1659	amide 1
									1543-1530	amide2
									11583	COOH protein
Casal	145	1988	Digilab FTS-15	absorption	FSD,2nd deriv	b lactaglobulin	aqueous	H2O only	1636-1680	b sheet antipar
									1626/1624	b strands exposed
									1664	turns
									1649	a helix and random
Dev	146	1988	Digilab FTS 15	absorption	2nd 4th derivatives	11 S globulin	aqueous	D2O	1240	amide 3
									1287	ahelix
									1317	CH2 CH2
									1400	carbonyl
									1455	CH2 CH3
									1476	Phe
									1518	TyrStr
									1548	amide 2
									1638	B sheet
									1660	disordered
									1687	Bturns and sheet
Navarro	147	1984	PE 283B & Nicolet MXS	absorption	primary spectra	lipid/protein ratio in sarcoplasmic reticulum			1651	a
									1630	b
									1659	random
								KBr	very dry	1740	phospholip ester carbonyl
							KBr	very dry	1660	Amide I

Name	ref	Year	Instrument	Method	deconvolution	protein	system	solvent	cm-1	conformation
Navarro	147	1984					KBr	very dry	1530	AmideII
							"	"	1660	Amide I Lysozyme native
							"	"	1670	Amide I Lysozyme denat
Nabedryk	148	1990	Nicolet 60sx	absorption	primary spectra	Bact.quinones	hydrated film	H2O	1650....	amide 1
Markovich	149	1989	Nicolet20sxc IR plan 1 micro	reflectance microscopy	primary spectra	membrane lipid	film	none	2995-2825	C-H stretch
Rial	150	1990	Nicolet 10DX	absorbance	n?th deriv	Uncoupling prot	sol CaF cell	H2O	1656	a
					"	"	"	D20	1654	a
					"	"	"	D20	1635	b sheet
					"	"	"	H2O	1687	antiparallel-b sheet
					"	"	"	D20	1672	antiparallel-b sheet
					"	"	"	D20 or H20	1624	border of b sheet
					"	"	"	?	1656	b turns or b antiparallel
					FSD & Curve Fitting	"	"	?	1615	C-C stretch,no struct info
					"			H20	1654	a-helix and random coil
					"			D20	1642	random coil and B sheet
					"			H20	1654	a-helix
									1633	b struc
									1673	b turns
								D20	1654	a helix only
									1672	b turns
									1664	b turns
El Antri	151	1990	Nicolet 10MX	abs,CaF2 cell	primary spectra	haemoglobin	solution	H2O	1687	antipar vibrations
								H2O	2554	a cysteine SH stretch
									2566	B cysteine sh stretch
									2592	b cysteine sh stretch 93
Simhony	152	1987	Nicolet 5 DX	absorp	primary spectra	BSA	paste on AgCl fiber	H2O	1648	amide 1
									1542	amide 2
									1458	phe
									1118	tyr
									937	a helix
Dong	153	1990	PE 1800	absorb 2cm				H2O	1624	b sheet
									1627	b sheet
									1632	b sheet extended chain
									1638	b sheet
									1642	b sheet

Name	ref	Year	Instrument	Method	deconvolution	protein	system	solvent	cm-1	conformation
Dong	153	1990	PE 1800	absorb 2cm				H20	1650	unordered
									1656	a helix
									1666	turn
									1672	turn
									1680	turn
									1688	turn

IR spectrum of Lactoferrin and its 4th derivative

Absorbance

1652

1540

Wavenumber (cm-1)

Nic/IR

Wavenumber (cm-1)

Nic/IR
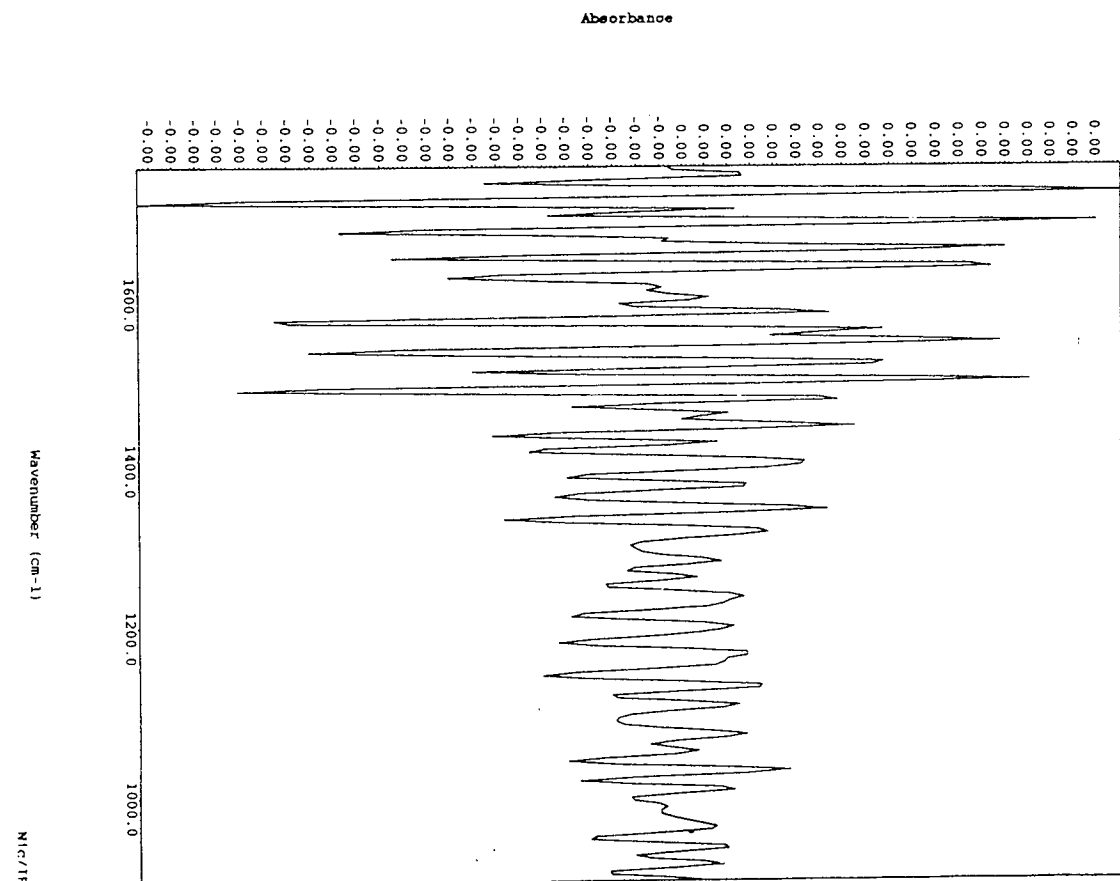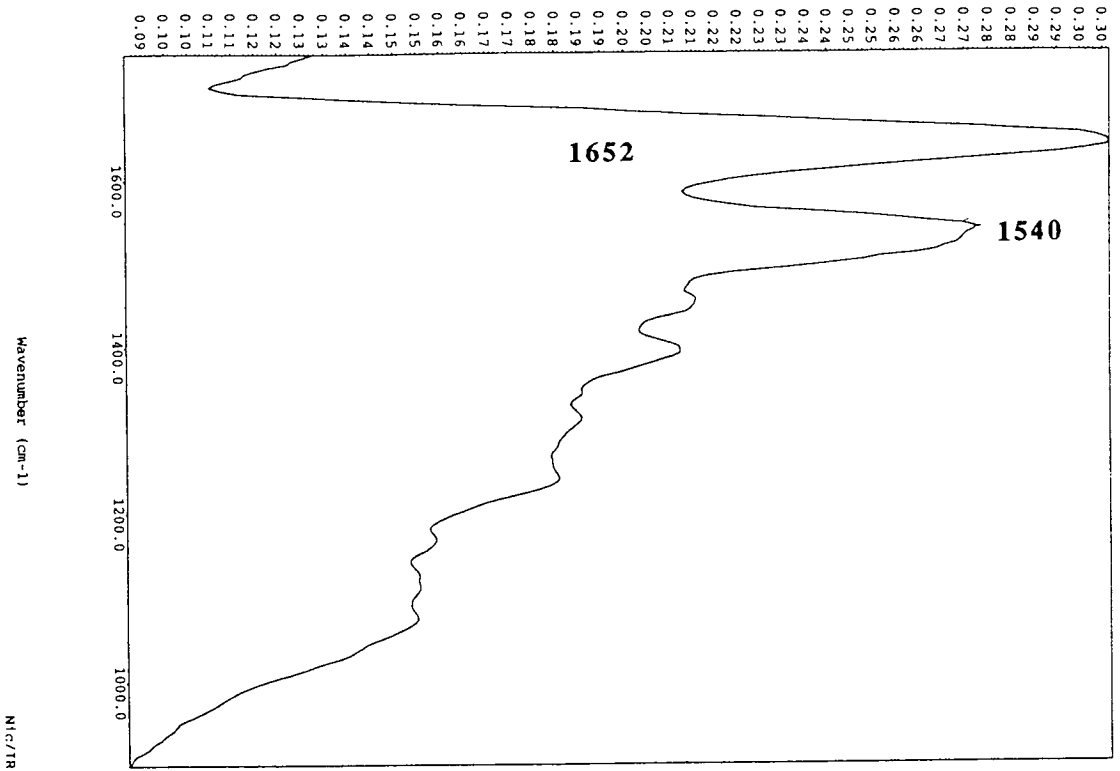
IR spectrum of Lysozyme and its 4th derivative

285
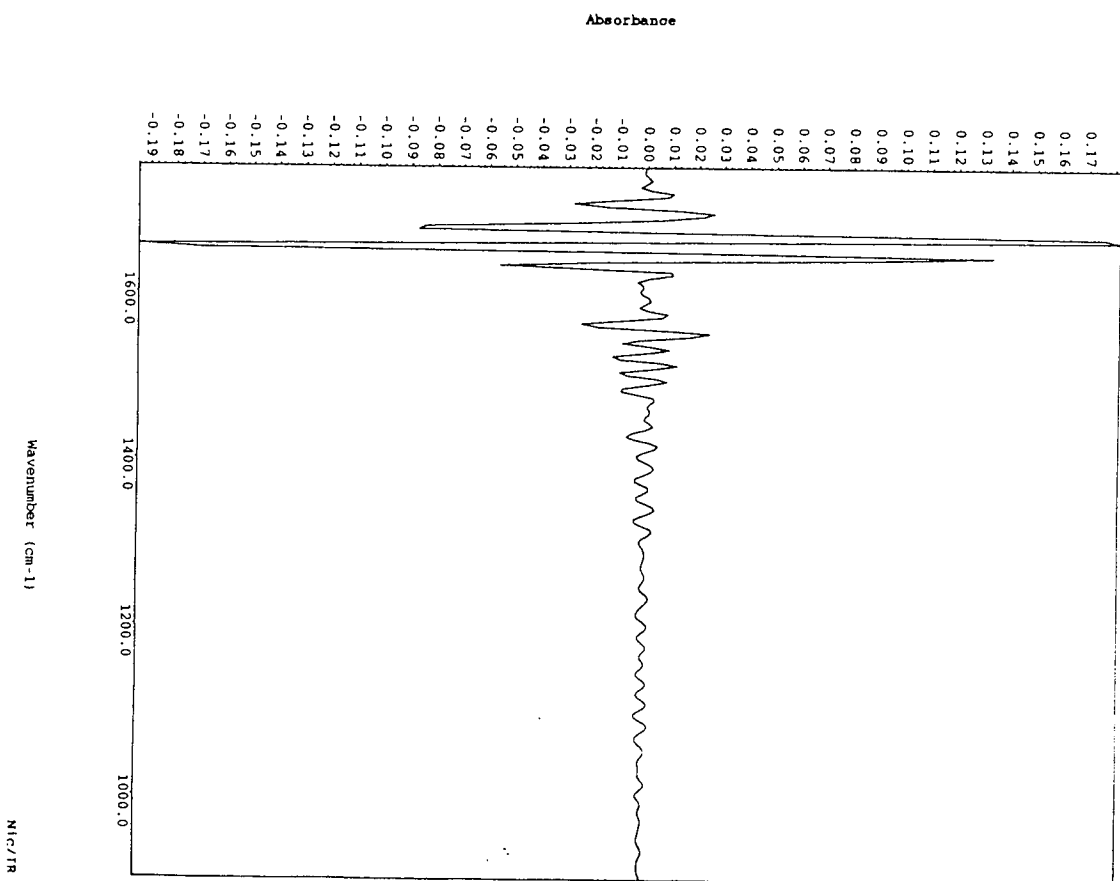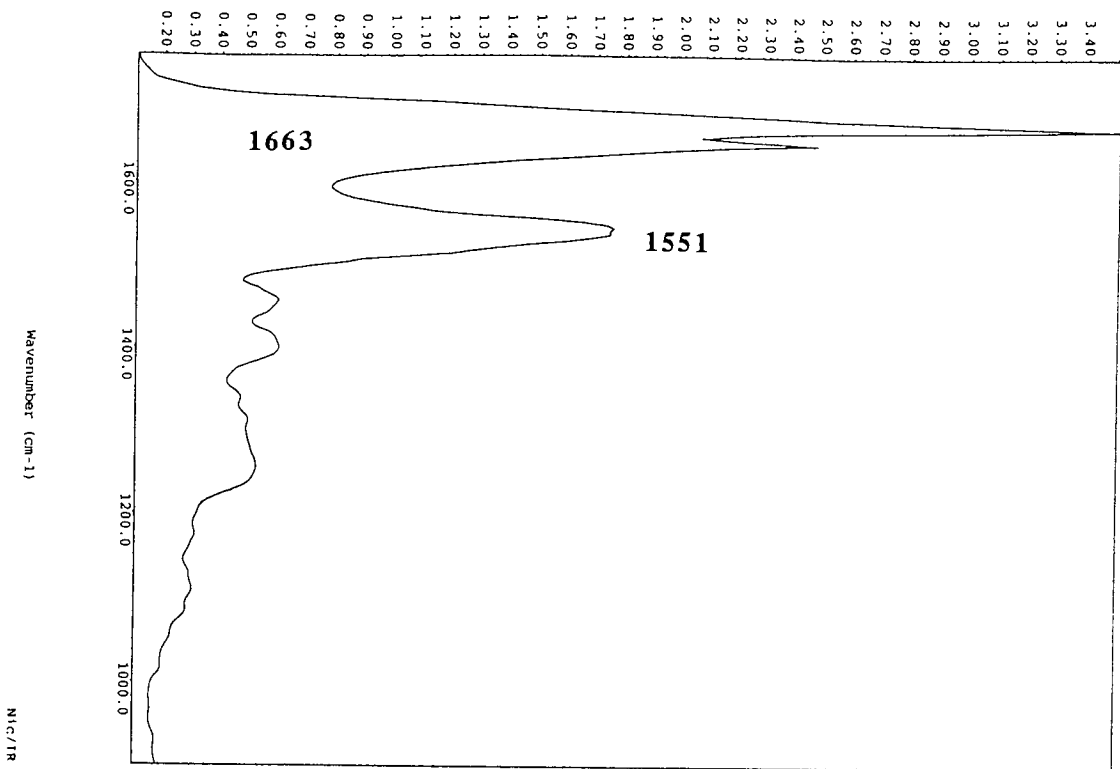
IR spectrum of Human albumin and its 4th derivative