Immunological and Biochemical Techniques in the Analysis of Tear Proteins

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Summary

This study is concerned with the analysis of tear proteins, paying particular attention to the state of the tears (e.g., non-stimulated, reflex, closed), created during sampling, and to assess their interactions with hydrogel contact lenses. The work has involved the use of a variety of biochemical and immunological analytical techniques for the measurement of proteins, (a), in tears, (b), on the contact lens, and (c), in the eluate of extracted lenses.

Although a diverse range of tear components may contribute to contact lens spoilation, proteins were of particular interest in this study because of their theoretical potential for producing immunological reactions. Although normal host proteins in their natural state are generally not treated as dangerous or non-self, those which undergo denaturation or suffer a conformational change may provoke an excessive and unnecessary immune response.

A novel on-lens cell based assay has been developed and exploited in order to study the role of the ubiquitous cell adhesion glycoprotein, vitronectin, in tears and contact lens wear under various parameters. Vitronectin, whose levels are known to increase in the closed eye environment and shown here to increase during contact lens wear, is an important immunoregulatory protein and may be a prominent marker of inflammatory activity.

Immunodiffusion assays were developed and optimised for use in tear analysis, and in a series of subsequent studies used for example in the measurement of albumin, lactoferrin, IgA and IgG. The immunodiffusion assays were then applied in the estimation of the closed eye environment; an environment which has been described as sustaining a state of sub-clinical inflammation. The role and presence of a lesser understood and investigated protein, kininogen, was also estimated, in particular, in relation to contact lens wear.

Difficulties arise when attempting to extract proteins from the contact lens in order to examine the individual nature of the proteins involved. These problems were partly alleviated with the use of the on-lens cell assay and a UV spectrophotometry assay, which can analyse the lens surface and bulk respectively, the latter yielding only total protein values. Various lens extraction methods were investigated to remove protein from the lens and the most efficient was employed in the analysis of lens extracts. Counter immunoelectrophoresis, an immunodiffusion assay was then applied to the analysis of albumin, lactoferrin, IgA and IgG in the resultant eluates.

Keywords: Tear proteins, contact lens deposits, vitronectin, closed eye environment, immunodiffusion assays, inflammation, lens extracts

Dedicated to Dad, Mum and Fiona

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α-	anti- (antibody)
APC	antigen presenting cells
AST	automatic stimulated tear
C	closed eye
°C	degree Celsius
CALT	conjunctival associated lymphoid tissues
CEE	closed eye environment
CIE	counter immunoelectrophoresis
CO_2	carbon dioxide
dH_2O	distilled water
DMEM	Dulbecco's modified eagles medium
DW	daily wear
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
EW	extended wear
Fab	fragment antigen-binding
FBS	fetal Bovine serum
Fc	fragment crystallizable
FDA	Food and Drug Administration
Fn	fibronectin
g	gram
GMA	glyceryl methacrylate
HBS	Hepes buffered saline
HCL	hydrochloric acid
Hf	Hageman factor (factor XII)
HMWK	high molecular weight kininogen
IEP	immunoelectrophoresis
(i)	pre-extraction absorbance

Ig	immumoglobulin
IgA	immunoglobulin A
IgD	immunoglobulin D
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
IL-1	interleukin 1
IL-6	interleukin-6
J chain	joining chain
kDa	kiloDaltons
L-glu	L-glutamine
Ln	natural log
LMWK	low molecular weight kininogen
MA	methacrylic acid
MAC	membrane attack complex
MALT	mucosal associated lymphoid tissues
2-ME	2-mercaptoethanol
MMA	methyl methacrylate
µg	micrograms
mg	milligrams
µl	microlitre
$\mu l min^{-1}$	microlitre per minute
ml	millilitre
µm	micrometres
mm	millimetre
mM	millimole
MTP	main tear proteins
N/A	not applicable
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
ηg	nanograms
nm	nanometres

O	open eye
OD	one day wear
OEE	open eye environment
PAI	plasminogen activator inhibitor
PEG	polyethylene glycol
PGI_1	prostaglandin I_1
PGE_2	prostaglandin E_2
PMMA	poly (methyl methacrylate)
PMN	polymorphonuclear
PVP	poly (vinyl pyrrolidine)
PTS	pseudotear solution
polyHEMA	poly (2-hydroxyethyl methacrylate)
RGD	Arginine-Glycine-Aspartic acid
RIA	radio immunoassay
RID	radial immunodiffusion
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sIgA	secretory immunoglobulin A
StDev	standard deviation
TBE	Tris/Boric acid/EDTA
TEMED	N,N,N',N'-tetramethylenediamine
TPA	tear specific prealbumin
TNF-α	tumour necrosis factor-alpha
Tris	Tris (hydroxylmethyl) aminomethane
TS	tear sample
2-ME	2-mercaptoethanol
UV	ultra violet
Vn	vitronectin
VP	N-vinyl-2-pyrrolidine
WHS	whole human serum
(x)	post-extraction absorbance

Chapter 1

Introduction

1.1 Introduction

The development of new biocompatible ocular prostheses relies on the understanding of the chemical and physical nature of tears and role of the ocular environment. Once inserted into the body, foreign polymers are rapidly coated with a biofilm which can alter the biocompatibility. There is no exception when it comes to the insertion of a contact lens. On a contact lens, this film can affect the visual performance of the lens and therefore shorten the life span of the lens if the film is not easily removed. This is a two-sided story, not only can the lens become spoilt with this layer of tear constituents, including lipids and proteins, but this absorption by the lens may denude the tears of important elements. Spoilation of the lens can be affected by a number of factors including, surface topography, chemical structure, the degree of hydration of the lens and also the nature of the spoilant, in this case the tear film.

The conjunctiva and cornea are covered by a structured gel commonly known as the precorneal tear film, which forms an interface between the air and the ocular tissues. The composition of tears include a complex mixture of proteins, enzymes, lipids, metabolites and electrolytes maintained by the effective interaction of the secretory, distributive and excretory processes of the lacrimal system. Tears, although minute in volume, demonstrate numerous important functions which serve to sustain a transparent, even layer over the cornea.

Tears and *in vivo* contact lens wear studies have the advantage of accessibility, lack of patient trauma and can be studied continually without detriment. This provides us with easy and safe analysis of the role of tears in contact lens spoilation. A full understanding of the structure of the tear film is important in assessing its interactions with contact lenses and to understand spoilation. Proteins, in tears, in spoilation, are of special interest because of their assumed potential for producing immunological reactions which are enhanced by the presence of a contact lens.

1.2. The Tear Film: Structure and Function

The tear film is a structured gel with a thickness ranging between 6-10μm and although difficult to determine, the tear volume has been estimated at 7.0±2μl.[1] There is a basic tear production rate of 1.2μl/min, with a range of 0.5-2.2μl/min which is influenced by sampling technique and rate of evaporation.[2, 3] In the normal eye, the precorneal tear film constitutes approximately 1μl, the conjunctival sacs (4-5μl) and the marginal strips (2-9μl).[4] The tear film can be grossly subdivided into three interactive layers which provides an interface between the outside environment and the underlying corneal epithelium. The three 'layers' are, the outer lipid layer, middle aqueous layer and the inner mucin layer but, as stated, they are not strictly distinct layers they may interact; mucins become diluted towards the aqueous layer and may combine with, and mask, the hydrophobicity of the lipids.[5] For the sake of simplification the layers will be dealt with individually, discussing their unique roles in the stabilisation and normal functioning of the human tear film. A full understanding of the complex structure and make up of the tear film is necessary in order to explain its interaction with a hydrogel contact lens biomaterial.

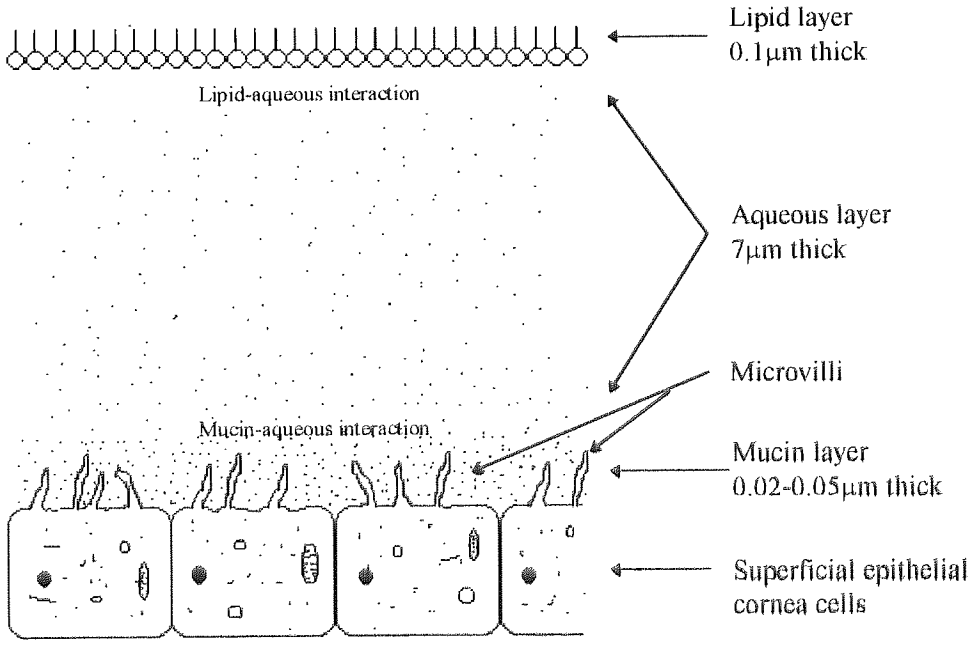


Figure 1.1. The structure of tears

1.2.1 Lipid Layer

The lipid layer is approximately 0.1μm thick.[6] This can depend on the degree of compression by the eye lids, which thins it further. During a blink action the lids can affect the thickness, as the upper and lower lids come together and the layer becomes compressed. The lipid layer comprises mainly of waxy esters, sterols, triacylglycerols, cholesterol, and a small amount of polar lipids and free fatty acids, however it has been demonstrated that there is considerable variation between individuals.[7, 8, 9,] The lipid layer is secreted by the meibomian glands in the lower and upper lids. The glands of Zeis and Moll may also provide a contribution to the lipid layer. This layer essentially behaves independently of the underlying aqueous layer, but rather is anchored at the orifices of the meibomian gland above and below, and does not follow the flow of tears from the lateral canthus to the puncta. The lipids do not clump together but orientate themselves so as to expose their hydrophilic ends to the aqueous layer. This layer is essential in the protection of the aqueous layer from polar lipid contamination that would inevitably lead to the premature rupture of the tear film. The main functions of the lipids are to prevent the evaporation of the tear film and to act as a lubricant for the smooth movement of the lids over the irregular corneal and conjunctival surfaces.

1.2.2. Aqueous Layer

The aqueous layer is the major component of the tear film, making up approximately 98% of the total thickness and measuring approximately 7μm thick. It is a complex dilute solution of both organic and inorganic species, with as many as sixty proteins detected,[10] combined with glycoproteins, glucose, urea and various electrolytes. Some of the proteins present are locally produced tear specific proteins and the primary source of these are the main lacrimal glands and to a lessor extent, the accessory glands of Krause and Wolfring. Other proteins detected in the aqueous tear are plasma/serum derived, the concentration of whom varies depending on the stability of the blood-tear barrier. This barrier can be affected by a number of factors including inflammation and eye closure, but even in the normal uncompromised eye

plasma proteins can still be detected. Other proteins may be synthesized locally by epithelial cells, e.g., the secretory component involved in sIgA transport.

As stated above more than sixty proteins have been detected in tears, with approximately twenty demonstrated to be derived from the lacrimal gland.[10] The concentrations of each protein varies between individuals, their state of health, and whether or not they are contact lens wearers. Determination of the proteins present depends on the tear collection method and the techniques used to analyse the tears. The following is a summary of the main tear proteins, discussing their functions in tears in an attempt to gain an insight into the affects of contact lens wear on the ocular environment and subsequent changes on the lens.

1.2.2.1. Lysozyme

Lysozyme was initially identified and described in 1922 by Alexander Fleming[11] as an antibacterial enzyme, isolated firstly in nasal mucus and later discovered in tears. It is secreted by skin and mucous membranes; in tears it is a lacrimal gland derived globular protein with a molecular weight of 14.6kDa. The average concentration in the unstimulated open eye tears is estimated at 1.3mg/ml[12] but it can range between 0.5-4.5 mg/ml[12, 13, 14] depending on collection and assay procedures. Human tear fluid contains the highest concentration of lysozyme as compared to other body fluids.[15] One of its main functions is in the innate immune response to bacteria, destroying certain classes of bacteria, mainly gram positive. Gram positive bacteria possess an outer coat of peptidoglycan compared with gram negative bacteria whose outer membrane is a lipid bilayer anchored to the peptidoglycan layer. Lysozyme degrades the N-acetylmuramic acid (NAM) - N-acetylglucosamine (NAG) backbone structure of the peptidoglycan and disrupts the bacterial cell wall. Gram negative bacteria may also be injured by lysozyme, especially if the structure of the outer membrane has previously been damaged or weakened, giving the enzyme access to the peptidoglycan.

Lysozyme concentration can serve as an indicator of tear dysfunction, the levels of lysozyme production provide an indication of lacrimal and accessory secretion and function. For example, in dry eye states such as in Sjögren syndrome, lysozyme levels are dramatically reduced.

1.2.2.2. Lactoferrin

Lactoferrin was first isolated from milk[16] as a reddish-pink protein with characteristics similar to, but not identical to those of transferrin, a family to which it now belongs. It is an iron-binding glycoprotein, with a molecular weight of approximately 82kDa, present in milk and to a lesser extent in other body fluids such as bile and tears; it was progressively discovered in most exocrine fluids. The main source of tear lactoferrin is the main and accessory lacrimal glands. The average concentration of lactoferrin in the open eye of unstimulated tears is 1.4mg/ml[17] measured by radial immunodiffusion with values ranging between 1-2mg/ml[12, 18] depending on collection and assay procedures. Lactoferrin is assumed to be involved in the defence of the eye against bacterial invasion.[19] Its proposed mode of action involves the inhibition of bacterial growth and colonization by the uptake of iron (an essential mineral for the growth of bacteria) from the environment, in order to deprive the microorganism of this essential bacterial nutrient. It has been suggested that lactoferrin is also involved in aiding the killing of gram negative bacteria by lysozyme, by altering the outer membrane and exposing the peptidoglycan for lysis, as mentioned above.[20] Other theories on its functionality have been put forward suggesting roles in the enhancement of natural killer cell function,[21] suppression of primary antibody responses [22] and exhibition of anti-complementary effects;[23] these theories have yet to be proven satisfactorily as most of these activities have only been demonstrated *in vitro*.

1.2.2.3. Tear Specific Prealbumin/Tear Lipocalin

Tear specific prealbumin is one of the four main tear proteins, however its function in tears remains largely unresolved. This may be partly due to the fact that since its initial discovery it has taken a long time to define the protein and determine its identity. In 1956 Erickson[24] carried out immunoelectrophoresis (IEP) on human tears against a rabbit anti-human tear and discovered a line of precipitation in the albumin region which persisted after absorption with normal human serum. Consequently it was named tear albumin noting that at this stage the idea that it may have been a modified version of serum albumin had not been ruled out.

In 1969 Bonavida[25] investigated the source and nature of tear albumin and demonstrated the existence of a prealbumin specifically produced by the lacrimal glands and thought to be absent in serum and other body fluids. It showed no cross reactivity with serum albumin and antibody to serum prealbumin (PA) failed to react with tears, it was therefore renamed specific tear prealbumin (STA/TPA). This incorporation of the word 'prealbumin' in the naming of this protein has led to some confusion over its true identity and is occasionally confused with serum prealbumin or albumin.

Ten years later work[10] was done on this protein under the name, 'protein migrating faster than albumin' (PMFA) which somewhat avoided the confusing association with the name prealbumin. They found that running it by IEP against rabbit anti-human tears demonstrated that two lines of precipitation occurred, a major line (in accordance with the Erickson work) with a migration faster than albumin towards the anode and a minor line exhibiting β-mobility near the well of application. SDS-PAGE of purified TPA again revealed two closely migrating bands, which gave molecular weights at approximately 17kDa and 22kDa.[26] Later an attempt to separate the major line precipitate (TPA_1) from the minor line precipitate was undertaken. The major anodic line was compared against serum PA and established that the proteins varied in molecular weight, at 21kDa estimated for TPA_1 versus 55kDa for serum PA and

unlike serum PA the purified TPA showed a predominance of acidic residues and a trace amount of tryptophan.[27]

In 1992 a computer assisted homology search revealed that the primary structure of TPA was highly similar to the lipocalins; a protein superfamily of small proteins (18-20kDa) which serve as carriers for hydrophobic molecules with various ligand specificities such as steroids, retinol and odorants. Among the twenty (approximate) members of this superfamily TPA showed the highest similarity with the von Ebner's gland protein of the rat, which is involved in taste.[28] Additionally, in contrast with earlier studies,[25] proteins reactive with an antiserum against TPA were found to be present in saliva, sweat, and nasal mucus, shown by western blot analysis, with similar size isoforms occurring in each.

The physiological role of the lipocalin varies widely according to the type of ligand they bind. It is speculated that tear lipocalins/TPA may serve as carriers for hydrophobic compounds of the tear film. The lipocalins were shown to bind retinol and some fatty acids *in vitro*,[28] noting that the tear lipocalins are unusual members of the lipocalin family in that they bind cholesterol. Tear lipocalins were shown to associate with fatty acids, fatty alcohols, phospholipids, glycolipids and cholesterol and it was suggested that by transporting certain lipids in aqueous tears they may prevent hydrophobic molecules from directly contaminating the mucous layer when thinned.[29] They may also function in aiding the even distribution of the lipids, however their physiological role may be more complex than simply to bind or transport lipids. Another function suggested is that they may have bacteriostatic activity on some gram negative bacteria and have the ability to inhibit leucine incorporation by *B. subtilis*.[26] This role is further enhanced by studies demonstrating that it interacts with lysozyme in external secretions.[30] But in conclusion, the true purpose of the tear lipocalin in tears has yet to be fully established.

1.2.2.4. Immunoglobulins

Immunoglobulins (Igs) are glycoproteins with antibody activity which means they combine specifically with the substance (antigen) that elicited their formation. They make up the humoral arm of the immune response which confers exceptional specificity in a world of vast diversity. They are bi-functional in that they can bind the antigen by means of the antigen binding fragment (Fab) and they can also initiate a variety of secondary, effector functions, by means of the fragment crystalizable (Fc) region of the antibody for example in complement activation (see Figure 1.2.). There are five classes of antibody in the human body, immunoglobulins G,A,M,E and D. They are similar in general molecular structure, but they have distinctly different amino acid sequences in the constant (C) region of their heavy (H) chain, this confers distinct functional differences, with each class assuming an individual role in the defence of the host against foreign or potentially harmful bodies.

Immunoglobulin M (IgM) is known as the first line of defence against attack and provides the main humoral immune response during the host's first encounter with a particular organism and which stimulates an increase in immunoglobulin G (IgG) and immunoglobulin A (IgA). It is also the most efficient antibody in complement fixation. Immunoglobulin E (IgE) and D (IgD) are present in the body in much smaller quantities. The function of IgD is poorly understood, but IgE, is known to be prominent in allergic responses, triggering the release of inflammatory mediators from mast cells. IgG is the predominant antibody of serum and circulates in the serum at an approximate ratio of 5:1 over IgA. IgA, on the other hand, is the main protective antibody of the mucosal and secretory immune system, which includes the external surface of the eye. In external secretion, such as in tears, IgA takes on the form of secretory IgA, a dimer. Despite the avascularity of the cornea, it possesses the highest concentration of immunoglobulins of all ocular tissues.[31] IgA and IgG are present in the cornea but IgM, a large molecule, cannot diffuse into the corneal stroma and thus is concentrated in the limbus.

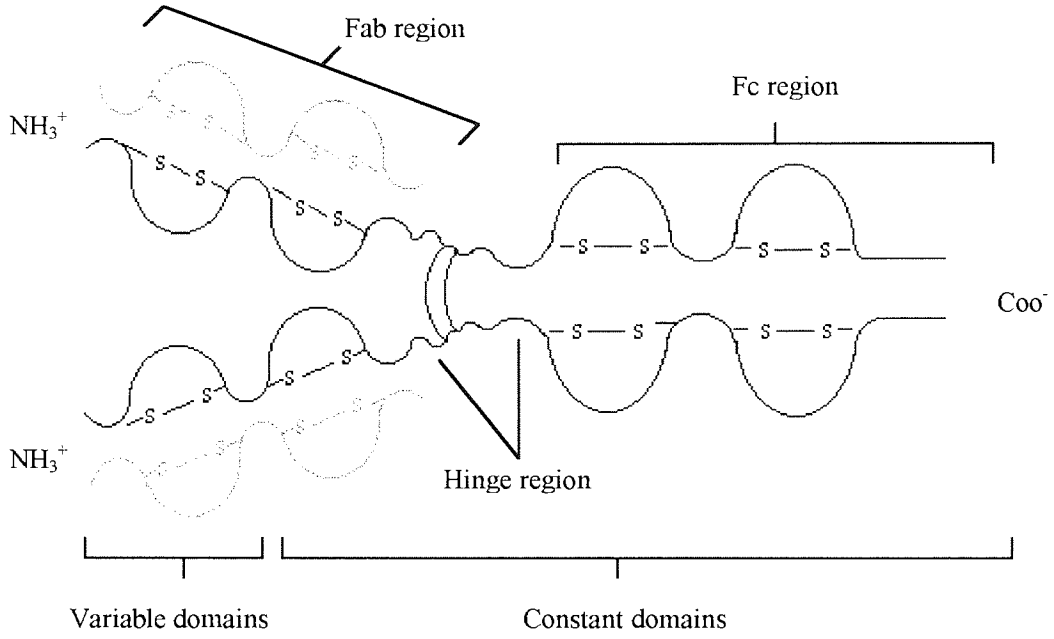


Figure 1.2. Outline of the structure of IgG. Demonstrating the general structure of an antibody and highlighting the fragment crystalizable (Fc) region which defines the function of the antibody and the fragment antibody binding (Fab) region which is the part of the antibody specific for the antigen. The red chains signify the light chains and the black chains signify the heavy chains.

1.2.2.4.1. Secretory Immunoglobulin A

Secretory immunoglobulin A (sIgA) is the most abundant and main antibody of the mucosal immune system, whereas IgG is the principle antibody in serum, sIgA is the focus of the seromucosa. In human serum, IgA exists as a four chain unit with a molecular weight of approximately 150kDa, whereas in external excretions, IgA (named sIgA, molecular weight 400kDa) is usually present in a dimeric form and possesses additional components for secretory function. The additional components are the J (joining) chain, a small acidic polypeptide produced by the plasma cells and, a secretory component, present in the form of a single polypeptide chain with a molecular weight of approximately 95kDa that is exclusively found in secretions. The J chain is thought not to be an absolute requirement for the polymerization of sIgA, but its presence does facilitate the polymerization of the basic units of IgA and IgM. The function of the secretory component is to enable sIgA antibodies to be

transported across mucosal tissues into the secretions. IgA (and the J chain) in tears is thought to be produced by plasma cells in the main and accessory lacrimal glands [32, 33, 34] but there is also evidence for the presence of IgA-containing human plasma cells in the conjunctiva.[34] IgA in the tear film is assembled from dimeric IgA, with the J chain, in the epithelial cells of the lacrimal gland. These epithelial cells synthesize the secretory component which wraps around the dimer and protects it from degradation.[32, 35] This sIgA moiety possesses a molecular weight of approximately 400kDa. The average concentration of IgA in open eye of normal tears is given at 0.29mg/ml,[12] but can range between approximately 0.1-0.6mg/ml,[26, 36] again depending on the collection method and assay used to analyse the tears. The functions of sIgA include the prevention of the adherence of bacteria to the mucosal surface,[37] agglutination of bacteria and anti-microbial neutralization and lysis.[38] The fragment crystalizable (Fc) region on IgA does not react with components of either paths of the complement cascade, neither does it bind C3b and therefore does not recruit inflammatory cells or mediators. This would suggest an anti-inflammatory role in the absence of an immune stimulus, which is an important characteristic to possess in the defence of the eye, an environment which has to deal with a constant barrage of insults from external sources that may induce an unnecessary excessive response.

1.2.2.4.2. Immunoglobulin G

Immunoglobulin G possessing a molecular weight of 150kDa constitutes approximately 80% of all the antibody in serum and is the effective memory antibody of serum. In a normal reaction to an invasive serum antigen, the host defence moves from an initial IgM response to an IgG response, but after secondary exposure to the same antigen, the IgG reaction is stronger and faster, this is due to the memory IgG antibodies circulating in the blood stream. In tears and other mucosal sites, the role of IgG of is not as significant, and consequently is found in normal human open eye tears only at low levels.[35] IgG lacks the extra chains of sIgA which enable transport across the epithelium, arising as it does in the mucosal surfaces possibly by passive diffusion. The average concentration of IgG in tears of the open unstimulated eye is

quoted at 0.13mg/ml.[12] The role of sIgA in the ocular environment is much better understood than that of IgG.

1.2.2.5. Albumin

Albumin in tears is serum derived.[32] It is one of the main proteins in serum with a concentration of approximately 50mg/ml, possessing a molecular weight of 68kDa. Albumin in serum is involved in the transportation of free fatty acids, stabilizing the osmotic potential and increasing the viscosity of blood. The role of albumin in tears is uncertain, but its presence is indicative of the permeability in the blood-tear barrier, inflammation, for example, affects the blood-tear barrier and results in serum leakage. Thus, albumin levels can be a good marker for the integrity of the blood-tear barrier and presence of other serum derived proteins. In the normal open eye the mean concentration of albumin has been estimated at 1.3mg/ml[12] but can approximately range between 3.9 - 0.1mg/ml,[33, 39] the higher estimated values are generally reported after epithelial damage or vascular leakage. In the attempt to ascertain the role (or roles) of albumin in tears the following observation has been made; it has been shown to increase the viscosity of a mucin solution, indicating some structural interaction between albumin and the mucins.[40] However, the reason for its presence remains unclear and requires further analysis.

Protein	Average concentration mg/ml	Molecular weight (kDa)	Tear/plasma derived
Lysozyme	1.3	14.6	Tear
Lactoferrin	1.4	82	Tear
sIgA (dimer)	0.3	160 (x 2)	Tear/plasma
IgG	0.13	150	Plasma
TPA/Lipocalin	1.23	21	Tear
Albumin	1.3	68	Plasma

Table 1.1. Summary of the main tear proteins: Concentration and source

1.2.2.6. Other Proteins

Depending on the method of identification, up to 60 proteins have been detected,[10] with twenty shown to be secreted by the lacrimal gland, others are serum derived, leaking from the circulation into the tear fluid and the rest are synthezised locally.

β-lysine has been described as an anti-bacterial substance known to be present in other bodily fluids and suggested to be present in tears, it has also been described as a non-lysozymal antibacterial factor (NLAF).[41] Its presence in tears is dubious.

Protein G was first described in 1980[42] and was suggested to be a new, separate entity in human tears. When separated and analysed by molecular weight on an SDS-PAGE run, in non-denaturing conditions, a species with a molecular weight of 31kDa was discovered, but was found to disappear in denaturing conditions.[42] This anomaly could be simply explained by the fact that the 31kDa species is a dimer of another tear protein. A lysozyme dimer would approximately yield a 31kDa entity, or it could be a new poorly understood protein. Mentioning protein G highlights our, as yet, incomplete understanding of tears and its components and illustrates the need for the further development of techniques in order to qualify and quantify tears.

Other proteins which have come to attention more recently are those involved in inflammation and the immune response. Their importance has come to light particularly in the closed eye environment, which is said to present a state of sub-clinical inflammation.[43] The proteins, which are thought to be mainly plasma derived due to an increase in vascular permeability, include those involved in the complement system, fibrinolysis, and the coagulation system, combining to interact in the immune response. Further explanations of these process are detailed later in this chapter under the heading of ocular immunology.

1.2.3. Mucin Layer

Historically the mucin layer was widely assumed to be an approximately 0.02-0.05μm thick layer but more recently it is thought not to be an exclusive layer but rather the mucins may also integrate throughout the tear film. The mucins are made up of glycoproteins, free protein and salts, the electrolyte content is similar to that of serum. Glycoproteins/mucins are a heterogeneous group of hydrated O-linked oligosaccharides, linked to a protein.[6] Ocular mucins are similar to the other mucins of the body, for example, in the respiratory and gastrointestinal tract, the thin layer of mucus that covers the epithelial cells in these and other tissues is defined as a viscous, sticky, water insoluble gel containing unique glycoproteins.[44] The main source of ocular mucin is the conjunctival goblet cells, but some mucus secreting cells have been discovered in the human lacrimal gland.[45] As the innermost layer, the mucin layer has direct contact with the superficial cells of the cornea and with the conjunctival epithelial cells. The superficial cells are covered with microvilli; adhering to the microvilli is a glycocalyx which interacts with the mucin layer to promote the formation of a stable smooth tear film on the corneal surface. Mucins are extremely hydrophilic and their primary function is to coat the hydrophobic corneal epithelium surface which enables the surface to become wettable by the aqueous tear. Mucins are thought to be fundamental in supporting the spread of the precorneal tear film; they lower the tear surface tension which enhances corneal wettability and thus facilitates the even spread of the film by blinking. Mucins may also contribute to the local immunity by providing a medium of adherence for IgA and lysozyme and by its adherence and thus clearance of many fine contaminants which the tear film frequently encounters.

1.2.4. Summary of Tear Functions

- Lubrication
- Formation of a smooth layer over the irregular cornea
- Maintenance of an antibacterial system for ocular surface
- Maintenance of corneal epithelium
- Washes away irritants/foreign bodies
- Serves as a vehicle for the influx of healing agents during injury, e.g., polymorphonucleocytes or fibronectin
- Facilitates the supply of oxygen to the cornea
- Enhances corneal wettability
- Retards evaporation
- Supports the immune response

1.3. Tear States

At this point it should be noted that tears are not simply a three layer unchanging entity, tears can be divided up into a number of different states which arise due to various internal and external stimuli. The tear state reflects obvious different tear protein compositions which can differ from patient to patient, but follow general patterns. It is very important, in tear analysis, to note the tear state being sampled as it can greatly influence on the outcome of protein levels detected.

- Basal tears:- Tears that are taken from the eye without stimulation and which involves sampling at a normal tear flow rate where no excess tearing should occur. The microcapillary pipette method of collection (see Chapter 2) is best suited for non-stimulated tear collection . Basal tears reflect the normal tear protein levels most accurately, a lacrimal gland secretion rich in lactoferrin, lysozyme and TSPA.
- Reflex tears:- Tears sampled by harsh abrasive methods, such as by the Schirmer filter strip test (see Chapter 2), demonstrate a reflex type tearing which possesses a greater quantity of plasma leakage proteins showing higher levels of albumin and

IgG and other leakage proteins.[46] The Schirmer test is believed to abrade conjunctival tissue and thus cause plasma influx.[47]

- Externally stimulated tears:- Tears stimulated through the use of external, non-contact irritation, for example, the use of ammonia or onions, which causes excess lacrimation and also stimulates a nasal response. Results from these tears show lower levels of plasma derived proteins, than by reflex tearing, as there is simply a general dilution of tears due to the excess watering.[48]
- Emotional tears:- These tears are also known as psychogenic lacrimation and as the first name suggests they are the tears of emotion, i.e., sadness and joy. Emotional tears are thought to be unique to humans but their purpose is largely unknown. Tears sampled in this state show protein levels higher than in stimulated tears and so define this state as a separate entity, a state of emotional irritation.[48]
- Open versus closed eye tears:- This definition must be made when taking a tear sample. The closed eye state has become known as a state of sub-clinical inflammation, induced by a reduced tear flow rate and loss of blink, with an increase in plasma leakage proteins and decrease in lacrimal gland secretion.[43,49] The state of the closed eye has not yet been clarified between a state of sleep and thus unconsciousness or simply one which requires a period of closed/shut eye with consciousness. This definition has and will prove to be hard to define.

1.4. Hydrogel Soft Contact Lenses

The greater our understanding of tears and the dynamics of its behaviour, under varying conditions, the greater will our understanding be on the interaction of contact lenses in the ocular environment. Deposits on contact lenses have long been implicated in limiting successful lens wear. Blurry vision, irritation and giant papillary conjunctivitis are often cited as adverse reactions arising as a result of tear deposition. Spoilation of the lens can be affected by a number of factors including, surface topography, chemical structure, and degree of hydration of the lens and also the nature of the spoilant, in this case the tear film. Various tear components deposit out of tears and onto the lens surface, including lipids, mucins, calcium and proteins. Extraneous substances like make up and air pollutants may also have a contributing role in the spoilation of the lens. The nature of the deposition, frequency and quantity largely depends on the surface properties of the lenses. The lenses are categorized in Table 1.2. In this study, it is the role of protein which remains at the fore in spoilation due to their assumed potential for stimulating, mediating and producing immunological reactions, be they to the detriment or well-being of the host

Hydrogel lenses have been categorized into four groupings, by the Food and Drug Administration (FDA), for the purpose of assessing the affects of accessory products on the lens materials. Lenses with a percentage water content above 50 were classified as high water content lenses and below 50 as low water content lenses. The more reactive surfaces were termed ionic lenses in contrast to the less reactive surfaces, called non-ionic lenses. The groupings are shown in the table below.

FDA Group	Lens Properties
I	Low water, non- ionic
II	High water, non-ionic
III	Low water, ionic
IV	High water, ionic

Table 1.2. FDA hydrogel soft contact lens group classifications

1.5. Structure of the Cornea

A knowledge of the structure and make up of the cornea is extremely helpful in understanding its role in association with tears in the defence of the ocular environment against potential pathogens. The following is a brief overview of the organization of the cornea, which is made up of the following layers:

- Epithelial layer with its basement membrane
- Stroma with its anterior modified zone of Bowman
- Descements membrane (basement membrane of corneal endothelium)
- Endothelium (mesothelium)

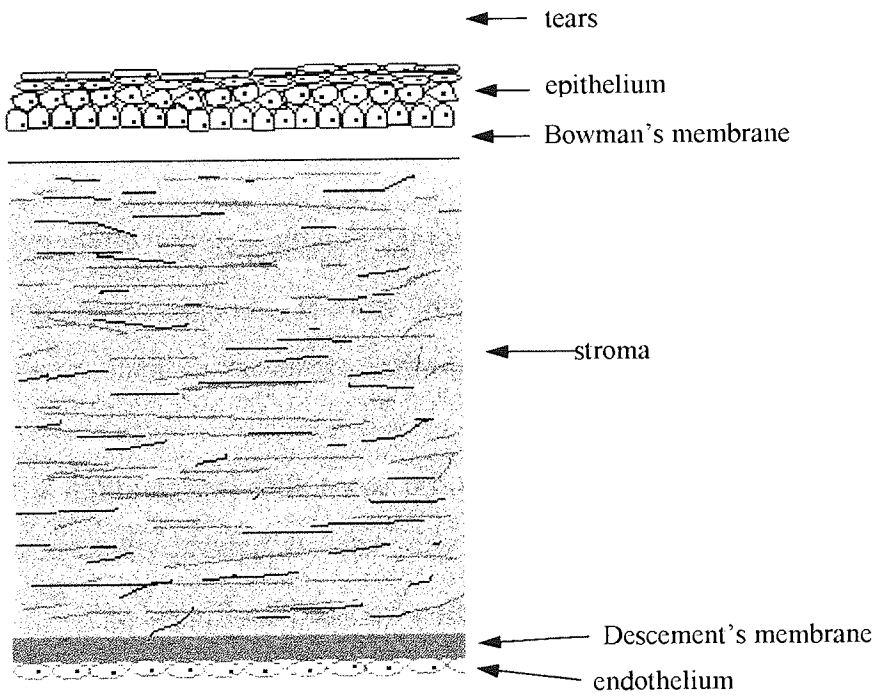


Figure 1.3. Transverse section of the cornea (Modified from [Wolffs Anatomy])[50]

1.5.1. Epithelium

For good health and normal vision, the corneal epithelium must provide an effective barrier against insult. It must be resistant to external battering such as rubbing of eyes, foreign bodies and the invasion of a contact lens. The epithelium must also act as a defence mechanism resisting the entry of microorganisms, bacteria, viruses and fungi, all of which have the potential to cause an epithelial defect which can ultimately destroy the cornea.

The epithelium is approximately 50 to 60μm thick accounting for roughly 10% of overall corneal thickness;[51, 52] for optical reasons this is kept uniform. The strength of the epithelium is enhanced by its compactness, there are no spaces between the cells and they possess interdigitating sides analogous to a lock and key. Desmosomes, intracellular junctions which are principally adhesive, add strength with their distribution around the cell. The epithelium secretes the basement membrane to which it is attached; this attachment occurs by means of hemidesmosomes, which are not as strong as that holding the membrane itself to underlying strata. Together the compactness, the intracellular junctions and the interdigitations all act synergistically to make the epithelium resilient to the severe challenges that it can encounter.

The epithelial layer is multi-layered with three cell types, the outermost layer being the superficial cells; centrally located are the wing cells and under these are basal columnar cells.[51, 52, 53] The superficial cells are constantly sloughed off and washed away during the blink and are replaced by the underlying wing cells which are in turn replaced by the basal columnar cells, in a continuous process of upward movement of the cells. The basal cells are attached to the basal lamina densa component of the basement membrane by hemidesmosomes via anchoring filaments and by intrinsic proteins such as laminin and fibronectin. The basement membrane is firmly attached to the underlying Bowmans' zone as well as to the basal cells, the former attachment being more substantial. Mechanical scraping of the epithelium, therefore, usually leaves behind the basement membrane together with the hemidesomesomal component of the basal cells.

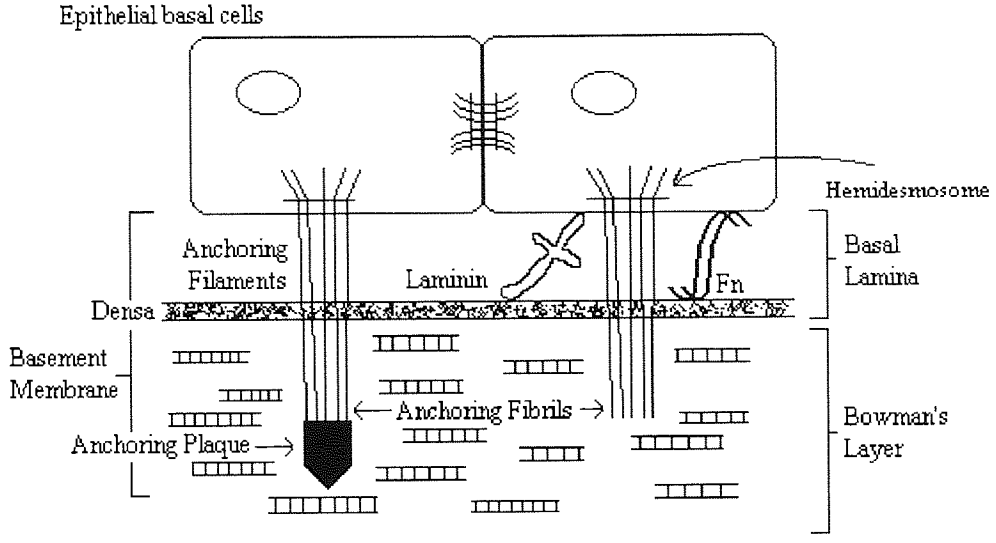


Figure 1.4. Structure of epithelial attachment to the underlying basement membrane

1.5.2. Stroma

The stromal layer is predominantly collagenous with an amorphous ground substance consisting of proteoglycans, glycoproteins, proteins and a sparse population of specialized corneal fibrocytes, keratocytes, which are dispersed through the tissue. A few wandering cells such as lymphocytes, macrophage and rarely polymorphonucleocytes may also be found. The stroma makes up approximately 90% of the corneal thickness and plays a major role in corneal transparency. This transparency is dependent on the orderly arrangement of the collagen fibres, lying parallel to the corneal surface and on the degree of hydration. The anterior portion is the Bowman's zone, below this zone the ground structure of the stroma consists mainly of mucoproteins and glycoproteins which fill all the spaces not occupied by fibrils or cells. The ground substance is approximately 4.5% of total dry weight of the cornea and consists of keratin sulphate, chondroitin- 4 -sulphate and chondroitin,[54] a secretory product of keratocytes. Nerve axons and their surrounding Schwann cells are located in the anterior and middle regions of the stroma.

1.5.3. Descements Membrane

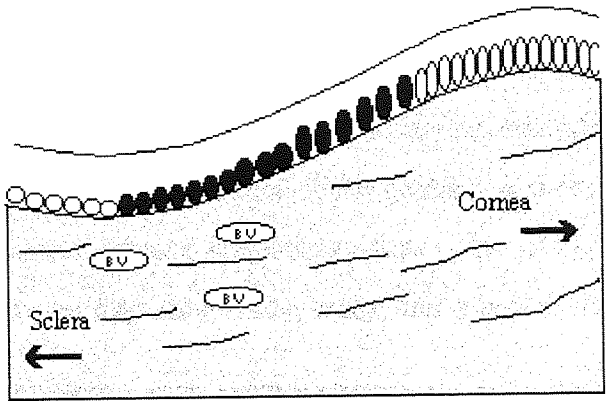
The Descements membrane or posterior limiting membrane is a basal lamina secreted, partially if not wholly, by the endothelium. It is comprised of type IV collagen, laminin and fibronectin and is produced throughout life, increasing in thickness with age. The thickness of this membrane in the adult eye is approximately 10-15 μm thick.[55] It is more resistant to collagenase destruction than the corneal stroma. The function of fibronectin in the Decements membrane was proposed to be its involvement in the adhesion of endothelial cells to the membrane.[56]

1.5.4. Endothelium

The endothelium is a monolayer of hexagonally shaped cells resting on the Descements membrane; it lines the posterior surface of the cornea and forms the anterior boundary layer of the aqueous cavity of the anterior chamber. The structural and functional integrity of the corneal endothelium is vital to the maintenance of corneal transparency. The two main functions of the endothelium are to pump ions and fluid out of the stroma into the aqueous humour; which maintains corneal hydration and transparency, and to form a stable barrier regulating the entry of aqueous humour fluids and dissolved solutes into the stroma.

1.5.5. The Limbus Region

The limbus region indicates a band of cells approximately 0.5-1.0 mm wide that interface between the cornea and sclera. They are the source of new basal cells; the limbus contains stem cells which differentiate into basal cells and migrate into the cornea. Blood vessels enter and leave the sclera in this region, small vascular loops develop from the vessels and extend approximately 1mm into the cornea, they represent the closest blood supply to the cornea.



BV=Blood vessels

Figure 1.5. The limbus region.

1.5.6. Conjunctiva

The conjunctiva starts as the transition from the skin of the lid margins and terminates at the transition of the conjunctival epithelium into corneal epithelium, with a termination of conjunctival stroma and the beginning of the Bowman's zone of the cornea. The conjunctiva is a thin translucent, non-keratinizing mucous membrane, the stroma of which is made up of loosely arranged collagen bundles, varying in compactness from superficial to deeper layers. It consists of fibroblasts, melanocytes and a small number of other cells such as macrophage, lymphocytes and plasma cells with occasionally some eosinophils and neutrophils. Goblet cells which mainly comprise such mucous membranes are not so high in number in the conjunctiva.

1.6. Ocular Immunology

The ocular environment is unique immunologically due to the fact that there is no lymphatic drainage apart from at the conjunctiva, and the normal cornea; like the brain and testes, is an immunologically privileged site. This privilege arises due to the presence of a special microenvironment and immunoregulation mechanism. Corneal avascularity is essential for optical clarity, but due to the absence of blood vessels, protection of the eye is provided by proteins in the tear film, blinking and by migrating lymphoid cells, macrophage and immune effector cells derived from the limbus region. The eye participates in all aspects of immune responses like any other tissue but the response is modulated by the cells and tissues of the eye; this privilege is lost when the cornea becomes vascularized. Among the many functions previously described for the various tear proteins, one of the greatest is their involvement in the defence of the eye against invading microorganisms. The protection of the ocular environment can be divided into three main areas: the physical, innate and acquired immunological factors.

The physical protection is controlled by the blink mechanics and those afforded by the flow of tear fluid. The motion of a blink is very important in washing away debris, in the words of F.J. Holly, "...the upper lid descends over the eye like a windshield wiper blade." Additionally the flow of tears, with excess tearing, over the eye can flush out debris, the drainage of the tear is aided by the blink action. The eyelashes also provide a form of protection by attempting to inhibit small particles from entering the ocular environment by brushing them away.

Innate immunity can be defined as a non-specific immunity and is fully functional at birth, before contact has ever been made with the particular organism, but lacks memory of previous encounters. When micro-organisms first enter the ocular environment they generally encounter the innate or natural protection of the host. It has long been demonstrated that tears contain anti-bacterial substances; the aforementioned lysozyme was one of the first anti-bacterials to be recognized,[11] which as described earlier, degrades the NAM-NAG backbone structure of

peptidoglycan and disrupts the bacterial cell wall of gram positive bacteria. This was followed by the discovery of lactoferrin, TSPA and possibly another protein β-lysine, whose presence as yet remains uncertain.

Acquired immunity is exclusive to vertebrates and allows us the ability to distinguish and specifically react with a seemingly infinite number of potential pathogens. It also has the capacity for memory, the second time the host encounters a particular organism the reaction is faster and stronger compared to the initial confrontation. It is the second line of defence and becomes activated if the physical or innate immunity fail to clear the organism. The acquired immunity of the ocular environment involves a complex series of events which was initially described with the discovery of antibodies, and in particular sIgA, an antibody important in all secretions of the body.

The acquired defence of the ocular surface is controlled by a complex series of interactive processes. It should be noted that the following description of the protection of the ocular environment must be considered in two ways; under normal functioning, immunoregulatory proteins and enzymes work synergistically to defend the host but the normal balance can be disturbed and the resulting response may be excessive and unnecessary. This is not exclusive to the eye as all over the body excessive inflammation or autoimmune responses can occasionally ensue resulting in autolysis, the mechanisms of which are relatively poorly understood. The insertion of a contact lens, a surface active biomaterial, could represent a cause of excessive host response, but this asks the question why in some instances the lens upsets the normal balance but in other instances it does not. The factors involved may be solely due to the biomaterial or a predisposition of the host, or it may be a combination of both.

The following description attempts to summarise the complexities of the host defence mechanisms, which must be understood in order to discern the origin and/or purpose, of the presentations of ocular pathologies and the possible adverse effects of contact lens wear.

1.6.1. Mucosal Associated Lymphoidal Tissues

The defence of the mucosal surfaces are governed by the mucosal immune system, commonly known as the mucosal associated lymphoidal tissues (MALT) which includes the conjunctiva, bronchus and gut.[57] As the name suggests it deals with the immune response of the lymphoid tissues that are associated with the mucosal surfaces as distinct from the systemic internal immune system. The systemic system differs from the MALT system in that the MALT relies on IgA as the main patrolling antibody, possesses T-cells with mucosa-specific regulatory properties and has a special trafficking system which transports induced cells from mucosal follicles to the diffuse mucosal lymphoid tissues underlying the epithelium. The MALT system receives antigen via the epithelium rather than through the lymphatic or blood circulation.[57, 58] The following description concentrates on the conjunctival system, the so-called conjunctival associated lymphoidal tissue (CALT), bearing in mind that the mucosal response as a whole is very similar in each case.[59]

The epithelium overlying the CALT is extremely specialized and antigen are preferentially taken up at this site. The underlying lymphoid aggregates consist of small and medium sized lymphocytes and frequently larger lymphocytes undergoing mitosis, there are no plasma cells are present, but the adjacent lymphatic channels are packed with lymphocytes. The CALT system requires specialized cells, Langerhans' cells and macrophage[57]. To process foreign and invasive substances they effect their response in association with specialized lymphocytes of the T and B-cells population.[60, 61]

Figure 1.6. Outline of the ocular surface defence mechanisms involving CALT.[59]

The Langerhans' cells are dendritic cells that reside in the epidermal and musocal epithelial surface. Their function is to process antigen and present antigen to other immunocompetent cells, for example T-cells, via epithelial surfaces.[38]

Ocular surface Langerhans' cells present antigen which can stimulates both arms of the immune response, sIgA activation and local T cell-mediated responses. The Langerhans' cells binding antigen can carry it via the lymphatic vessels to draining lymph nodes, leading to host sensitization. Thus antigen presentation via ocular surfaces is accomplished by two systems, CALT and Langerhans' cells.[59] These antigenic signals evoke a T and B-cell response in the regional lymph nodes where they migrate via the bloodstream to the mucosal site. The B-cells migrate to the sites beside the lacrimal and accessory gland epithelia where they differentiate into immunoglobulin producing plasma cells, sIgA, the predominating antibody,[57, 58] and to a lessor extent, IgG, IgM and IgE production.

T cells on the other hand arrive in sub-mucosal sites of the conjunctiva,[59] and as a result of antigen presentation by the antigen presenting cells (APCs), they produce cytokines which enhance the proliferation of the immune response. The dendritic cells appear to be more efficient in the induction of T cell mediated response than macrophage.[59]

1.6.2. Conjunctiva

Changes in the stability and permeability of conjunctival blood vessels may occur in response to injury, which results in the release of antibodies and cellular immune factors into the tears from the bloodstream. Antigen presenting cells (Langerhans' cells) are normally resident in the conjunctiva and are able to present antigen to conjunctival lymphocytes. They do not reside in the normal central epithelium, but do infiltrate it during corneal inflammation.

1.6.3. Inflammatory Mediators Generated by the Activation of Plasma Systems

The complement, kinin, blood coagulation and fibrinolytic mechanisms are physiological processes that occur through sequential cascades of enzyme activation from their inactive form, which results in the advancement of the cascade. Although each mechanism has its own distinct category, they can interact and overlap and incorporate other cell membrane proteins. The cascades are directly or indirectly involved in immunologic effector responses. Further, the recent discovery of complement proteins, vitronectin (a complement inhibitory glycoprotein) and plasmin (a member of the fibrinolytic cascade), in the closed eye demands the understanding of the sequence of events and the inflammatory mediators involved. It also demonstrates the need for the further analysis of tears for other plasma leakage proteins. One such protein investigated in this thesis was kininogen, a member of the kinin family : important inflammatory mediator.

1.6.3.1. Complement Cascade

The complement cascade involves a group of plasma and cell membrane proteins that play a key role in the host defence process. It is an enzyme system consisting of 11 proteins which can be activated by the two antibodies IgM and IgG which combine with the antigen that elicited its activation; the antigen-antibody complex combine with the initial protein of complement C1 and the cascade progresses. Other factors that can activate complement include microbial polysaccharides and some endotoxins.[38] The cascade, as the word itself suggests, involves many series of reactions, each reaction cascading on from the next. This system evolves to assist the host defence mechanism by damaging invading organisms and producing inflammation of the tissues. The final stage complement proteins, the so-called membrane attack complex (MAC) attaches to the membrane of the potential pathogen, enzymatically breaks down the membrane, which creates a hole, which then results in cell rupture and lysis and eventually death.[38, 62] Generally each complement protein is broken down into two fragments (*a* and *b*), the *b* fragment continues on the cascade while the *a* fragments are potent mediators of inflammation. This effect is achieved through the alteration of local vascular permeability, chemotactic attraction of leucocytes and the modulation of inflammatory cell function at the site of complement activation.

A complex system as it is, the complement cascade requires tight regulatory control to prevent excessive complement destruction, autolysis or excessive inflammation. This is achieved by a variety of proteins, an example is the presence of a C1 inhibitor which recognizes activated CLr and CLs on C1 and destroys this activation, it also demonstrates kinin Hageman factor inhibitory properties, an illustration of the cross-over nature of these systems. Another inhibitor of the complement system is the S-protein (vitronectin).[38] This glycoprotein interacts with the C5b67 complex, as it forms in the fluid phase, and interacts with its membrane-binding site in order to prevent the conventional binding of C5b67 to biologic membranes. Following binding of S protein to the fluid phase, C5b67 binding of C8 and C9 to the fluid phase

complex can proceed to form the MAC but the complex can not insert into lipid membranes and does not lyse cells.[63]

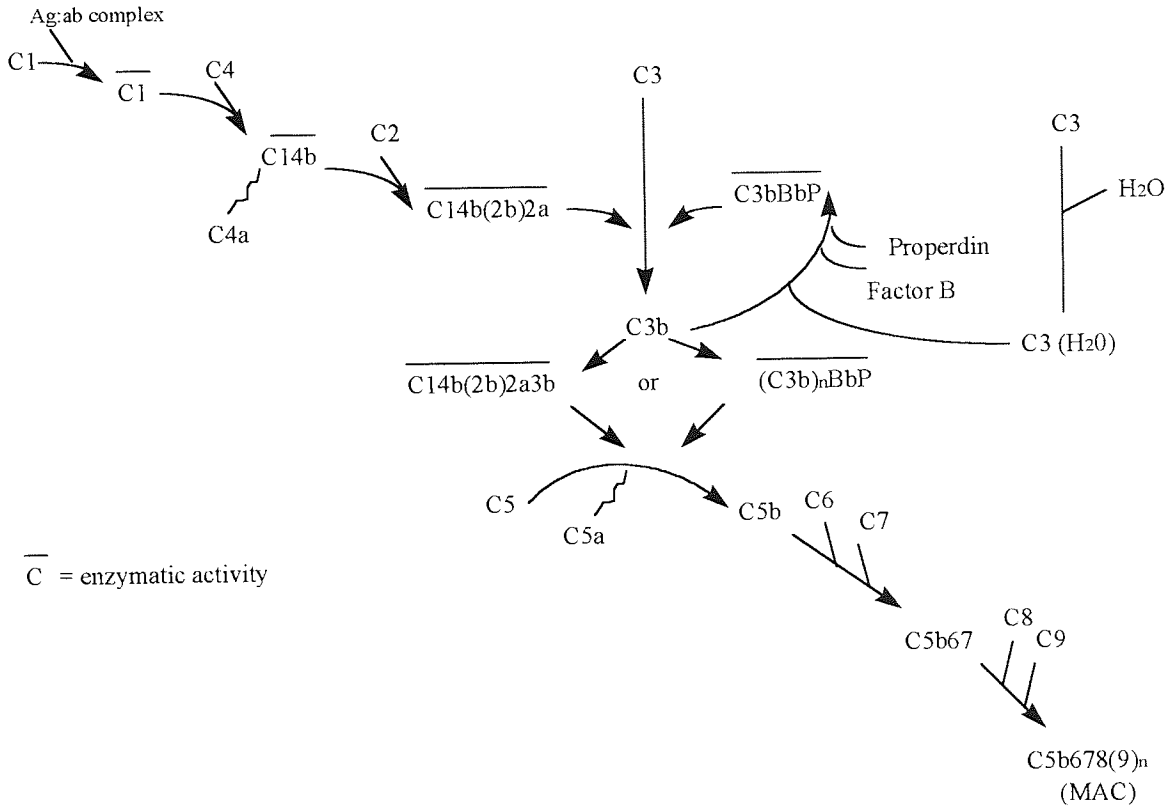


Figure 1.7. The complement pathway[38]

Individual components of the complement system have been discovered in tears but the majority of the complement system has yet to be detected.[23, 64, 65] One study investigated C1q, C3, C4, C5, C9 and Factor B (alternative pathway) in tears, with only C3, C4 and Factor B detectable in open eye tears, but all the components tested were detected in the closed eye tears.[65] All these complement enzymes were found to be functionally active.

1.6.3.2. Fibrinolysis

The end product of the fibrinolytic pathway is an enzyme plasmin, a potent proteolytic enzyme with a broad spectrum of activity. Plasmin is formed from the activation of the proenzyme plasminogen by either tissue or plasma activators. Tissue activators are found in most tissues (except the liver and placenta), plasminogen activator is also produced by macrophage. Triggering of fibrinolysis occurs when plasminogen activator, plasminogen and fibrin are in close proximity, where both the plasminogen and its activator bind to the fibrin clot. Plasmin attacks fibrin at a number of sites, producing many various fragments, which have the capacity to polymerise and compete with fibrinogen for thrombin in the coagulation cascade and act as inhibitors of clot formation, which may prevent the clot from being removed.

Tear plasmin activity has been demonstrated in the eye where corneal disease has been detected[66] and in corneal ulcers where an obvious increase in plasmin levels occur,[67] leading to the suggestion that elevated plasmin levels are somehow important in the pathogenesis of ocular infection and disease. Plasmin has also been detected in tears of patients during contact lens wear,[68] and hence is suspected to contribute to the development of corneal epithelial pathologies associated with contact lens wear.

Plasmin, plasminogen and plasmin inhibitor, α_2-antiplasmin, have all been detected in closed eye tears after overnight sleep and are presumed to be derived from plasma through the blood-tear barrier.[69]

Plasmin, a serine protease, has the ability to digest a variety of proteins. In tears, its primary role involves extracellular proteolysis following trauma, infection and inflammation. Plasmin is pro-inflammatory, with the capacity to activate the complement system,[70, 71] generate factors chemotactic for polymorphoneucleocytes[71] and cleave vitronectin, which renders it unable to bind the C5b-9 complex of complement, preventing its inhibition and thus up regulating local destruction.

1.6.3.3. Kinin Cascade

The kinin system is the second most important mediator in the blood system, the role of the kinin system is in the generation of inflammation, which advances to give the end product Bradykinin. Kinins are peptide mediators that are produced in an inflammatory episode. The consequences of kinin activation include an increase in vascular permeability, vasodilation, pain, smooth muscle contraction and an ability to stimulate arachidonic acid metabolism.[38] Initiation is achieved on contact with a variety of negatively charged surfaces which results in Hageman factor (Hf) (coagulation factor XII) activation and cleavage. This induces kinin cascade activation (through the activation of kallikrein,[72] activation of the intrinsic coagulation system and initiation of fibrinolysis. Hf is the core protein in this activation, the other important proteins are high molecular weight kininogen and prekallikrein. Kallikrein, an intermediate enzyme, can activate the complement system, and acts as a positive feedback activator during the interaction of Hf with an artificial surface.[38]

Kininogen is a single chain glycoprotein that, on proteolytic cleavage, gives rise to Bradykinin and is bound to the activating surface, presumably near the Hageman factor. Two forms of plasma kininogen occur, a high molecular weight kininogen (HMWK), at 120kDa and a low molecular weight kininogen (LMWK), at 68kDa. HMWK possesses coagulant activity which depends on the ability of cleaved HMWK to bind anionic surfaces and to associate with prekallikrein.[73] This highlights the importance of the negatively charged contact system activation.

The physiological role of the kinin generating system is uncertain and only a few cases of kinin presenting diseases are understood. It is only in recent years that its potential role in inflammation in a variety of pathologies has gained high interest and importance. In light of the potential of kinin for the presentation of complications, the anionic nature of contact lenses and the recent interest in plasma derived proteins during lens wear and in the closed eye environment, investigation into the presence of kinins in lens wear was assessed.

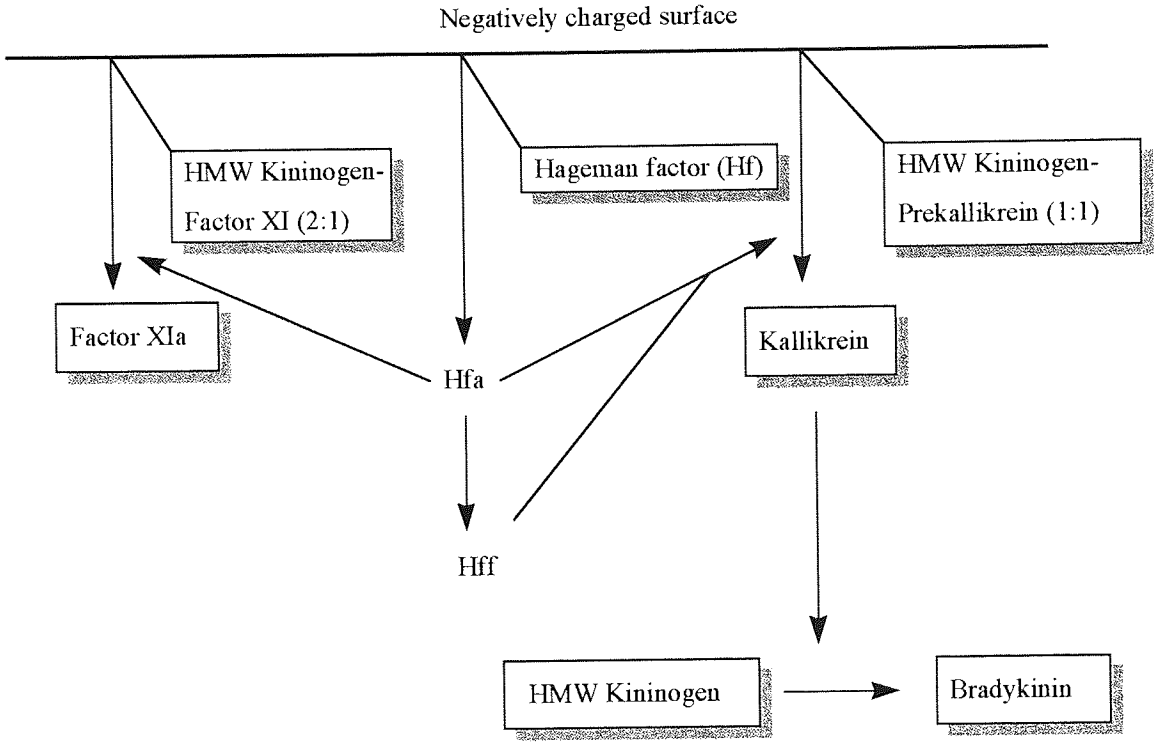


Figure 1.8. Simplification of the kinin pathway.[38] *Hfa = Hageman factor activated, Hff = Hageman factor fragmented*

1.7. Aim and Scope of the Research

This thesis was based on the analysis of proteins in tears and their interactions with contact lenses, which involved the use of a variety of analytical techniques in the measurement of proteins in tears, on the contact lens, and extracted from the lens.

Chapter 2 was important in explaining the experimental techniques employed throughout the research and details the parameters of each method.

Chapter 3 was concerned with a novel cell-based assay which was used to detect the ubiquitous and important inflammatory regulatory glycoprotein, vitronectin, on the surface of contact lenses *in vitro* and *ex vivo*. The effects of lens material, wear modalities and patient influence were all investigated.

Chapter 4 involved the design and optimisation of the immunodiffusion assays for a variety of applications in tear and contact lens eluate analysis.

Chapter 5 applied the immunodiffusion assays in the estimation and investigation of the closed eye environment through the analysis of tear samples taken immediately on waking. The difficulties encountered in this study are detailed.

Chapter 6 was concerned with the factors affecting the deposition of proteins on contact lenses and in particular the individual nature of the proteins involved in this deposition. Various techniques, including the immunodiffusion assays, were utilised. The presence of the kinin family of inflammatory mediators, and in particular kininogen, was assessed in relation to contact lens wear.

Chapter 7 was used to correlate all the information gathered throughout this thesis, and to assess the consequences and conclusions.

Chapter 2

Materials and Methods

2.1 Introduction

This chapter outlines and summarises the main techniques employed in tear analysis and in contact lens spoilation studies. The details of each assay, where necessary, are further highlighted in their respective chapters when used for a specific experiment.

2.2. Tear Collection

Tear collection is by no means an exact science; various and numerous methods have been employed over the years to carefully extract tear samples from patients and volunteers. The consequent result of this is that there are many tear statistics that can not be directly correlated with each other, and today there still remains no universal method of sampling.

2.2.1. Tear Film Distribution

There are various points in the eye from which tears can be collected, as shown in Figure 2.1. The approximate volumes in each area of the eye are as follows: preocular tear film (1μl), conjunctival sacs (4-5μl) and the marginal strips (2-9μl).[4, 74]

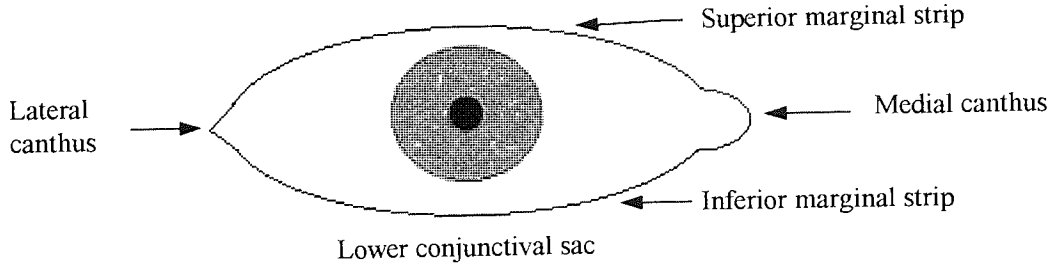


Figure 2.1 Tear collection points of the eye

2.2.2. Choosing the Most Suitable Tear Collection Method

Before attempting tear analysis, the problems and logistics of tear collection were initially addressed. It was important to choose and apply one method which could fulfil, or attempt to fulfil, the following criteria.

- Non-stimulating
- Allow tear state required
- Suitable for analyte under investigation
- Free from patient discomfort
- Reproducible
- Ease of performance
- Provide sufficient quantity for investigative assay

A critical approach and list of the pros and cons of the widely used methods are listed below, including a description of the glass microcapillary method, the protocol of choice in this research. The decision to utilise the microcapillary method will become apparent when describing the other sampling methods, which can be crude, cause patient discomfort or cause excess unwanted tear stimulation, but more importantly it provides the closest fit to the chosen criteria.

To assume one technique could fulfil all criteria would be folly, as no individual technique was going to sustain all the optimum conditions. An appropriate example was that of vitronectin which, along with some other plasma proteins, has a high affinity for glass, rendering collection with glass microcapillaries ineffectual. The solution therefore was to define which criteria were most important and to choose the method that best executed these requirements. In conclusion to obtain a 'truer' tear, demonstrating a lack of excess tearing was preferable, which allowed the control of the desired tear.

2.2.3. Schirmer Test

A strip of filter paper usually 35mm long and 5mm wide (Clement Clarke Int. Ltd. 4701001) is inserted into the lower conjunctival sac and the tears are adsorbed by the strip wetting approximately 5-6mm. It is a simple and inexpensive test, is reliable in itself but is harsh and results in the production of reflex tears. The end of the strip is rounded to avoid excessive irritation but its presence in the inferior fornix and the fact that the strip is inserted dry must make it a traumatic technique, which can cause excess watering, thereby diluting normal tear protein levels and causing plasma leakage contamination i.e., reflex tearing.[46] An example of this is shown by the increase in albumin levels collected by this method.[47] This collection method also has the disadvantage that the tears have to be extracted from the paper for analysis, thus creating a second tear collection variable, the tears can be extracted by various methods and to various efficiency degrees. The Schirmer test is preferentially used to assess tear flow rates and is an extremely useful test in estimating lacrimal gland function but again this has to be effected by the harshness of the test.

2.2.3.1. Personal Observation

Before attempting to perform the Schirmer test on volunteers a self-test was performed and was found to be a very uncomfortable experience and excess reflex tearing was noted throughout, creating an almost pseudotear. It was decided that this test would be abandoned, due to the fact that it conformed to very few of the pre-set criteria.

2.2.4. Filter Paper Method

The filter paper is placed directly on the eye and allowed to become wet with tears and may then placed directly on an agarose gel radial immunodiffusion assay. This collection method is similar to the Schirmer test in that filter paper is used and direct contact with the eye is necessary, but whereas the Schirmer test can also calculate tear flow rate this method simply absorbs the tear film. The disadvantage of this collection method is, again, that the tearing is stimulated due to the irritation of the paper on the

eye. Tears collected by this method were found to cause an increase in lysozyme and lactoferrin levels compared to basal tears, which may be due to an induced flow rate.[75]

2.2.4.1. Personal Observation

To perform this type of collection the filter paper can not be handled as this can contaminate the paper, thus insertion and removal of the paper from the eye must be done by means of tweezers, an idea that neither appealed to me nor my volunteers.

2.2.5. Sponge Collection

This method as the name suggests uses an absorbent triangular sponge, 1.5cm in length, which is used to collect tears along the inferior marginal strip to the medial canthus. The sponge opens out as the tears are absorbed, the capacity of which can reach in excess of 1ml. The main disadvantage with the sponge is the requirement for larger volumes of tears, a volume less than approximately 5µl barely visibly wets the sponge, and, during the sampling, evaporation of tears moving up the sponge is a problem. Another drawback is that the tears collected must then be extracted from the sponge which can create more problems and variation. One primary advantage of the sponge would be in the collection of tears from young children where the use of glass microcapillary tube would be dangerous.

2.2.5.1 Personal Observation

It has been found in this laboratory that this technique was more suitable for lipid analysis, made easy by methanol extraction and high performance liquid chromatography analysis. The sponge was also useful in sampling proteins which had an affinity for glass, e.g., vitronectin, which would render glass microcapillary collection useless. With regard to patient discomfort, the sponge does not cause discomfort but excess stimulation remains a consequence. Additionally, on obtaining a tear sample, the problems of extraction, and thus secondary variability, ensue.

2.2.6. Glass Microcapillary

This collection method uses narrow bore microcapillary pipettes (The Binding Site, Birmingham AD041), to collect 1-7μl of tears from the lateral canthus and/or inferior marginal strip. It is a relatively time consuming method but there is little conjunctival irritation and collection in this manner demonstrates a 'truer tear', rich in lacrimal proteins, with minimal plasma leakage contamination. The main advantage attributed to the microcapillary pipette technique is that it allows control over the type of tears collected, whether the tears required are stimulated or non-stimulated, for example. Evaporation is also not as prevalent though by this method. The concentrations of typical plasma proteins (albumin and IgG) are higher in tears collected by the Schirmer test,[46, 75] with the 'truer tear', comprising mainly of lacrimal gland derived proteins resulting from carefully taken microcapillary tears.[76]

2.2.6.1. Personal Observation

It was found that, with care and patience, tear samples could be collected without any excess flow or greater obvious rate of tear flow. Additionally, tear samples approximating 1-2μl could be obtained, with patience, from those in the group who displayed some degree of dry eye, without stimulation, thus avoiding the creation of a non representative group of volunteers.

During the course of the work many tear samples were required, with the result the microcapillary collection method was the only technique I was willing to use on a regular basis. It was atraumatic and willing volunteers were generally only to be found when this technique was employed. This was a major influence on the choice of technique, but it also fitted all but one of the criteria set for this particular study.

The only criteria that remained unobtainable by the microcapillary method was in the analysis of proteins with a high affinity for glass, e.g. vitronectin, but as discussed in Chapter 3, this limitation was overcome and used to an advantage in the development of an on-lens assay. The affinity for glass could be equated with an affinity for a

contact lens, thus when attempting to analyse proteins with an affinity for glass, deeming the microcapillary method redundant, an on-lens assay could be employed as an alternative. As stated above, the sponge method could also be used to overcome this problem.

2.2.6.2. Collection of Varying Tear States by Glass Microcapillary

To obtain psychogenic lacrimation (emotional tears) or non-contact, externally stimulated tears, by ammonia inhalation for example, the glass microcapillary collection method is the optimum choice. Both these tear states present excess tearing, the microcapillary method thus, without contact or causing interference with the eye, can collect these tears without detriment, quickly and efficiently. More recently it has become apparent that the most frequently used method for collection of closed eye tear samples is by a self collection method which requires the use of glass microcapillary tubes. This method remains the only true method which can be self applied, without patient detriment or discomfort. The microcapillary method allows the greatest form of tear state control, which is extremely important when considering the vast diversity of tear states presented.

In summary, it seems that the optimum method in tear collection would be to use the microcapillary sampling technique universally and in this study it was the method of choice.

2.2.6.3. Automatic Excess Tearing by Microcapillary

One important point to note was that for some subjects, obtaining a stimulated tear sample, by microcapillary, was inevitable. This section of the population demonstrated a faster flow rate of tears during sampling. Without contact or any other external stimulation, the subjects' eye presented an initial excess tearing as a nervous reflex, at a flow rate approximating close to in excess of $5\mu l min^{-1}$. This section of subjects sampled were described as demonstrating automatic stimulated tears (AST).

Two 5µl non-stimulated tear samples were taken, with a 10 minute interval between sampling, at a rate of $1\mu l min^{-1}$. A 10µl sample of an AST was also taken in approximately 2 minutes. The counter immunoelectrophoresis (CIE) assays shown in Figures 2.3. and 2.4., demonstrate the differences that are apparent between this section of people and those who do not automatically present excess tearing.

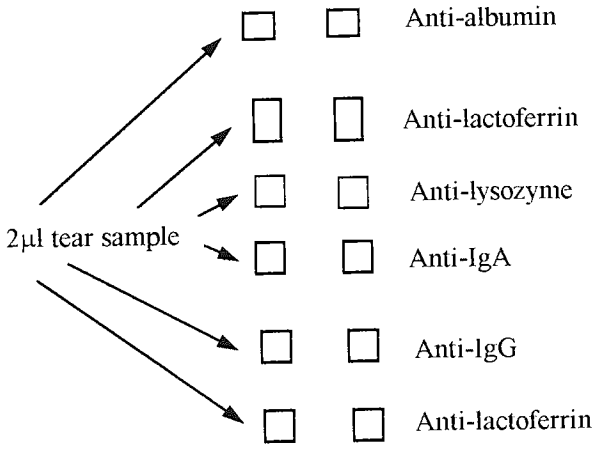


Figure 2.2 Well key for non-stimulated versus stimulated tears shown by counter immunoelectrophoresis. (Figure 2.3. versus Figure 2.4.)

Figure 2.3. Non-stimulated tear sample (TS-1) run by counter immunoelectrophoresis

Figure 2.4. Automatic stimulated tear sample (TS-2) run by counter immunoelectrophoresis

The technique of CIE will be explained in more detail later in this chapter under immunodiffusion techniques, but the basic principle of the CIE relies on the visible precipitation of antibody : antigen complexes. It is a detection assay with semi-quantitative properties. A comparison of the two assays above confirm the difficulties encountered due to the many variables in tear sampling and show the differences between AST samples and non-AST samples collected by microcapillary. The antibody concentrations and quantities were kept constant in both assays which allowed the individual protein results to be directly comparable between gels.

The non-stimulated sample showed clear defined lines of precipitation between the antibody and its respective antigen for all proteins tested. It was evident in the AST sample that all the proteins displayed a degree of dilution, as shown in the reduction in the intensity of each precipitation line, from the non-stimulated sample. The most obvious differences were apparent with the immunoglobulins, both IgA and IgG concentrations were greatly affected and diluted, but were still detected. The intensity reduction of the lactoferrin lines were not so potent but nevertheless evident. Albumin also shows a degree of dilution. Consequently, it was important to note the tear flow characteristics of each subject when analysing protein concentration results.

2.3. Cell Culture Techniques

Cell culture techniques were utilised in this research to develop an on-lens, probe assay. Cells, with integrin receptors for a variety of extracellular proteins, provided the visual aid to investigate the adhesion of the glycoprotein, vitronectin on *in vitro* and *ex vivo* contact lenses.

2.3.1. Cell Line

The cell line used in the cell culture assays was 3T3 Swiss mouse fibroblasts, first cultured in 1963[77] and were obtained from ICN Flow (Paisley, Scotland). The fibroblast cells were utilised due to the fact that they possess integrin receptors for both fibronectin and vitronectin, the two adhesion proteins of interest in the on lens-assay. While some cell lines grow in suspension, others like fibroblasts require a suitable substrate to which to adhere and spread for growth, these cells are 'anchorage dependent' and they form a monolayer that reaches confluence when all the available space on a substrate is taken.

2.3.2. Isolation and Culture

On receipt of the 3T3 primary culture, the cells were seeded into 75ml flasks and initially suspended in Dulbecco's Modified Eagles Medium (DMEM) giving a final volume of 25mls, and on reaching confluence were passaged as described below. The cells were then frozen down after the first passage in liquid nitrogen and brought up as required. After the first passage the primary culture becomes known as a cell line and can be propagated and passaged several times. With each progressive passage the cell fraction with the ability to propagate more rapidly, gradually take over, with the slower growing cells declining in number and becoming diluted out. By the third passage the cell line becomes stable, supporting a population of fast growing, hardy cells suitable for assay requirements.

2.3.3. Determination of Cell Growth Phase

Before attempting to use the 3T3 cell line for investigative experimental purposes the growth state of the cells was predetermined. Gaining an insight into the growth characteristics allowed an understanding of the performance of the 3T3 cell type under the varying assay conditions, and demonstrated when the cells were at an optimum for use, and also predicted when a passage was required. For example, generally when a cell line reaches the stationary phase the growth fraction of cells is reduced and the cells may become more differentiated, with some cells shown to secrete more extracellular matrix proteins. Various parameters can affect the growth phase such as the tissue culture plastic, medium type, plasma concentration and handling.

The growth cycle typically obeys a pattern of lag phase followed by an exponential phase through to a stationary phase and onto an eventual decline phase. The lag phase immediately proceeds passage and is a phase of adaptation. The exponential phase is the period of rapid growth with a 90-100% growth fraction, confluence is reached toward the end of this phase and is optimal for use since the population is at its most uniform and the viability is high. The stationary phase following confluence demonstrates a massive reduction in cell proliferation and the growth fraction can fall to below 10%. Cells may also begin to grow on top of each other forming multi-layers due to the limited space. If the cell suspension is allowed to proceed further the cells will eventually reach a decline phase due to limited space and nutrients.

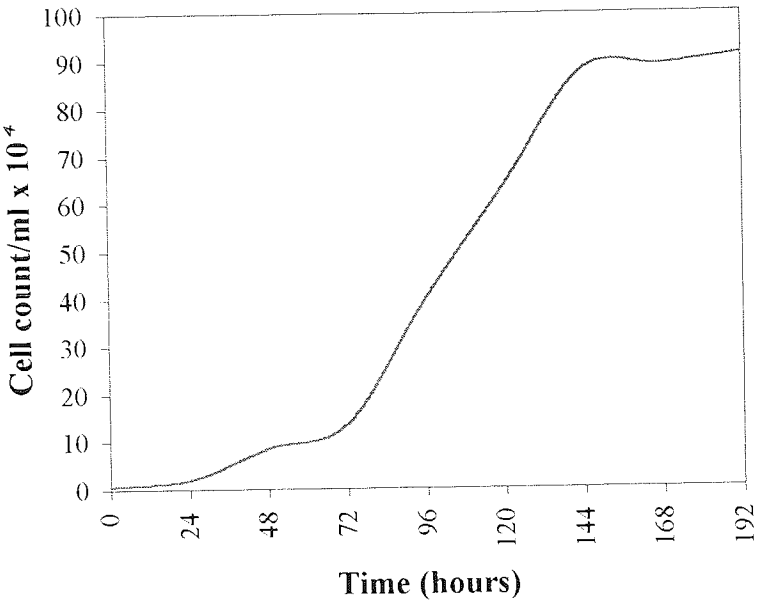


Figure 2.5. An example of a 3T3 fibroblast growth curve demonstrating the lag phase, exponential phase and stationary phase.

2.3.4. Cell Viability

To determine the cell viability prior to each assay, one drop of cell suspension was added to one drop of 0.4% trypan blue stain. The dead cells with disrupted membranes took in the blue stain while the viable live cells remained clear in colour. A % cell viability was calculated from the following equation:

$$\frac{\text{Total Cells - Dead Cells}}{\text{Total Cells}} \times 100 = \% \text{ viability}$$

2.3.5. Passage of Cells

Cell culture stocks were passaged regularly according to the growth rate determined previously, were not used at a high passage number; the cells were not used beyond passage 8 to ensure that their ability to maintain their phenotype was not compromised. On reaching confluence the cells were ready for the cell culture assays. The passage of cells and all cell culture assays were carried out in a laminar flow hood according to sterile tissue culture protocol.

2.3.5.1. Materials

Centrifuge
Incubator
Laminar flow cabinet
Microscope
Haemocytometer and cover slides
Pipette filler
Sterile disposable plastics - pipettes, universal and centrifuge tubes and tips.

2.3.5.1.1. Chemicals at 37°C

DMEM-complete media (GibcoBRL 41966-029)
Fetal bovine plasma (FBS) (GibcoBRL 10106-169)
Trypsin/EDTA (Sigma T-4049)
L-Glutamine (L-Glu) (Sigma G-7513)

2.3.5.2. Method

Initially the DMEM-complete media was prepared by adding 50ml of FBS and 5ml of 200mMol/L-Glu solution to a 500ml bottle of DMEM. When the cells reached confluence, which was evident by the formation of a monolayer of cells covering all of the bottom of the 75ml tissue culture flask, the old medium was decanted from the flask by means of a 25ml pipette. Approximately 3ml of trypsin/EDTA, warmed to

37°C in the incubator, was added to the flask with a 5ml pipette, swirled gently and was aspirated off. A further 5ml of trypsin/EDTA was added and the flask was returned to the incubator for 1-5 minutes until the single layer of cells was visibly detached. The solution becomes cloudy and the cells can be seen sloughing off the surface with a gentle shake. The trypsin/EDTA was then aspirated off and 20ml of DMEM was added to stop the reaction of the trypsin/EDTA, 10ml of the well mixed, medium and suspended cells were taken from the flask using a pipette, put into a centrifuge tube and centrifuged at approximately 1500-2000 rpm for 5 minutes until a pellet of cells had formed. The supernatant was carefully removed with a tipped pipette, the cells were resuspended in 10ml of fresh medium and evenly mixed. A tiny drop of cell suspension was dropped onto a haemocytometer slide (Figure 2.6.), to be counted.

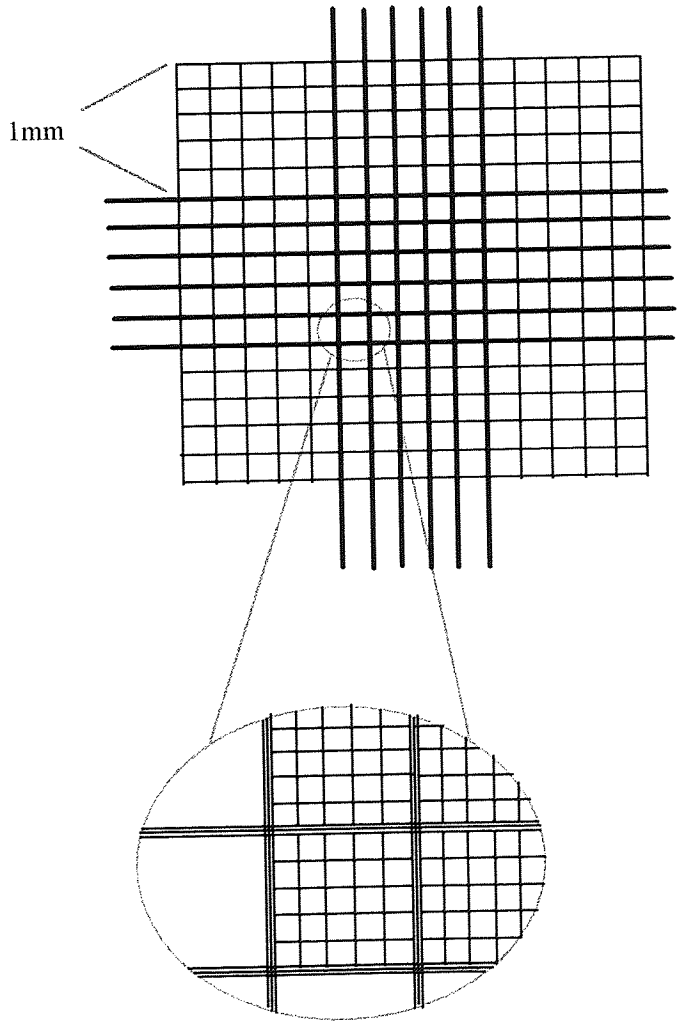


Figure 2.6. Haemocytometer grid for counting cells

The cells were counted in 5 (1mm x 1mm) squares with the average count taken and used to calculate the density required, the final cell count was usually adjusted to $0.5x10^4$ cells/ml to continue the cell line. Complete medium was aliquoted into a new 75ml culture flask to make the volume of the calculated cell suspension and medium up to a total volume of 25ml. The flask was then incubated at 37°C in an atmosphere 5% CO_2 concentration with the flask lid slightly open.

2.4. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was used in the analysis of tears and contact lens extracts, in the results chapters the advantages, disadvantages and limitations of this technique are discussed where applicable.

Most biological macromolecules are electrically charged and will therefore move in an electrical field; the direction of motion is obviously dependent on the sign of the charge. The transport of particles through a solvent by an electrical field is called electrophoresis. The rate of migration or mobility through the electrical field depends on the strength of the field, on the net charge, size and shape of the molecules and also the ionic strength, viscosity and temperature of the medium in which the molecules are moving.

The most commonly used technique of electrophoresis is SDS-PAGE combining the use of sodium dodecyl sulphate with a polyacrylamide gel. SDS separations differ in the fact that the migration is determined by molecular weight and not by the intrinsic electrical charge of the polypeptide. All samples to be tested were treated with an SDS / 2-mercapatoethanol (2-ME) mixture. SDS is an anionic detergent that denatures proteins by wrapping around the polypeptide backbone which allows the protein a net negative charge which is proportional to is length. This is combined in the treatment buffer with 2-ME which works by breaking disulphide bonds, whereby the molecules then assume a random coil formation.

Polyacrylamide gels help to reduce diffusion common to agarose gels, which results in sharply separated components with maximum resolution and they are tougher gels than agarose gels. Acrylamide monomers polymerise into long chains with a crosslinker providing the covalent links, the composition of the gel can be managed in order to attain optimum conditions for the samples to be analysed. This was achieved by controlling the pore size of the gel, which can be modified to control the passage of the proteins in the system. The porosity can be regulated by two means. Firstly, this could be achieved by means of applying various total percentage of acrylamide, i.e., the sum of the acrylamide monomer and the N,N`-methylene-bis-acrylamide crosslinker (or bis). This is designated the %T, and as a general rule, as the %T increases the pore size decreases. Secondly, adjusting the pore size can be managed by varying the amount of crosslinker, expressed as a % sum of the monomer and crosslinker and is expressed as the %C. Trial and error has determined that at a 5%C, the smallest pore size is created regardless of %T and increases above and below this percentage.

In summary a 10%T and 2.7%C gel, as was used for the separating/running gel in tear analysis, has a 10% weight/volume of acrylamide plus bis, with bis accounting for a 2.7% of the total weight of acrylamide.

A thin block of gel was prepared with migrating macromolecules passing through the narrow passages in the gel, the smaller molecules can move through the gel with less hindrance and thus migrate quicker through the gel. The migration rate increases as the molecular weight decreases. SDS-PAGE was utilised to analyse tear samples and contact lens extracts and its ability to do so was assessed.

2.4.1. Materials

2.4.1.1. Hardware

The electrophoresis apparatus utilised in this research was an SE600 vertical slab unit (Hoefer Scientific Instruments) which was attached to PS1500 DC power supply (Hoefer Scientific Instruments). Hoefer Scientific also supplied all the glass plates, spacers, combs, cams, seals, clamps and dual gel casters.

2.4.1.2. Solutions

(see Appendix I for all recipes)

1. Monomer solution
2. Separating gel buffer
3. Stacking gel buffer
4. 10% SDS
5. Initiator
6. Running gel overlay
7. Treatment buffer
8. Tank buffer
9. Water-saturated n-butanol

2.4.2. Preparation of Separating and Stacking Gels

(All volumes used below are given when 2 gels were made simultaneously)

Glass sandwiches were set up in the dual gel caster using 1mm glass spacers in preparation for the gel. Initially the separation gel is added, this fills the gel sandwich approximately 3cms from the top. The separating gel was made up in a vacuum flask to the following recipe:

20mls monomer	solution (1)
15mls separating gel buffer	solution (2)
0.6mls 10%SDS	solution (4)
24.1mls dH_2O	

The flask was stoppered and a vacuum was applied via the side arm on the flask for approximately 3 minutes, swirling the flask continuously. This removes oxygen which inhibits polymerisation. 300µl of the ammonium persulphate initiator (5) and 20µl of TEMED catalyst (Sigma T-8133) were then added and mixed, taking care not to generate bubbles. The separating gel was poured by means of a syringe into the sandwich to approximately 4cm from the top, removing unwanted bubbles by dipping a spacer into gel. A 0.5 cm layer of water-n-butanol (9) was pipetted onto the top of

the settling gel. This was used to obtain a smooth surface at the interface between separating and stacking gels and to keep out oxygen which would inhibit the polymerisation.

A sharp liquid-gel interface was then visible after approximately 1 hour, which gave the appearance of 2 distinct lines indicating that the separating gel had fully polymerised. The water-n-butanol overlay was decanted and the surface of the gel was washed three times with distilled H_2O.

The stacking gel was then prepared and as above for the separating buffer, the mixture was prepared in a side arm vacuum flask, in the following proportions.

2.66mls monomer	solution (1)
5.0mls stacking gel buffer	solution (3)
0.2mls 10%SDS	solution (4)
12.2mls dH_2O	

After applying a vacuum for approximately 2 minutes the following solutions were added.

100µl initiator	solution (5)
10µl TEMED catalyst	

A 1mm well dividing comb was inserted into the top of each sandwich and the newly made gel was poured into the sandwich by means of a syringe right to the top. The gel was allowed to set, polymerisation was completed when the wells become visible to the eye between comb prongs, the combs were then removed and the tank buffer (8) was loaded onto the top of the gel, filling the wells preventing evaporation.

2.4.3. Preparation and Treatment of Analytes

The tear samples/lens extract or analyte was prepared prior to running the gel using a treatment buffer (7) which contained SDS employed to confer a net negative charge to the protein proportional to is length, and 2-ME to break the disulphide bonds. An equal volume of analyte was mixed in an eppendorf with an equal volume of treatment buffer, and boiled in a waterbath for approximately 2 minutes, and a tiny drop of dilute phenol red dye was then added using a Hamilton syringe. The dye was added in order to trace the movement of the analytes down the gel during a run.

2.4.4. Loading and Running the Gels

With the tank buffer covering both gels the analytes were ready to load; 20µl of each analyte was added to the bottom of each well by means of a Hamilton syringe taking care to avoid air bubbles. Loading the analytes was undertaken as quickly as possible in order to avoid lateral diffusion in the wells which could lead to cross-contamination of the analytes.

For every gel run at least one molecular weight standard was run concurrently, without which analysis of the gel would be difficult. With the upper tank buffer chamber in place over the gels; sealed tightly preventing the mixing of the upper and lower buffer chambers, the gel sandwiches were then placed in the lower buffer tank. The gel tank was then attached to the powerpack, the upper buffer chamber was connected to the cathode and the lower buffer chamber to the anode. The voltage and power switches were set to the highest values and the current was adjusted to 25mA/cm per gel. The gel tank was placed on an stirrer, which, by means of a flea, allowed the buffer to circulate. The system was then cooled down by running cold water through a closed heat exchange system in the tank. The analytes were carefully monitored as they progressed from the stacking gel through to separating gel and down the gel; completion of a gel run occurred when either the dye neared the end of the gel or when a set assay time was reached.

2.4.5. Post Gel Run

2.4.5.1. Solutions

(see Appendix I for all recipes)

10. Coomassie Brilliant Blue stain
11. Destain
12. Fixative
13. Gel storage solution

2.4.5.2. Stain and Destain

The gels were removed from the sandwiches and fixed, (12), overnight. Post fixing the gels were stained in Coomassie brilliant blue (10) for approximately 2 hours. Coomassie blue is a dye which forms non-covalent bonds with proteins. The gels were then destained (11) until all the background stain was eliminated, this generally required several washes with fresh destain. The gels were then stored in 25% methanol overnight and transferred to storage in distilled water. A gel can be stored for at least 4 months in distilled water without the stain fading. Storage of the gel in 25% methanol overnight helped prevent the initiation of fungal growth during storage. During this time the gels were either photographed, scanned or measured by densiometry for analysis.

2.4.6. Analysis of Gels : Densitometry

Analysing the gel by defining the movement of the analyte post run, relied on the clear performance of the molecular weight standards run simultaneously in the gel.

Densitometry of certain gels was undertaken on a 2202 Ultrascan Laser Densitometer (LKB Bromma). It was a useful technique utilised in the analysis of the gel electrophoresis runs, the intensity of the resulting bands were measurable and were defined by the property that the greater the intensity of a band the greater the quantity of protein present. Densitometry was used as a semi-quantitative instrument with gels that were run with a set of concentration gradient standards; an intensity versus

concentration curve was obtained from which unknown analyte concentrations could be concluded. Comparisons between gels was not applicable. Densitometry also proved useful in measuring the distance of protein movement on the gel.

2.5. Immunodiffusion Gel Assays

Immunodiffusion development was undertaken for tear analysis to exploit the high specificity of these assays, but specifically to utilise the fact that small volumes of analyte are sufficient for quantification and qualification. This factor is extremely important in tear analysis where sample volumes are very small. The average tear sampling volume is 1-3µl. They are relatively cheap assays, simple to perform, provide reproducible results and suit the interdisciplinary laboratory with little need for extensive or expensive equipment.

The purpose of all immunodiffusion techniques is to detect the reaction of an antibody and antigen by precipitation of reaction in a semi-solid medium such as agarose. They are the simplest and most direct means of demonstrating antibody/antigen reactions. There are many variations of the immunodiffusion theme such as double, single or electrophoretic diffusion methods, depending on the number of reactants and variables involved. Immunodiffusion assays are dependant on pH, buffer electrolytes and temperature which require adjustments for optimal assay conditions, but most importantly they are dependant on the concentration of antigen and antibody in relation to each other. The lack of equilibration between the antibody and antigen can lead to erroneous results or misinterpretation, an example of this is the prozone effect which occurs in the zone of antibody excess where sub-optimal precipitation can occur. Maximal precipitation occurs in areas of equivalence with decreasing amounts in zones of antigen or antibody excess as shown in Figure 2.7.

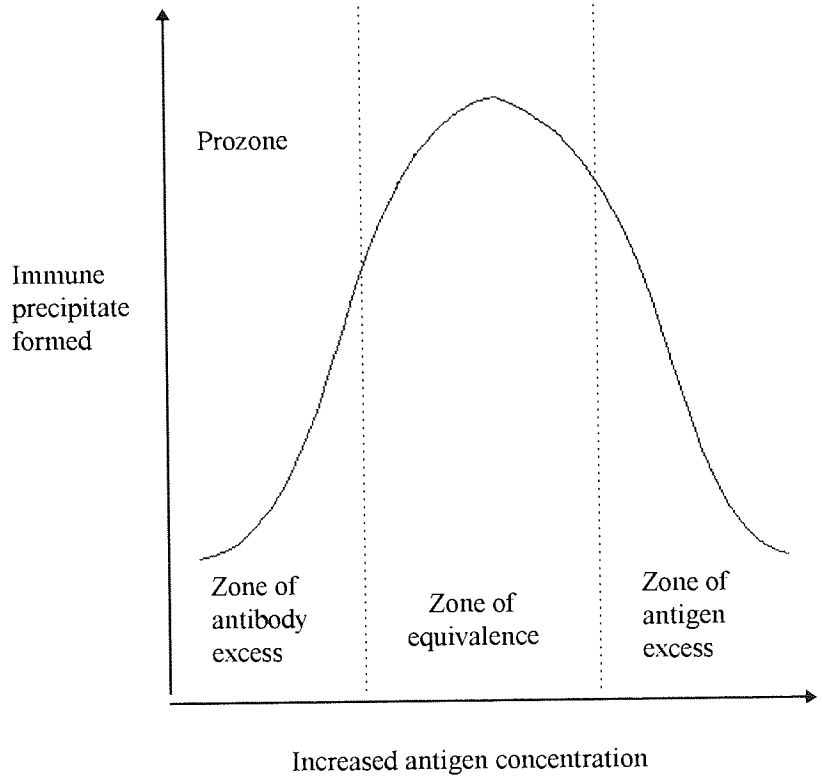


Figure 2.7. Immunoprecipitation curve. The amount of antibody is kept constant.[38]

The principle of an immunodiffusion reaction is the visible assessment of the precipitation of antibody : antigen complexes in solution or by diffusion through a semi-solid medium. The gel diffusion reactions require a high water content gelatinous supporting medium which allows the migration of the antibody and antigen freely through the matrix. Agar, a seaweed extract, provides a favourable medium: it is a mixture of two polysaccharides, agaro-pectin and agarose. Agaro-pectin contains the sulphate and carboxyl groups of the agar, whereas the agarose is neutral, free from ionised groups, thus it is more advantageous to use an agarose medium free from agaro-pectin which demonstrates a less pronounced electroendosmosis. Agarose is also more resistant to acids than agaro-pectin and can be used in electrophoresis at lower pH.

In 1949 the technique of immunodiffusion was developed and modified as a semi-quantitative assay for biological fluids by Ouchterlony,[78] this assay, still in use today, initiated many diversifications of the immunodiffusion technique. The modified and developed assays fall into three general categories, single double or electrophoretic diffusion or can be combined. In single diffusion either the antibody or the antigen is fixed and the other is allowed to diffuse into it, in double diffusion both reactants move towards each other. The single or double diffusions can be coupled with electrophoresis to enhance sensitivity and speed up the reaction time.

Five immunodiffusion assays were developed for tear analysis as shown in Table 2.1.

Assay	Principle	Movement
Ouchterlony	Double diffusion	Radial
Radial immunodiffusion	Single diffusion	Radial
Immunoelectrophoresis	Double diffusion + electrophoresis	Radial
Counter-immunoelectrophoresis	Double diffusion + electrophoresis	Linear
Rocket electrophoresis	Single diffusion + electrophoresis	Linear

Table 2.1. Principles of immunodiffusion assays utilised in the study of tears and their interactions with contact lenses

2.5.1. Preparation of Agarose Gels

2.5.1.1. Materials

(see Appendix I for solution recipes)

Waterbath at 56°C
Heated stirrer
Glass slides (8cm x 8cm)
Melinex sheets (7.5cm x 11cm)
Microscope slides
Pyrex conical flask and 100ml bottle
Glass pipette
5mm immunodiffusion cutter (ICN 421753)
Surgical blade and ruler
Coomassie Blue Stain (10)
Destain (11)

Agarose (Sigma A-0576)
Polyethylene glycol 8000 (PEG) (Sigma P-2139)
1 x TBE buffer (14)
Antibody
Antigen standards and analytes
Vacuum pump
Centrifuge tubes

2.5.1.1.1. Immunochemicals

Goat anti-human whole human serum (Sigma H-9640)

Human Antigen	Anti-Human Antibody	Origin
Lactoferrin (Sigma L-0520)	Sigma L-3262	Rabbit
Lysozyme (Sigma I-6394)	Binding Site PC073	Sheep
IgA (Sigma I-0633)	Sigma I-1261	Goat
sIgA	CalBiochem 411423	Mouse
IgG (Sigma I-2511)	Sigma I-1011	Goat
Albumin (A-3782)	Sigma A-7544	Goat
Lipocalin	---	---
Prealbumin (Sigma P-7528)	Sigma P-5414	Goat

Table 2.2. Immunochemicals employed in immunodiffusion assays

2.5.1.2. Gel Preparation

1.2g of agarose and 3g of PEG 8000 was weighed out and diluted in 1 x TBE buffer. Various different buffers were used for the different assays but as a general rule TBE buffer was proven to be optimum, sustaining clear, sharp bands of precipitation. The solution was boiled on the heated stirrer for approximately 5 minutes or when the agarose and PEG were visibly dissolved, was then poured into a pre-warmed bottle and incubated in a waterbath at 56°C (the minimum temperature at which the gel remains molten), ready for use.

2.5.1.3. Pouring the Gel

10ml of molten agarose was poured into a pre-warmed 12ml centrifuge tube and immediately poured onto the glass slide or melinex sheet. It was important to place the slides and sheets on a level surface to ensure an even flow of the gel. The gel was then allowed to set for approximately 15 minutes. When pouring radial immunodiffusion and rocket electrophoresis gels a predetermined amount of antibody was mixed with the molten agarose in the centrifuge tube and poured as an even gradient onto the slide or sheet.

Wells and/or troughs were cut into the gel when set, the wells were cut by means of a 5mm immunodiffusion cutter and troughs were cut out with a surgical blade using a ruler as a guideline. The cut wells and troughs were removed by means of a glass pipette attached to a vacuum pump and discarded. Various examples of the basic set-ups for each assay are shown in Figures 2.8.- 2.11. Wells are not required for counter immunoelectrophoresis, the template shown in Figure 2.11. for counter immunoelectrophoresis was cut into a melinex sheet. This sheet was placed on the gel allowing the reactants to diffuse down into the gel and could be reused for many gels.

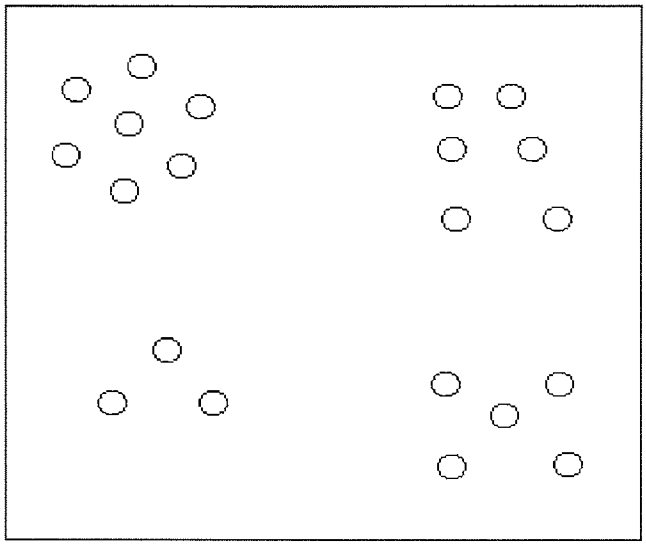


Figure 2.8. Template patterns for Ouchterlony

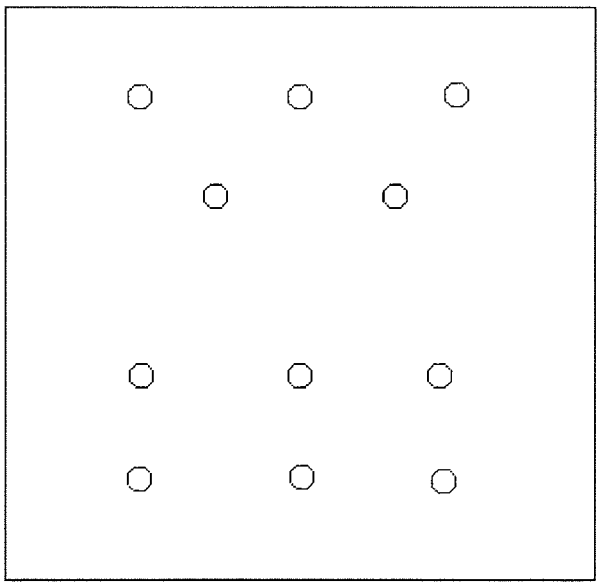


Figure 2.9. Template for RID

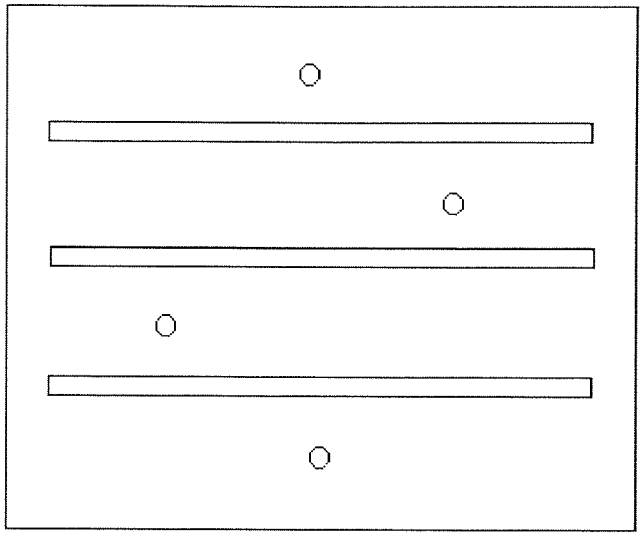


Figure 2.10. Template patterns for IEP

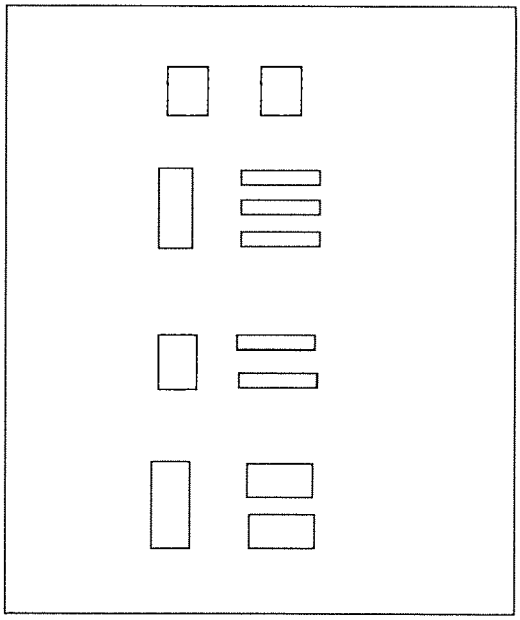


Figure 2.11. Templates patterns for CIE

2.5.1.4. Post Gel Run (Stain and Destain)

On completion of each assay (post diffusion or electrophoresis) the gels were immersed in 0.9% saline overnight to wash all unbound protein. The gels were then dried out by placing filter papers on top of the gel with a weight on top of the filter paper and placed by a window for approximately 24 hours. The filter paper and weights were removed and the gels were stained in Coomassie Blue stain (10) for 2 hours and transferred to destain (11) until all background stain removed - this generally required more than one fresh destain solution. The gels were then ready for analysis and cataloguing, the ring precipitates in RID gels were read by means of a jewellers eyepiece (Flubacher + Co.).

2.5.2. Immunodiffusion Assay Methods

The next section outlines a brief summary of the principles and methodologies of each immunodiffusion assay applied in tear analysis and contact lens studies, in an attempt to assess their overall application.

2.5.2.1. Ouchterlony[78, 79]

Ouchterlony was used to investigate the specificity of all immunoreactants prior to using them in future quantitative assays. It also acted as a means of testing whether a particular antigen from tears could be quantified by these gel methods. This assay allows great diversity, has many applications and due to the relative ease of performance has helped in the study of tear proteins.

This is a simple but extremely useful assay based on the principle that when an antigen and antibody diffuse through the agar, they form stable immune complexes which can be visually analysed. It is a qualitative technique and is used in the estimation of antigen in biological fluids. This method involves the use of agarose plates with wells cut for both the antibody and antigen, the two reactants diffuse into the gel, an immunoprecipitate forms at the point of equivalence. The immunological relationship between two antigen can be assessed by setting up precipitation reactions adjacent to

one another. Three basic types of precipitation line interactions occur; if a single line of reaction results, this proves the purity between the antibody and antigen, a number of reaction lines suggests the occurrence of a polyspecific reaction. The three basic patterns of reaction are shown in Figure 2.12.

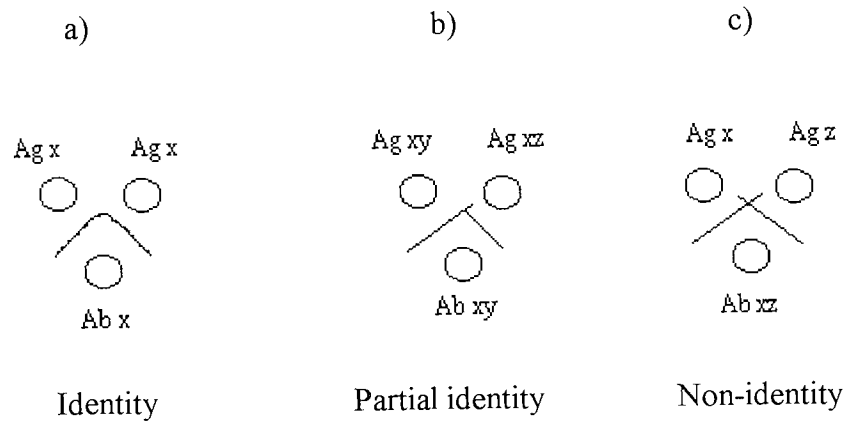


Figure 2.12. Ouchterlony patterns of reaction

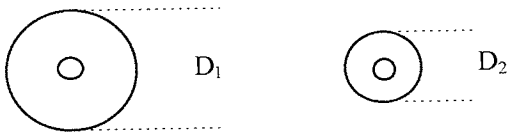
a) A reaction of identity wherein the antigen against the specific antibody are the same, this is shown by the lines of precipitation which join at a point of intersection.

b) A reaction of partial identity which is typified by a 'spur' formation wherein one of the lines of precipitation intersects the other. This 'spur' formation has been interpreted by many to demonstrate partial identity between the test antigen; the antigen have a common determinant x, but individual determinants y and z.

c) A reaction of non-identity wherein the antigen against the reference antibody are different and thus the resultant lines crossover each other.

2.5.2.2. Radial Immunodiffusion (Mancini's Assay)[80]

In 1965 Mancini introduced a novel technique for employing single diffusion for the accurate quantitative determination of antigen; it was based on the principle that a quantitative relationship exists between the amount of antigen placed in a well cut in the antibody containing agar and the resulting ring precipitates. When antigen diffuses from the well, initially it is present in a relatively high concentration. As the antigen diffuses radially the concentration continuously falls until a point is reached at which the reactants are nearer optimal proportions and a ring of precipitate is formed. A relationship exists between the antigen concentration and the area or diameter (D) of the precipitate, the higher the concentration of antigen, the greater the diameter of the ring.



Analyte 1 Analyte 2

Figure 2.13. Titres of the analytes proportional to diameter of the ring

If the antigen rings displayed a small diameter this was due to the use of excess antibody in the supporting gel, decreasing the volume of antibody increased the antigen ring diameters relatively and made the examination of results easier. For each tear analyte, trial assays were performed to determine the quantity of antibody optimal for each assay. Through the incorporation of standards of known antigen titres, on the same plate as the analyte, a calibration curve was obtained and used to determine the amount of analyte in each tear sample tested. Diffusion time can take anywhere between 24-48 hours to reach completion and is much slower than electrophoretic diffusion methods. The detection limit of the Mancini technique has been estimated to be in the range of 1-3μg of antigen.

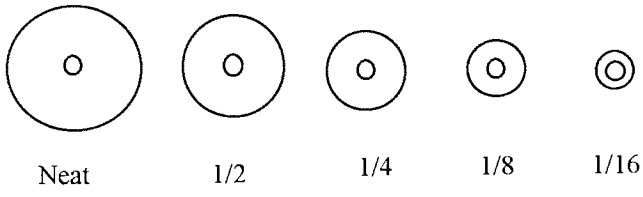


Figure 2.14. Radial immunodiffusion depicting the serial dilution of standards from which unknown analyte titres can be calculated

After several days when the migration of the precipitates has reached completion, the diameters of each standard were read with the jewellers eye piece and from these values, a standard curve was created, plotting the antigen concentration versus diameter.

2.5.2.3. Rocket Electrophoresis (Laurell's Assay)[81]

Laurell's assay commonly known as rocket electrophoresis (RE) was devised in 1966 and is also a quantitative method, but which involves electrophoresis of the antigen into an antibody containing gel. The precipitation arcs have the appearance of a rocket, hence the name, the lengths of which are proportional to the antigen concentration incorporated into the wells. Like most electrophoretic methods this is a rapid method but one drawback to this assay is that the antigen must move to the positive pole on electrophoresis. It is therefore suitable for proteins such as albumin, transferrin etc., but immunoglobulins are not suitable and thus are more conveniently quantified by radial diffusion. The Laurell assay can be used for the quantified estimation of antigen in solution down to approximately 0.5μg/ml.

Agarose gel was prepared with the required volume of antibody in the matrix, 5μl well holes were punched in the gel according to the chosen template and as shown in each particular assay. The individual tear samples were applied across the row of wells in the antibody containing gel. The volume of antibody required for each assay was previously determined, through trial and error, and the volume used in each

individual is listed with each assay. Samples of known concentrations of analyte/antigen to be estimated were added in varying dilutions to the wells, along side the set of analytes under investigation.

RE was performed on the Paragon Electrophoresis System (Beckman) which was modified for immunoassay purposes.

Electrophoresis was allowed to continue until all the antigen excess was consumed and the precipitation arcs were stationary, the movement of the analytes were monitored by adding a drop of Coomassie blue dye in one of the wells. Each run continued for between 30-60 minutes. The relationship between antigen concentration and arc length is linear and in theory only two antigen concentrations are required for calibration, but in most assays a series of concentration standards were used covering the desired concentration range.

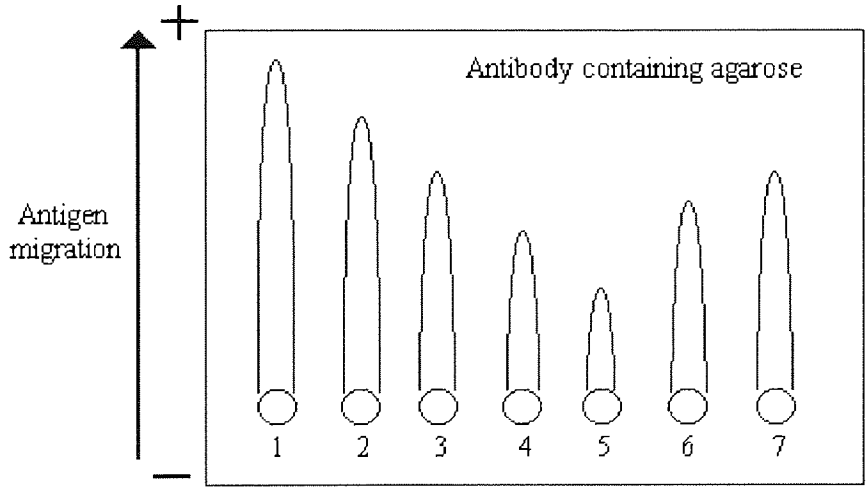


Figure 2.15. Outline of rocket electrophoresis. Wells 1-5 represent progressively decreasing standards, 6 and 7 are the unknowns which can be calculated from the resulting standard curve.

2.5.2.4. Immunoelectrophoresis

Immunoelectrophoresis (IEP) also involves the separation of proteins in an electrical field, but combines the properties of electrophoresis and radial immunodiffusion. This is a valuable assay for the identification of antigen by their electrophoretic mobility particularly in complex mixtures such as in tears.

Identification of particular antigen from the mixture relies on prior knowledge of the electrophoretic movement of each antigen which has come about over many years of examination. The electrophoretic movement falls into 5 categories of identification as shown in Figure 2.16.

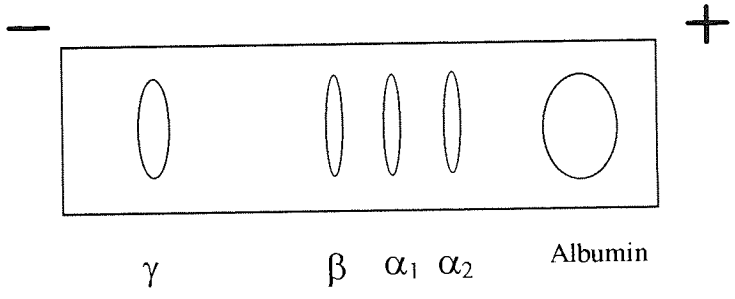


Figure 2.16. Electrophoretic zones of mobility

Using the template designed for IEP the antigen or tear sample was applied to a well cut in the gel and electrophoresis was performed on the modified Paragon System (Beckman) which separated the antigen mixture into individual fractions by electrophoretic mobility. The movement of the tear samples was monitored by the addition of Coomassie blue stain. On completion of the run, 30-60 minutes, approximately 30μl of antibody was loaded into pre-cut troughs in the gel which ran parallel to the movement of the antigen mixture. A double diffusion ensued, generally overnight, the antigen diffused radially from their new position in the gel, moved toward the antibody and when they reached the point of equivalence, formed a precipitate. The precipitate takes the form of an arc due to the electrophoretic movement.

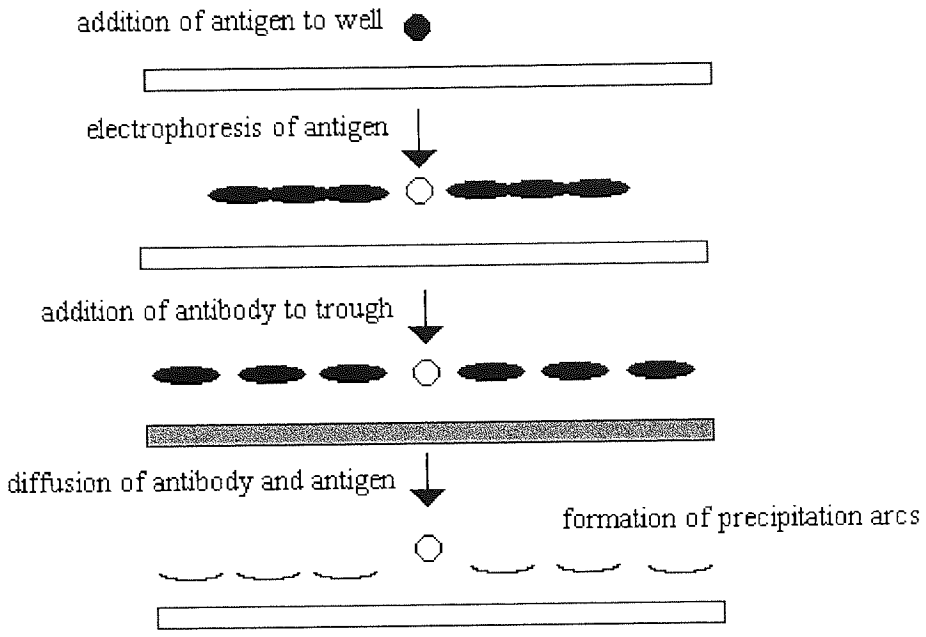


Figure 2.17. Summary of the protocol for immunoelectrophoresis

Each protein forms an arc of precipitation with its corresponding antibody in a particular zone, relative to the protein movement. Some typical electrophoretic patterns are shown in Figure 2.18. below.

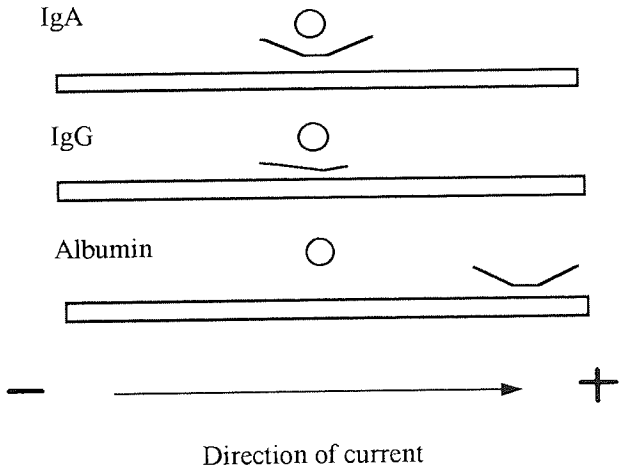


Figure 2.18. Typical electrophoretic patterns

2.5.2.5. Counter Immunoelectrophoresis

Counter immunoelectrophoresis (CIE) is a double electro-immunodiffusion in one direction, the principle of which is demonstrated in Figure 2.19. CIE involves the antigen and the antibody being driven towards each other in an electric field, where they then precipitate at an intermediate point between their origins in the gel. CIE can produce visible lines within minutes and is approximately ten times more sensitive than the standard double diffusion techniques. Detection, thus is in the range of 0.1-0.3μg/ml of antigen.

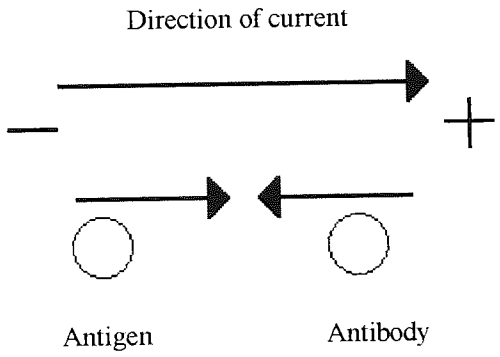


Figure 2.19. Principle of counter immunoelectrophoresis

CIE, unlike the other immunodiffusion methods does not require pre-cut wells in the gel, instead a previously prepared, template cut, melinex sheet was placed on top of the gel, the analytes and their respective antibodies were carefully dropped in the template holes and allowed to diffuse into the gel over varying periods of time depending on the assay performed. On completion of diffusion, the melinex sheet was removed and the gel was electrophoresised for 30-60 minutes on the Paragon electrophoresis system. Immediately after electrophoresis, lines of precipitation become evident, but to improve the detection the gels were Coomassie blue stained.

2.6. Quantitative Ultra Violet Spectrophotometry (UV)

A U2000 spectrophotometer was used to study the deposition profile of proteins and the factors that affect the rate of this deposition on a variety of contact lenses. The measurement of proteins by UV was based on an assay originally described in the analysis of deposited proteins on contact lenses.[82] UV spectrophotometry takes advantage of the fact that most proteins display an absorbance peak at 280nm due to intrinsic aromatic amino acids which can be measured and translated into protein concentration. The concentration of protein eluted into solution during storage could also be calculated.

2.6.1. Materials

The essential components of a UV spectrophotometry are shown in Figure 2.20. and summarised below:

- Two lamps:- Usually a halogen or tungsten lamp, providing a wavelength output from 350-900nm and a deuterium lamp with a 200-400nm output range.
- Absorption cells:- The U2000 uses two such cells, a reference cell and analyte cell. The analyte cell contained the sample under investigation and the reference cell was used to subtract unwanted background absorbance values. The UV spectra range requires the use of quartz cuvettes.
- Wavelength filter or monochromator:- As the name suggests selects the required wavelength.
- Detector:- A photosensitive detector used to measure the quantity of light transmitted by the analyte in the cuvette.
- Display monitor:- An on screen display of the results, linked up to paper source for a hard copy.

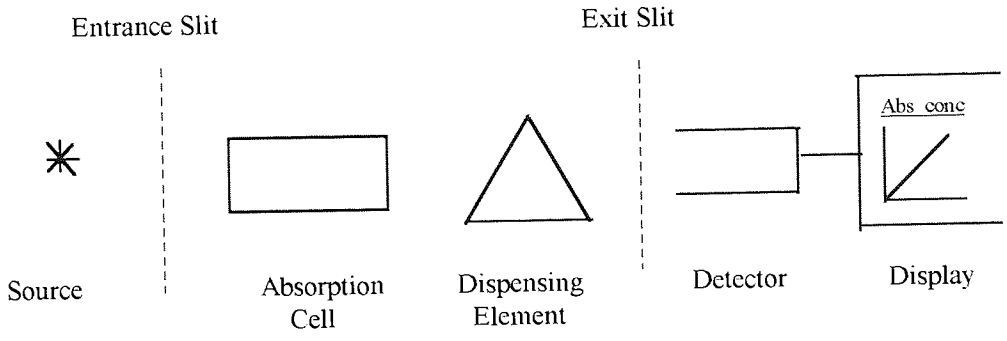


Figure 2.20. Schematic representation of UV spectrophotometer

2.6.2. Method

Each lens to be analysed was inserted carefully into the bottom of the quartz cuvette in solution, in an upright position facing the light beam against the wall of the cell. The set up of each lens was kept constant and standard in order to improve reproducibility. The lens containing cuvette was then put into the absorbance cell sitting on an 8mm high rubber mount, used to ensure the light beam hit the lens centrally. The solution in which the lens was generally measured was distilled water and prior to each new lens reading the instrument was readjusted to auto zero with only distilled water in the cuvette and distilled water in the reference cell. The absorbance measurement and calculated concentration was obtained for each lens or solution by simply the touch of the start button.

The absorbance of each lens plus deposition could be read directly from the instrument, but to calculate the relative concentration a linear calibration was performed. This was achieved by setting up a calibration graph of absorbance versus concentration using a set of standards of known concentration of the analyte or analytes under investigation. Consequently, the absorbance of the unknown analyte was measured and the concentration could be calculated from the calibration graph. The calibration curves obtained for each protein and mixtures of proteins, as required, were stored in the memory of the instrument for use in the clinical and extraction studies.

Each on-lens protein measurement is a combination of both protein and lens absorbance; background lens absorbance can create a varied range of absorbance values which can create erroneous results when not taken into account. To minimise the effects of lens absorbance a blank lens could be inserted in the reference cell which would then be automatically subtracted from the reading displayed. However, this was not the method of choice due to reproducibility problems and will be discussed in more detail in Chapter 6.

2.7. Analysis of Proteins in Tears, on the Contact Lens Surface and in Lens Extracts

This chapter has detailed the techniques that were employed in the study of proteins in tears, with and without contact lens wear - a range which encompassed immunological, biochemical and surface techniques. The main advantage found in the use of these methods was their ability to be used in conjunction with each other, as a back-up or a validation of a particular result. An example of this synergy was employed in the analysis of lens extracts. The proteins removed from *ex-vivo* various lenses by a chemical extraction method were analysed by CIE to investigate the individual nature of the spoilant, and UV spectrophotometry was used to define the efficacy of the extraction and determine whether CIE should be performed on a particular extract. SDS-PAGE was also utilised in the analysis of the overall picture of the eluate.

Assay	Probe	Analyte Proteins	Sensitivity	Comments
Cell Culture	Cells	Vitronectin	+++	On-lens detection, not suitable for tear analysis
SDS-PAGE	Molecular sieve, Coomassie Blue	MTP¤	+	Troublesome method, requirement for ~10µl sample volumes, poor detection
Ouchterlony*	Antibodies	MTP	++	1-3µl sample required against upto 6 antibodies
IEP*	Antibodies	MTP	+++	1-3µl sample required, interpretation difficulties
CIE*	Antibodies	MTP	+++	1-3µl sample required, important in lens extract analysis
Laurell*†	Antibodies	Albumin	+++	1-3µl sample required, anionic antigen only
RID*†	Antibodies	Lactoferrin, IgA, IgG, Albumin	++	1-3µl sample required, effective quantitative technique
UV	Radiation at 280nm	Total Protein	+++	Versatile, single protein analysis on *ex vivo* lenses not possible

*Table 2.3. A summary of the experimental techniques employed in the analysis of proteins in tears and their interaction with contact lenses. * Limited by availability of antibody. † Limited by availability of purified antigen. ¤MTP= Main tear proteins*

Chapter 3

Vitronectin Adsorption on Contact Lenses:

Locus and Significance

3.1. Aim

The aim of the study was to evaluate the potential influence of the contact lens, the lens material, patient variance and wear regimes on vitronectin adsorption onto the contact lens and consequently to attempt to understand the role of vitronectin in the post-lens micro-climate. The development of a novel cell based, on-lens assay has made the detection of vitronectin in tears and its interaction with contact lenses more accessible.

3.2. Introduction

There has been a growing interest in the factors involved in the inflammation of the ocular environment and this interest has escalated in the area of contact lens wear, where the vast array of contact lens materials could aggravate or modulate the normal host immune response. Vitronectin, which has been detected in tears,[83] is a prominent inflammatory regulatory protein and adhesion molecule. It has become a potential inflammatory marker and thus has been focused on intently in contact lens wear. Vitronectin levels have been shown to rise in the closed eye environment which creates what is now well known to be a state of sub-clinical inflammation, distinguished by the increased presence of plasma protein leakage. The presence of vitronectin in tears is poorly understood and its concentration increase in the closed eye remains an unexplained phenomena, but its interactive role with plasmin and complement and the elevated levels in the closed eye suggests its possible importance in inflammation.

The insertion of a contact lens onto the eye has the potential to cause inflammation, because it is foreign to the host and is a surface active biomaterial. Vitronectin is a very sticky adhesive protein with the ability and affinity to stick to many synthetic compositions including contact lenses. Both of these factors justify the principle behind the development of a novel on-lens cell assay which can detect vitronectin on a contact lens and may prove very important in realising vitronectin's potential as an inflammatory marker.

The potential influence of vitronectin in bacterial pathogenesis has recently been recognised in other mucosal sites of the body. Vitronectin has been shown to promote the adherence of *Neisseria meningitidis* to human umbilical vein endothelial cells via the Arg-Gly-Asp (RGD) amino acid sequence,[84] and another study has shown vitronectin to bind to *Staphylococcus aureus*, *streptococci* and *Escherichia coli*.[85] Some extracellular host proteins are known to prevent the invasion and colonization of microorganisms, i.e., lactoferrin, lysozyme and the complement proteins, but it is quite possible that other factors, such as vitronectin, may be used by the invading potential pathogen to promote its attachment and spread in the host. Bacterial adherence to host cells or extracellular components is a prerequisite for colonization and activation of the immune system. This adherence, utilised to the advantage of the bacteria, may be mediated by vitronectin to the detriment of the host, but on the other hand vitronectin may be mediating bacterial adhesion in order to aid the ingestion and clearance of the microbe by phagocytosis.

In order to develop and realise the need for this assay we must understand the structure and diverse functions that vitronectin possesses in order to ascertain the true potential and possible roles of vitronectin in the ocular environment.

3.3 The Structure of Vitronectin

Vitronectin, previously known as epibolin, s-protein and spreading factor, is an adhesive glycoprotein present in the circulation and in a variety of tissues: it promotes the spreading and attachment of a wide variety of cells. It is a member of a family of cell adhesion molecules which mediate cell adhesion through a common RGD containing sequence.[86] Vitronectin possesses an amino acid sequence at the N-terminus which demonstrates a sequence homology with somatomedin B, a plasma protein. It has been suggested that the somatomedin B plasma protein may not be a separate entity but may simply be derived from vitronectin by limited proteolysis.[87]

Vitronectin, a single chain glycoprotein was run under non-reducing conditions by SDS-PAGE and shown to have a molecular weight of 75kDa. Under reducing conditions two breakdown bands were evident, yielding bands at 65kDa and 10kDa.[88] Monoclonal antibodies directed against vitronectin recognised both the 65kDa and 75kDa entities.[88] Both forms of vitronectin can be found in the circulation but only the single chain species is produced by hepatocytes. Although the major source of vitronectin is primarily the liver,[89] platelets,[90] megakaryotes[91] and monocytes/macrophage[92] may also synthesize vitronectin. The concentration of vitronectin in plasma is approximately 0.2-0.4mg/ml.

Conformational changes in structure have been noted. A folded structure was described[93] which can be affected through various binding functions, denaturation or proteolysis. They may modify the folded structure or give rise to an extended form. The extended form seems to be predominant in circulation, allowing the molecule to perform its multifunctional properties.

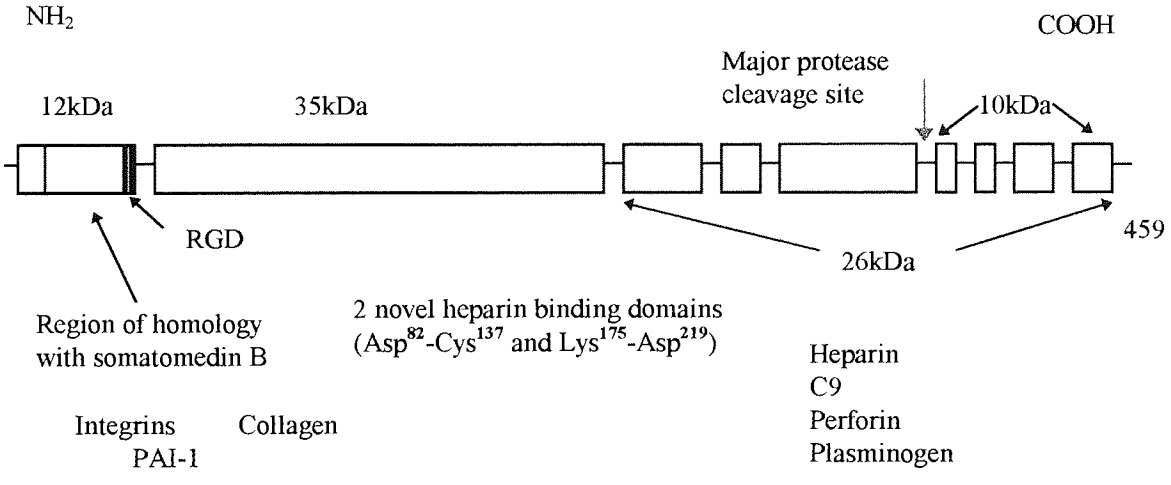


Figure 3.1. Extended structure of vitronectin highlighting its binding domains[90, 94, 95]

3.4. Vitronectin and Ligand Assisted Functions

Vitronectin is involved in several physiological processes; its function is dependent on its binding to various matrix and cellular components. Binding to various biological components can serve to activate or aid vitronectin deposition or alternately, vitronectin can aid in stabilizing or activating a variety of macromolecules. The following is a summary of the main vitronectin-binding dependent functions which were described initially in plasma. Consequently its activities in the ocular environment may seem irrelevant, but it must not be assumed that its multifunctional behaviour is limited systemically. Increasingly, more and more plasma-derived proteins are being discovered in the ocular environment and thus their involvement to any degree in association with vitronectin can not be dismissed.

3.4.1. Vitronectin and Complement

The s-protein, now more commonly referred to as vitronectin, was shown to be involved in the modulation of the complement system.[63] This system, which can be activated by antibody-opsonized cells or microbes, works to lyse or damage that which activated it, and this is done by means of the end membrane attack complex (MAC). Vitronectin binds to the C5b67 complex of complement. This resulting vitronectin-C5b67 complex is water soluble and loses its ability to insert into cells. The C5b67 complex can still bind C8 and C9 proteins of the system to form C5b6789 (MAC) but cell lysis does not occur. This prevention of cell lysis by MAC inhibition may serve to protect innocent peripheral cells, limiting unnecessary host cell damage under normal circumstances. Vitronectin was also shown to block perforin pore formation induced by cytotoxic T-cells.[96] Both of these immune mediating roles appear to be dependent on the heparin domain but the mechanisms remain unclear.

3.4.2. Vitronectin and Integrins

The integrins are a family of adhesion molecule receptors with structural and functional homologies, are heterodimeric complexes that are present on cell surfaces and which can bind to extracellular matrix components, including vitronectin. The integrins are made up of two non-covalently linked α and β chains which recognize the RGD sequence on many extracellular components and are important in the regulation and migration of cells and in tissue organization. Various different integrin subfamilies have been identified, linked by a common β subfamily. Vitronectin is recognized by the β_3 subfamily which is expressed by most cell types. The RGD sequence for cell attachment is adjacent, on the C-terminus side, to the somatomedin B domain.[94] Vitronectin is, therefore, very important in the modulation of cell attachment, spreading and migration and thus in aiding the healing process.

3.4.3. Vitronectin and Thrombosis

As the s-protein, vitronectin was also shown to regulate the blood coagulation system by inhibiting the rapid inactivation of thrombin by anti-thrombin III in the presence of heparin. Vitronectin does this by forming a tri-molecular complex with the thrombin and the anti-thrombin III.[63]

3.4.4. Vitronectin and Fibrinolysis

Vitronectin can also stabilize type-1 plasminogen activator inhibitor (PAI-1) through binding to it by means of the somatomedin B domain at the N-terminal of the fragment.[87] PAI-1, an inhibitor of plasminogen activator, a key enzyme in the initiation of fibrinolysis. It is synthesized in an active form but is very unstable in solution and decays rapidly.[97] By binding to PAI-1, vitronectin stabilizes it, allows it to remain active and thus it may play an important role in the control of anti-proteolytic activity.[98] When vitronectin binds to PAI-1, its cell-attachment properties do not appear to be affected.[99]

3.4.5. Vitronectin and the Contact Lens

Vitronectin has a high affinity for plastic surfaces and it affects the spreading of various cultured cells *in vitro*. An example of this behaviour was shown in tissue culture conditions with medium containing bovine serum. Only vitronectin was adsorbed in high enough concentrations from the medium onto the substratum to mediate adhesion, spreading and growth of bovine corneal epithelial cells.[100] Vitronectin also has the ability to stick to glass which is important to consider when taking tear samples as the vitronectin may be adsorbed out of tears and onto the collecting microcapillary tube. Thus the sponge collection method is preferable when analyzing tears for vitronectin.

More recently, vitronectin has been demonstrated to adsorb onto contact lenses through the development of a novel on-lens cell based assay.[101] Considering its role and now established capacity as an inflammatory marker, this area was pursued further in order to investigate the potential influence of the contact lens and the lens material on vitronectin-mediated inflammatory processes.

3.4.6. Vitronectin and Tears

The presence of vitronectin has been demonstrated in the open unstimulated tear at an approximate concentration of 0.75±0.3μg/ml,[83] but its presence in tears was given greater attention due to the observation that there was a notable increase in vitronectin levels in tear samples after overnight eye closure. The concentration in the closed eye was shown to rise to a concentration of 3.65±2.17 μg/ml,[83] which constitutes an almost 50-fold increase in vitronectin going from the open to the closed eye tear.

The concentrations determined in this paper require particular scrutiny and should only be quoted with the following clarification. The problem lies in the fact that these tears were sampled by means of glass microcapillary pipettes and while noting that vitronectin has a high affinity for glass, there was no mention of the use of an extraction method to

overcome this fact. The tear samples were then pooled and stored in plastic eppendorfs, which may have further depleted the tears of vitronectin. The resultant mean figures given are also representative only of a population of six volunteers. But the fact remains, that under an identical collection method, there is an obvious increase in vitronectin going from the open eye, basal tear to the closed eye environment tear solution.

The closed eye, as stated above, takes on a state of sub-clinical inflammation. Recognizing the anti-inflammatory properties of vitronectin, such as inhibition of complement lysis and plasmin mediated inflammation, its role in the closed eye may be extremely important in controlling or co-controlling this dynamic situation. (Chapter 5)

3.5. Fibronectin

Fibronectin shares with vitronectin the role as a multifunctional adhesive glycoprotein responsible for attachment, spreading and migration of different cell types, but fundamentally it is essential for the adhesion of almost all types of cells.[102] It is this shared function in corneal maintenance and the fact that fibronectin has also been detected in tears, that the role of fibronectin in the adsorption onto contact lenses must also be investigated. Additionally, there exists the possibility that fibronectin may be pulled from the corneal bed. The aim of this section of work was not to study fibronectin as such, but to examine its influence on the assay and analyse its performance on *in vitro* and *ex vivo* lenses in comparison with vitronectin.

3.5.1. The Structure and Function of Fibronectin

Fibronectin is normally a 450kDa molecular weight glycoprotein with two chains, but three alternatively spliced forms have been demonstrated, the significance of which is not yet understood.[103] It can be found in plasma as a soluble protein and in the extracellular matrix as an insoluble protein.[104] Fibronectin contains approximately 5% carbohydrate[103] and it appears that this may function primarily to stabilize specific regions of the molecule against proteolytic attack, for example during inflammation when plasmin (a proteolytic protein) levels rise.

Fibronectin can bind to numerous biological macromolecules such as fibrin, collagen and heparin which are major components of the cornea. It mediates the adhesion and spreading of cells to cells, and cells to extracellular matrix.[103, 105]

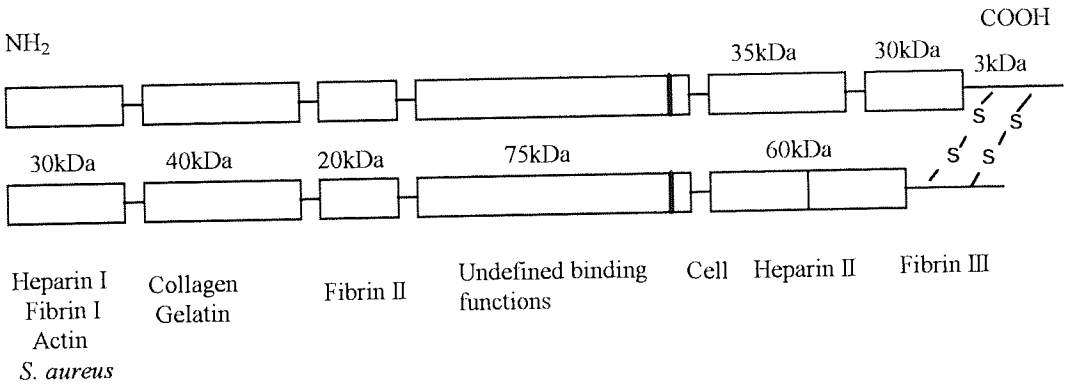


Figure 3.2. Binding domains of fibronectin[104, 105, 106, 107]

3.5.2. Fibronectin and Healing

Fibronectin has been shown to be present at corneal wound sites but is not detectable in the normal cornea,[108] suggesting therefore that detectable levels of fibronectin during wound healing dissipate when the normal cornea is restored. It is also reported to be a chemoattractant for corneal epithelial cells,[109, 110] and that it can enhance phagocytic activity by leucocytes and macrophage.[107] Additionally, fibronectin binding to fibrin from the blood may be important in the initial stages of wound healing in the damaged cornea, enabling corneal cells at the periphery of an injury to migrate across the defect. A fibronectin film enhances the migration of corneal epithelial cells in cultured corneal blocks and *in vivo*.[111] A thin fibronectin and fibrinogen film can form across the corneal wound and the epithelial cells migrate into the area. The presence of fibronectin is essential for wound contraction as fibroblasts draw the collagen fibrils together using fibronectin as the anchoring point.[111]

3.5.3. Fibronectin and Tears

Fibronectin was originally discovered in tears in a study on the eye after surgical trauma.[112] Tear samples were taken before surgery and for twelve days after surgery. The mean value quoted before surgery was 280ηg/ml, a baseline fibronectin level. After surgery, the levels gradually rose and then declined to baseline levels after twelve days. More recently, work was done to evaluate and compare fibronectin concentration levels in the open eye versus the closed eye. In the open eye, base values were given as 19±24ηg/ml, with the closed eye tear samples rising to 4127±3222ηg/ml.[113] The difference in open eye values between the two authors was explained by the latter as being due to their more sensitive probes. However as both used ELISA (enzyme linked immunosorbent assay) the difference may have been in some way due to the fact that the first study involved pre-operative patients.

The role of fibronectin in tears and in the cornea is assumed mainly to be in the healing process, confirmed clinically through the addition of fibronectin drops to the eye, which have been effective in resurfacing corneal epithelial cells in patients with persistent epithelial defects.[114]

3.6. Vitronectin Cell Adhesion Assay

As previously described vitronectin has many roles; it may be an inflammatory marker, has an increasingly observed role in bacterial pathogenesis and demonstrates many adhesive properties. There is also an observed connection between vitronectin presence and raised plasmin levels in contact lens wearers. On this basis a vitronectin assay was designed to assess this connection and the role of vitronectin in contact lens wear. Parameters analysed included contact lens materials, wear regimes and patient variability. Vitronectin's affinity for plastic and glass surfaces was exploited in the design of the assay to detect its adsorption onto the contact lens. In order to detect the adsorption of vitronectin onto the contact lens surface, a probe to visualise and trace its presence was required. This was achieved by utilising cells as a probe, taking advantage of the cell binding domain on vitronectin. The cells employed in this study were 3T3 Swiss mouse fibroblasts, which posses integrins receptors for both vitronectin and fibronectin and are a resilient and easily maintained cell line.

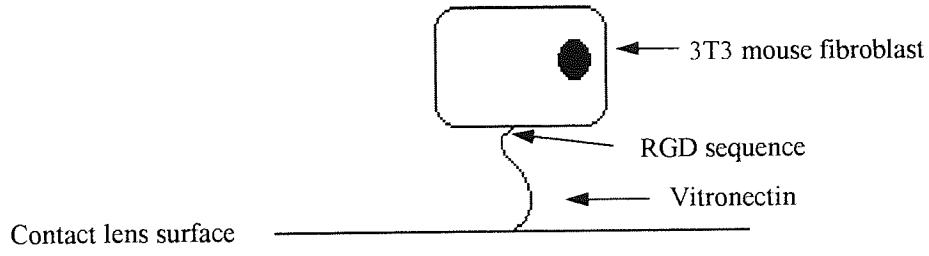


Figure 3.3. Diagrammatic outline of vitronectin mediated cell adhesion on a contact lens

The cell assay involves the selective inhibition of the adhesion molecules, by the use of antibody blocking techniques, to validate the significance of vitronectin on the lens surface. An anti-vitronectin (anti-Vn) antibody control was incorporated into the assay to block the action of vitronectin and used as a comparison against the vitronectin standard control wells in order to assess the adhesion of vitronectin onto the lens. An anti-fibronectin (anti-Fn) antibody was also used as a direct comparison against the anti-Vn antibody and vitronectin standard controls. The purpose of this was to either eliminate or accept the role of fibronectin as an adhesion molecule in contact lens wear and to further validate the dominant adhesion of vitronectin onto the contact lens. The use of an IgG antibody as a control was to negate the action of an arbitrary antibody in the system.

Comparative studies were set up in order to obtain *ex vivo* and *in vitro* spoilt lenses. The worn lenses were all aseptically removed prior to the assay. The following is a general explanation of the assay developed to analyse the involvement of vitronectin in contact lens spoilation. The protocol outlines the original assay[101, 106] and includes the modifications employed to improve the assay.

3.6.1. Materials

(as listed in Chapter 2, see cell culture)

3T3 Swiss mouse fibroblast cells (ICN Flow - Paisley, Scotland)

3.6.1.2. Chemicals at 37°C

HEPES buffered saline (HBS) (Sigma H-0887)
Dulbecco's modified minimum essential medium (DMEM) (GibcoBRL 41966-029)
1x dilution of trypsin/EDTA (Sigma T-4049)
Stock bottle of 1% gluteraldehyde (Sigma G-6257)
Stock solution of 50mM magnesium chloride

3.6.1.3. Immunochemicals

Rabbit IgG (polyclonal) (Sigma-I5006)

Rabbit anti-human fibronectin (polyclonal) (Sigma F-3648)

Rabbit anti-human vitronectin (polyclonal) (Gibco BRL A104)

Human Vitronectin (Sigma V-8379)

Human Fibronectin (Sigma F-2006)

3.6.1.4. Clinical Materials: *In vitro*

Prior to *in vitro* doping assays the appropriate lenses were incubated in specific μg/ml concentration of vitronectin or fibronectin according to assay requirements. Each contact lens was placed in 0.5ml of protein solution and stored at 4°C for 24 hours. The lenses were then washed with excess solution and were ready for analysis.

3.6.1.5. Clinical Materials: *Ex vivo*

Lens Name	Manufacturer	FDA Classification	% water content	Ionicity	Composition
Vistagel	Vista Optics	Group I	38	Non-ionic	HEMA
Vistagel	Vista Optics	Group I	42	Non-ionic	HEMA, VP
Vistagel	Vista Optics	Group II	60	Non-ionic	HEMA, VP
Vistagel	Vista Optics	Group II	75	Non-ionic	HEMA, VP
Acuvue	Vistakon	Group IV	58	Ionic	HEMA, MA
Precision UV	Pilkington Barnes-Hind	Group II	74	Ionic	VP, MMA

Table 3.1. Characterization and details of the lenses employed in on-lens cell based assays. HEMA = 2-hydroxyethyl methacrylate, MA = methacrylic acid, VP = vinyl pyrrolidine, MMA = methyl methacrylate

3.6.1.6. Lens Wear Modalities

All *ex vivo* lenses employed in the wear modalities studies were Acuvue lenses, worn under the following protocols:

Wear Modality	Protocol	Mean Hours of Wear
One Day Disposable	1 Day	8
Daily Wear	14 Days-Removed at night-Cleaned with ReNu	198
Extended Wear	1 Week- Continuous wear	168

Table 3.2. In vivo Acuvue lens wear modality protocols

All worn lenses were removed aseptically prior to the assay and stored in saline or ReNu, as required.

3.6.2. Method

The contact lenses to be assayed were placed individually in the wells of a 24 multi-well tray according to the requirements of each individual assay. As the vitronectin standard control, a well containing the lens and HBS only was included. A second well containing 300μg/ml solution of rabbit IgG antibody in HBS was prepared as a control for the effects of an arbitrary antibody in the system, whose effects should be negligible.

Each lens was rinsed with 1ml of HBS three times to clean the lens of any loosely bound material; this was done by means of aspiration of the HBS in the wells. To each of the wells, with the exception of the IgG wells, in to which 1ml of IgG solution was added, 900μl of HBS was added. 100μl of the anti-Fn and anti-Vn antibodies were added to

their respective wells which already contained 900µl of HBS. To the vitronectin standard control, 100µl of HBS was added to make it up to a 1ml end volume.

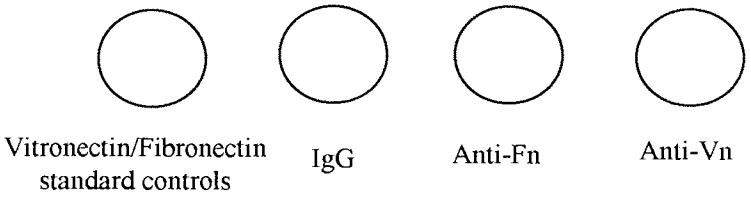


Figure 3.4. Vitronectin assay well key

Well Name	Contents
Vitronectin standard control	900µl HBS + 100µl of HBS
IgG	1ml IgG (300µg/ml HBS)
anti-Vn	100µl anti-Vn + 900µl HBS
anti-Fn	100µl anti-Fn + 900µl HBS

Table 3.3. Summary of vitronectin assay well contents

The tray was then placed into the incubator for 60 minutes at 37°C at 5% CO_2. During this hour the tray was agitated gently approximately every 10 minutes to prevent the antibodies from settling on the centre of the lens and to enhance their distribution.

After about 40 minutes of this incubation, the cells were prepared for the assay. The cells were passaged by the usual method up to the point of post-centrifugation. The supernatant was taken off and the pellet was resuspended in a known volume of HBS, for example, 10ml. The cells were then counted using the haemocytometer slide and

adjusted to give a final count of $5x10^4$ cells/ml. 1ml of the adjusted cells was then added to 19ml of HBS. A test to ensure cell viability was undertaken at this point by adding a drop of trypan blue to a drop of cell solution and counting the dead (blue) cells versus the total cell count.

On completion of the incubation period, the tray was removed from the incubator and the solution carefully removed from each well, taking care not to touch the lens with the pipette tip. 1ml of cells and 100µl of 50mM magnesium chloride was added to each well, giving a final concentration of 5mM magnesium chloride per well. The tray was again incubated at 37°C for 60 minutes. After the incubation period, the cell solution from each well was aspirated, and the lenses were rinsed three times in 1ml of HBS. At this point the lenses were removed from the assay plate to a new 24 well micro tray - this was to avoid later complications when analysing lenses with the microscope; some cells were inclined to stick and rest on the bottom of the wells due to natural gravitational pull and competitive binding that tissue culture plastic could create. This step avoided misinterpretation. As a final wash step, the lenses were then rinsed through once more with 1ml of HBS, which was then taken off. As a fixative, 1ml of 1% gluteraldehyde was added to each well. The cells attached to the lenses were then ready for counting.

3.6.2.1. Counting Cells on Contact Lenses

The cells were counted on the lenses fixed in gluteraldehyde on an Olympus (CK2) microscope under a x10 magnification. When counting the cells on the surface of the individual lenses, four different zones of view on the lens were defined as shown in Figure 3.5., in order to gain an overall view of the lens surface. In each zone four counts were read and an average was taken, with the exception of the centre zone in which only one count was feasible. The field of vision in which each individual count was taken was defined by an internal graticule in the eye piece of the microscope. The eye piece graticule measures 1cm x 1cm, which when read under a x10 magnification allowed a field of vision of $1mm^2$. An average of the four counts in three fields and single count in centre zone was calculated and used the counts presented.

- Centre (c)
- Off Centre (oc)
- Middle Zone (mz)
- Periphery (p)

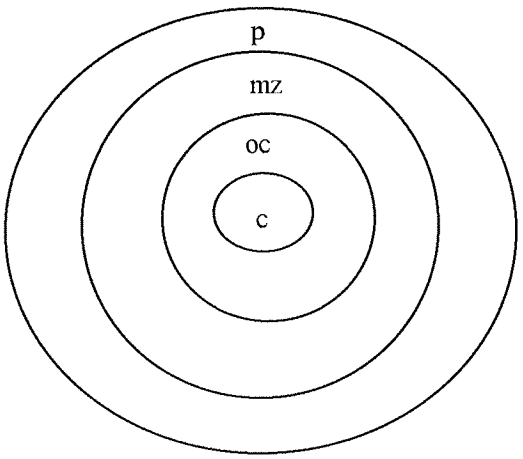


Figure 3.5. Zones of view on the contact lens

It must be pointed out at this point that this aspect of the assay is subject to experimental variation between personnel. The fields of choice were arbitrary and may be read manually or by image analysis, the former method was employed in this research. The parameters of each count will change from laboratory to laboratory, thus whilst the results of the following experiments are presented as average cell count per field it must be noted that these values depend on the set parameters of this group of experiments.

3.7. Results

3.7.1. Demonstration of the Adhesion of Vitronectin to Contact Lenses *in vitro*

The basis of this chapter and assay relies on the fact that vitronectin adheres to contact lens surfaces and that fibroblast cells with integrin receptors for vitronectin, adhere to the contact lens using vitronectin as the binding ligand. Therefore, an initial experiment was performed to prove that vitronectin adsorbed out of solution onto the contact lens surface and that it was traceable by a fibroblast cell assay. Four Group I unworn polyHEMA lenses were doped for 24 hours with a 20μg/ml solution of vitronectin. A duplicate set of unworn, non-doped polyHEMA lenses, were assayed as a control. The choice of a Group I set of lenses in this assay was used to take the assay to the limits. Group I lenses are known to display lower levels of spoilation over various wear regimes compared to Groups II and IV, and thus could be classed as the lowest threshold for all lenses to be analysed.

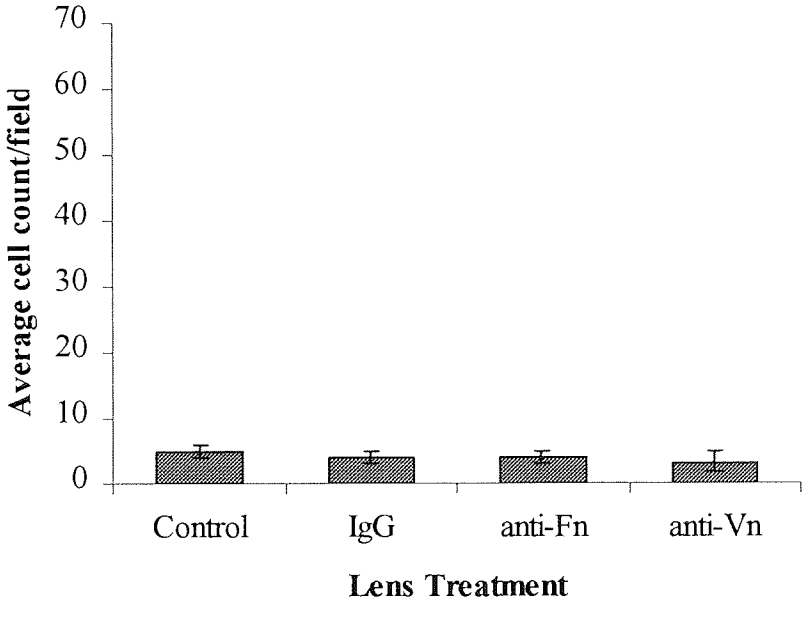


Figure 3.6. Cell adhesion on non-doped polyHEMA lenses in vitro

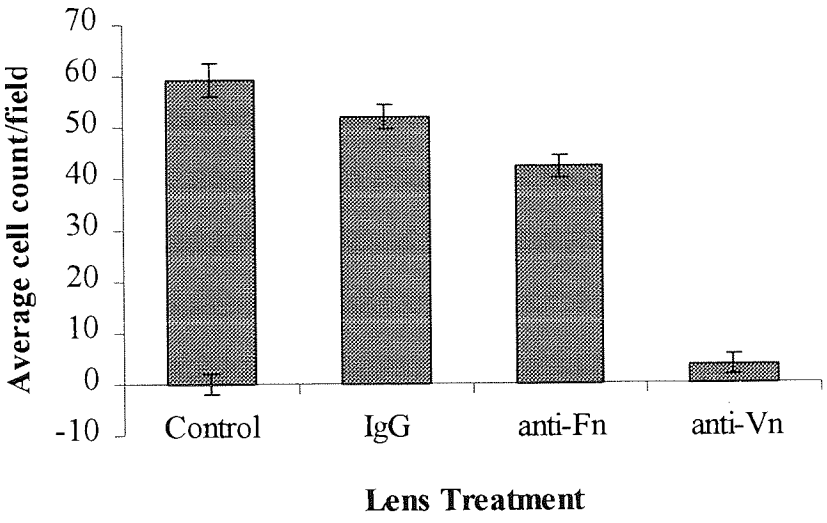


Figure 3.7. Cell adhesion on vitronectin doped polyHEMA lenses in vitro

The validation of vitronectin-dependent adhesion of 3T3 fibroblasts to contact lenses was confirmed on two levels. Firstly, a comparison of results between the non-doped (vitronectin negative, Figure 3.6.) lenses and the doped (vitronectin positive, Figure 3.7.) lenses, shows vitronectin-mediated adhesion of cells to the polyHEMA lenses. The cell count rises from an average of 3 cells per field on the non-doped lenses up to an average of 60 cells per field on the doped lenses. The non-doped lenses displayed a basic background cell adhesion count which was likely to be removable with further wash steps. The IgG control wells displayed a similar great difference between the doped and non-doped lenses, indicating the role of vitronectin in adhesion.

Secondly, the action of the anti-Vn antibodies on the vitronectin-doped lenses, which functioned to bind to the adsorbed vitronectin on the lenses, highlighted the fact that without free vitronectin on the lens surface the cells were unable to bind directly with the lens. Only a background level of cells adhered to the anti-Vn lenses, similar to the

numbers present on the non-doped lenses. The antibodies blocked the adherence of the cells to the lenses via vitronectin, thus proving that the cells could not employ an alternative ligand for mediated adhesion. The use of anti-Fn antibodies served as another control. The cell counts were a little lower than the vitronectin standard control and IgG, but in essence displayed little effect on adhesion inhibition.

3.7.2. Analysis of Fibronectin Mediated Cell Adhesion *in vitro*

Both vitronectin[83] and fibronectin[112] have been reported in tears. They are immunologically unrelated and biochemically different, but due to the fact that their cell attachment activity is similar, an assay was carried out to assess the individual performance of each adhesion ligand under these assay procedures. It would be inadvisable to assume that vitronectin is solely responsible for both adsorption onto the contact lens and the cell-mediated adhesion.

An initial assay was undertaken to determine a suitable and standard concentration for *in vitro* doping of Acuvue lenses by fibronectin. From a doubling dilution range of standards, a concentration of 12.5μg/ml of fibronectin was selected taking into account the requirement for a satisfactory cell count and cost effectiveness. This concentration which was similar to those used for vitronectin doping experiments, but which is far greater than those to be found in tears but taking in the possible influx of corneal derived fibronectin. The 12.5μg/ml concentration was employed as the baseline for the experiment which assessed fibronectin on Acuvue lenses.

The designated, control, anti-Vn and anti-Fn, Group IV Acuvue lenses were doped in 12.5μg/ml of fibronectin for 24 hours. The aim of this assay was to assess the potential of fibronectin to adsorb onto the lens surface out of solution. Its ability to be visually analysed using cells as a probe was positively determined in the previously mentioned fibronectin concentration selection assay.

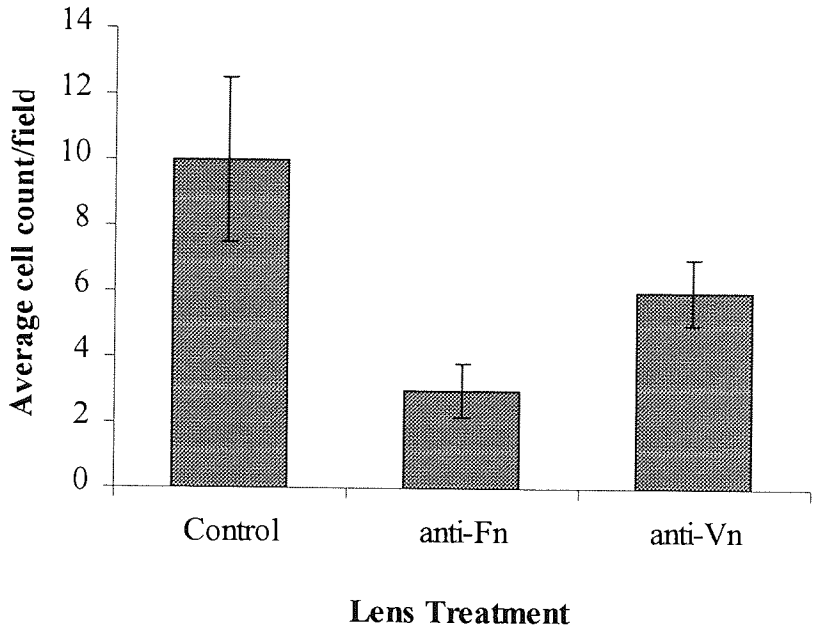


Figure 3.8. Cell adhesion on fibronectin doped Acuvue lenses in vitro

Fibronectin demonstrated poor adsorption out of solution onto the contact lens, displaying low cell counts compared with that of vitronectin-mediated adhesion at similar doping concentrations. Acuvue lenses were utilised in this assay due to the fact that Group IV lenses are prone to greater levels of spoilation than Group I lenses and so the expected low levels of fibronectin-mediated adhesion on contact lenses could be better evaluated. Even with the use of Acuvue lenses the cell counts were low. Thus, it could be concluded that vitronectin, and not fibronectin, is the dominant adhesion molecule at work in cell-mediated adhesion on the contact lenses, demonstrable by the low cell counts on the fibronectin-doped control lenses. A comparison between the control lenses and the anti-Fn lenses would suggest a minor role for fibronectin in the adhesion, but at such low levels of cell counts this comparison is not statistically significant.

3.7.3. Effect of Vitronectin Concentration on Cell Adhesion on polyHEMA lenses *in vitro*

The next experimental step was to evaluate the effect of vitronectin concentration on cell adhesion. The aim was to assess vitronectin over a broad concentration range, which encompassed its concentration determined in tears. For this assay only the vitronectin standard control lenses were required. No antibodies were used - the anti-Vn antibodies employed to prove the role of vitronectin in the mediation of the adhesion of cell onto contact lenses, were now clarified *in vitro*. *In vitro* polyHEMA contact lenses were used in order to keep the lens type consistent with the initial experiment and to reduce experiment parameter variability. The four concentrations of vitronectin used to dope the lenses were: 20μg/ml, 10μg/ml, 5μg/ml, and 1μg/ml. Four non-doped lenses were also analysed as background controls.

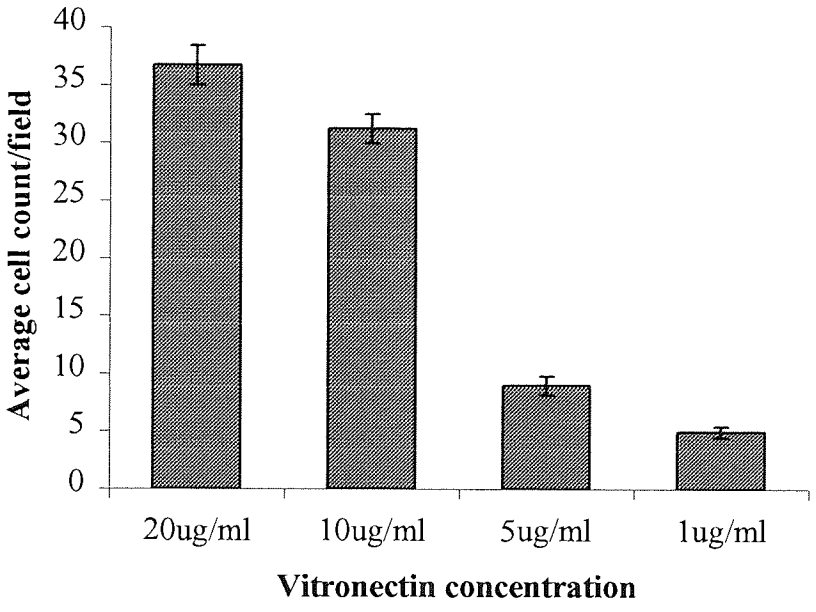


Figure 3.9. Cell adhesion with varying concentrations of vitronectin on polyHEMA lenses in vitro

Figure 3.10. below represents a concentration curve derived from an average of the results of the cell counts shown in the previous figure, to show the average cell count versus vitronectin concentration to scale. This curve, however, can not be used as a means of directly calculating vitronectin concentration against cell count as each assay is a separate entity and can only be used as a general guideline.

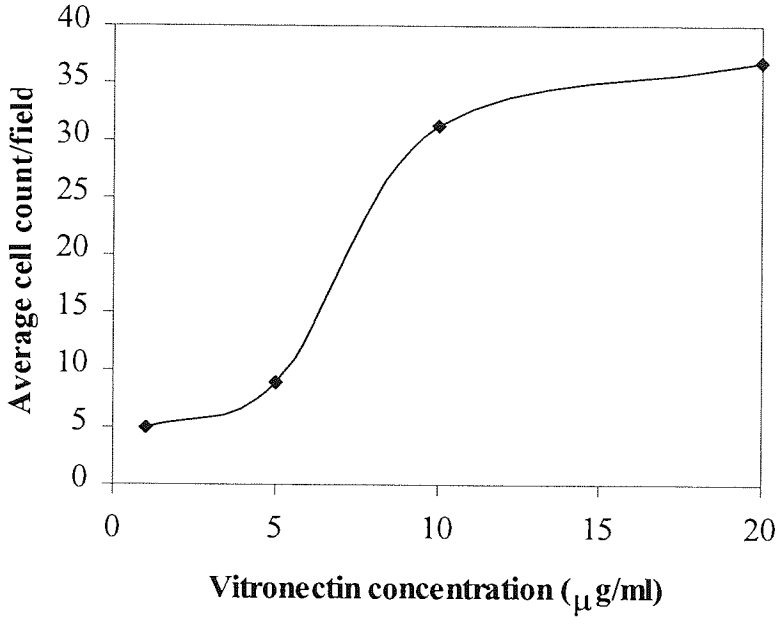


Figure 3.10. Relationship between vitronectin concentration and on-lens cell adhesion

The results demonstrate that cell adhesion is directly dependent on vitronectin concentration. The higher the concentration the higher the cell count. Previous studies[106] have shown 20μg/ml to be an optimum concentration for the efficient performance of the assay, but for this research 10μg/ml was deemed to be optimum and most cost effective for *in vitro* doping assays. A plateau of saturation was not reached within this range of standards, but concentrations above 20μg/ml are well out of the range that would be encountered for in vitronectin tears. It is predicted the curve would continue to rise gradually, reaching a plateau when vitronectin density versus free space on the lens would become a limiting factor. The four non-doped controls (not shown) displayed similar results as displayed in the initial assay; a low level cell count which may be taken as background.

3.7.4. Effect of % Lens Water Content on Vitronectin-Mediated Cell Adhesion on polyHEMA *in vitro*.

The next two experiments were designed to assess the influence of the lens on the adsorption of vitronectin to its surface. This assay was carried out to investigate the effects of changing water content on the binding of vitronectin to the contact lens materials, and thus the adhesion of the cells onto the contact lenses. The lenses used were all Vistagel non-ionic lenses with water contents of 38%, 42%, 60%, and 75% Only the vitronectin standard control and IgG lenses were tested. All the lenses were doped with vitronectin at a concentration of 10μg/ml for 24 hours. This concentration of vitronectin was chosen as the optimum, as discussed above, to avoid the use of greater concentrations of this expensive commodity.

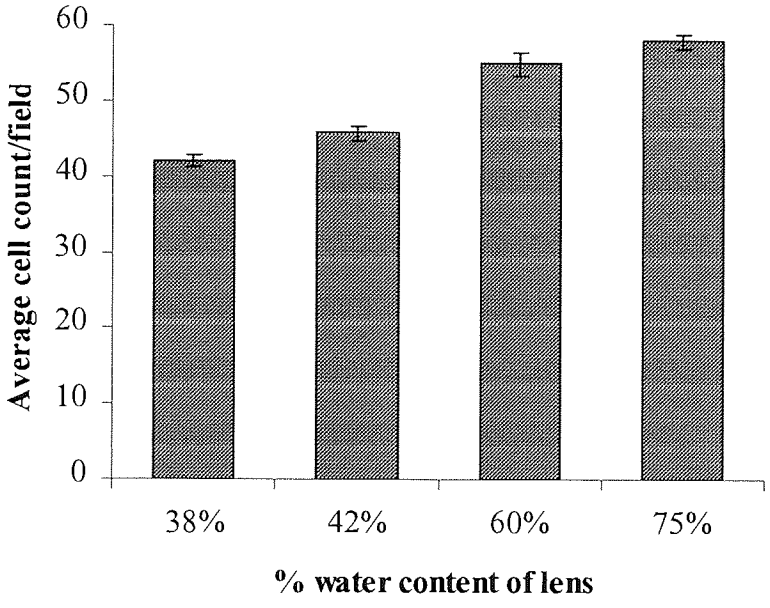


Figure 3.11. Vitronectin-mediated cell adhesion on varying water content control polyHEMA lenses in vitro

The results of the IgG control lenses displayed a similar trend as above, with almost identical levels and are not shown. Figure 3.12. presents the percentage water content results to scale, providing a clearer picture of the effect of water content on vitronectin adsorption onto the lens surface. It illustrates the fact that water content does not have a huge affect on these non-ionic lenses, but there is a progressive increase in adsorption with water content, with an assumed tendency to reach a plateau of adsorption.

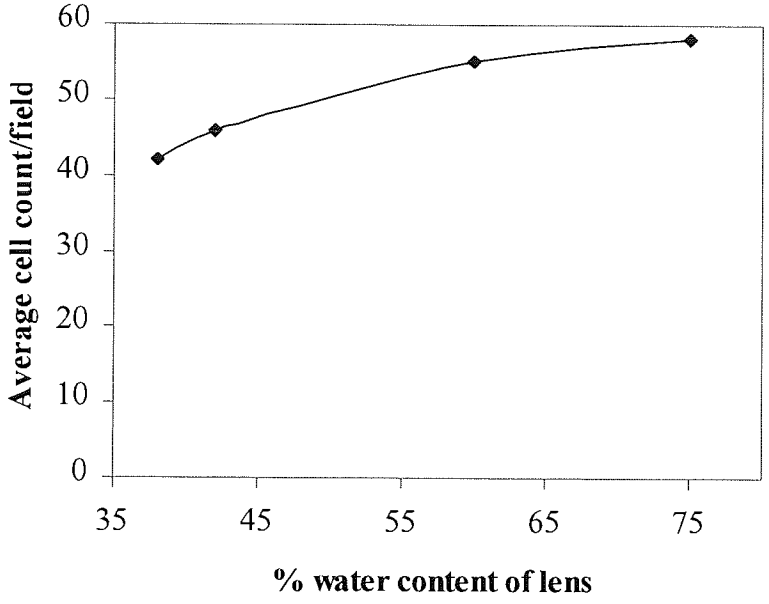


Figure 3.12. Relationship between water content of a non-ionic contact lens and vitronectin adsorption

The concentration of vitronectin adsorbed onto the contact lens was directly affected by the water content of the lenses; the higher the water content of the lens the higher the adsorbance of vitronectin onto the lens surface. Figure 3.12. highlights the fact that the differences in cell count from 38% up to 75% was not vast. Vitronectin is known to have a high affinity for glass, a property applied in the isolation and purification of this glycoprotein from serum and other sources. Glass is an inorganic ionic material with a surface energy several times higher than hydrogels and other polar polymers. This fact correlates well with the observed steady increase in the absorption of vitronectin as the polarity increases with water content (and ionicity). In this assay the surface energy of the lens remained the same while the polar component increased, further demonstrating vitronectin's affinity for more polar materials. This experiment analysed the effect of water content on non-ionic lenses, consequently it was important to assess the effect of ionicity on vitronectin adsorption - which is shown in the following experiment.

3.7.5. Analysis of the Effects of Ionicity on Vitronectin Adhesion *ex vivo*

To complement the water content study, this assay was designed to assess the effect of lens ionicity on the adsorption of vitronectin onto a contact lens. This was achieved using Group II (Precision UV) versus Group IV (Acuvue) lenses. Group II lenses are non-ionic and Group IV lenses are ionic; both are high water content lenses. All lenses were worn on a daily wear basis according to protocol.

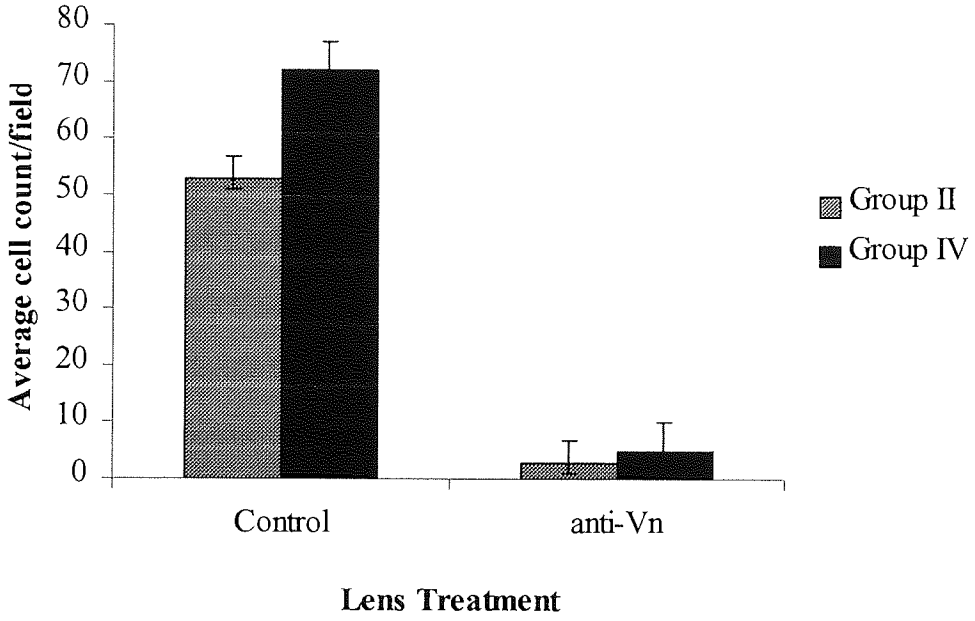


Figure 3.13. Relationship between the ionicity of a contact lens and vitronectin adsorption.

This assay further demonstrates the well-known spoilation characteristics of ionic versus non-ionic lenses. The ionic lenses presented a greater number of cells than can be seen on the non-ionic lenses. It was important to ascertain if the spoilation pattern by vitronectin on the contact lens was similar to that seen for a total protein spoilation. The results demonstrated that ionicity and water content did have an important influence on the adsorbance of vitronectin, producing the similar patterns, with higher adsorption for higher water contents and with ionic lenses.

Precision UV lenses possess a higher water content (74%) than Acuvue lenses (58%). This may suggest that the ionicity of the lens overrides and was more important than the water content with regard to vitronectin spoilation.

3.7.6. Analysis of Vitronectin-Mediated Cell Adhesion on *ex vivo* Daily Wear Acuvue Lenses

The following assays investigate the absorption of vitronectin out of tears onto a contact lens *in vivo* analysing the effect of wear time and wear modalities, and to assess the overall performance of vitronectin on *ex vivo* lenses. Acuvue lenses were worn on a daily basis and on final removal, stored in saline. The lenses were removed aseptically from the saline and placed in the 24 well plate. All the lenses were worn by the same asymptomatic patient for one week.

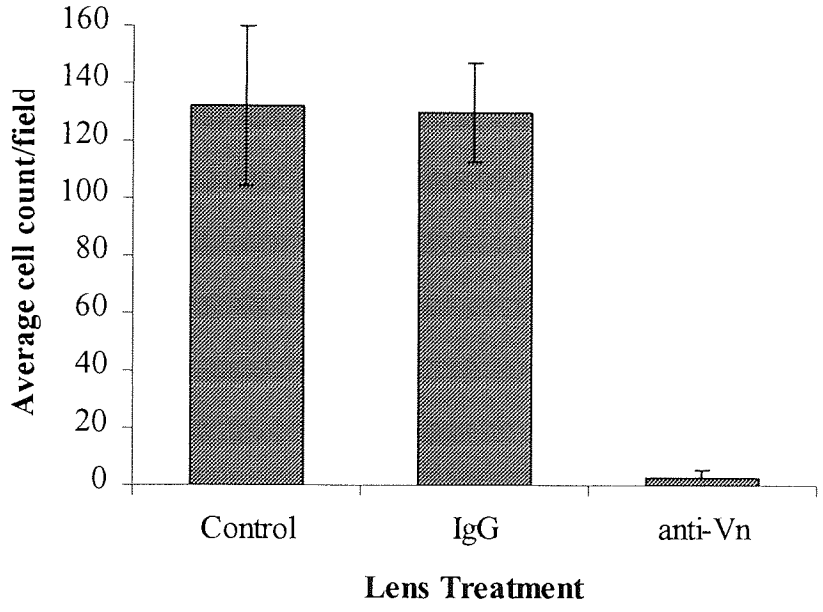


Figure 3.14. Vitronectin-mediated cell adhesion on daily wear Acuvue lenses ex vivo

3.7.6.1. Efficacy of ReNu in the Removal of Vitronectin from *ex vivo* Contact Lenses

The purpose of the assay was to assess the effectiveness of ReNu; considered to be one of the most efficient commercially available multi-purpose care system,[115] in the removal of adsorbed vitronectin from the lens surface. A second set of daily wear Acuvue lenses were tested but on removal the lenses were stored in ReNu and prior to the assay the lenses underwent the rub and rinse method. This served to determine the effect of ReNu on the adhesion of vitronectin to the contact lens whilst in storage and attempted to assess the reversibility of the adhesion.

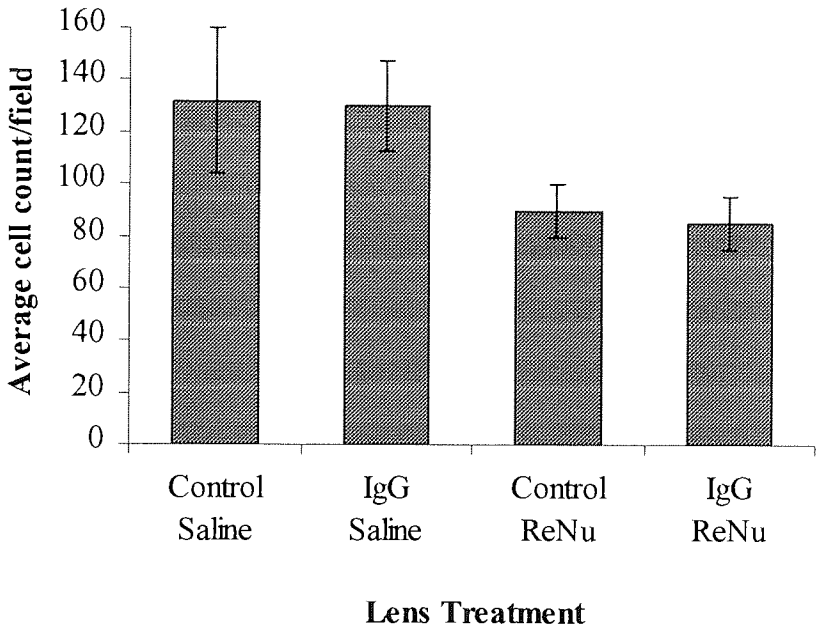


Figure 3.15. Analysis of the effect of ReNu on vitronectin adsorbed onto Acuvue lenses ex vivo

The first experiment on the daily wear lenses demonstrated high levels of vitronectin adsorption out of tears, with a massive reduction evident on the anti-Vn treated lenses. The results of the ReNu experiment displayed a small drop in cell count between the lenses stored in saline and the lenses stored in ReNu. ReNu was considered to be one of the best and most efficient multi-purpose care systems in the removal of proteins,[115] but this assay demonstrates that this care system is not wholly effective in reversing the adhesion of vitronectin from the contact lens. Essentially, this suggests that vitronectin is not removed by multi-purpose solutions and thus will remain on the lens throughout the life of the lens. Whether there is an initial layer of vitronectin formed which is always retained on the surface or is a constant competitive binding through proteolysis or some denaturation remains unknown.

3.7.7. Analysis of Vitronectin-Mediated Cell Adhesion on *ex vivo* Extended Wear Acuvue Lenses

The aim of this experiment was to determine vitronectin in extended wear, and more importantly, in overnight wear. As stated in the introduction of this chapter, vitronectin has received great attention in the closed eye environment - its concentration levels previously shown to increase 50-fold from the open eye to the closed eye.[83] Therefore, it was interesting to analyse the concentration of vitronectin on extended wear lenses which are in the eye overnight. Extended wear Acuvue lenses were worn for one week and stored in saline until the assay was performed.

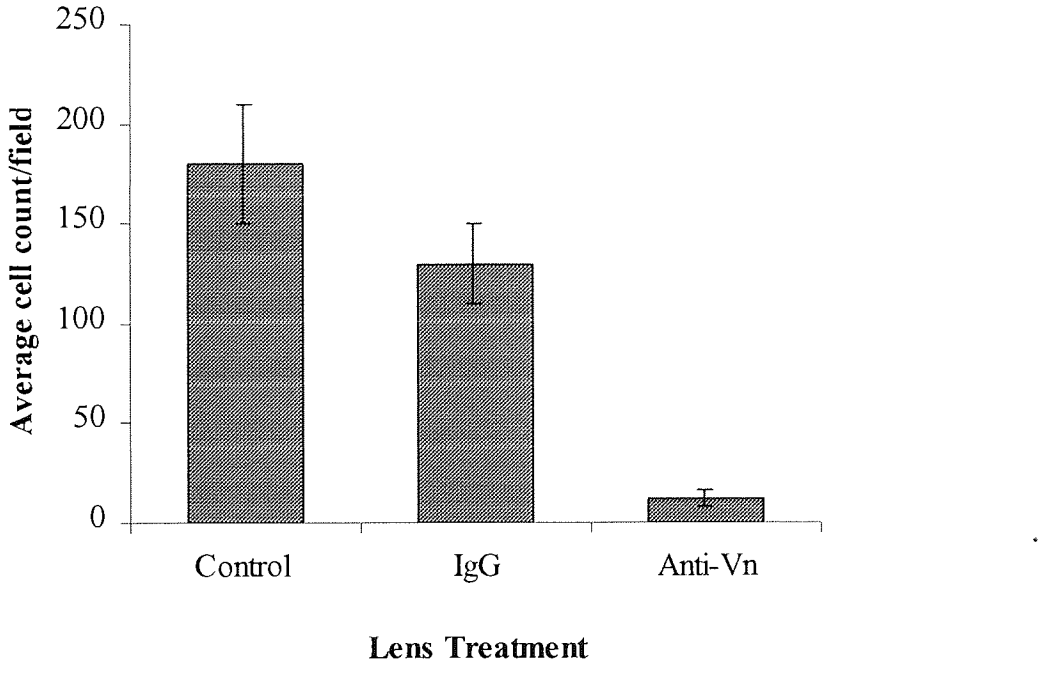


Figure 3.16. Vitronectin-mediated cell adhesion on extended wear Acuvue lenses ex vivo

3.7.8. A Comparison of Vitronectin-Mediated Cell Adhesion on One Day, Daily and Extended Wear Acuvue Lenses *ex vivo*

The aim of the next set of assays was to correlate the daily (DW) and extended wear (EW) results, with the inclusion of one day (OD) disposable lens counts, to demonstrate the time variables involved. The comparison between daily wear and one day disposables provided information on vitronectin adsorption over time; 1 day versus 14 days wear.

The daily wear lenses were worn for approximately 30 hours less than the extended wear lenses. However, the main difference lies in the wear regime, daily wear lenses, as the name suggests, were worn by day and removed at night, extended wear lenses, on the other hand, were worn overnight. Overnight wear was the key factor, and served as an interesting means of analysing vitronectin in the aforementioned closed eye environment; an environment recently identified as having sub-clinical inflammatory properties.

All the lenses were worn to protocol and as stated in the previous assay, the extended wear lenses were given a final wash with ReNu in order to standardize the storage procedures applied in the daily wear lenses.

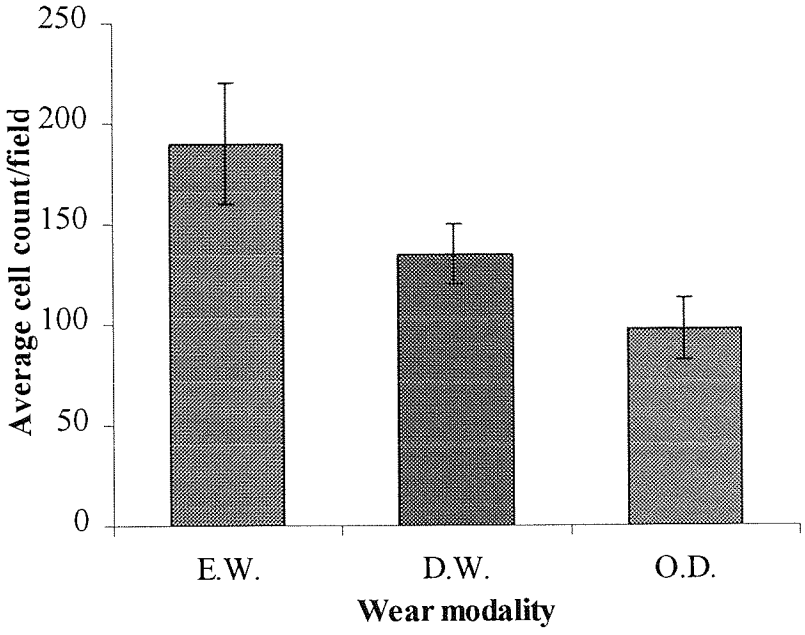


Figure 3.17. Vitronectin-mediated cell adhesion with varying wear regimes on Acuvue lenses ex vivo

These results demonstrated two points of interest. The first was the demonstrable difference in vitronectin concentration after 14 days daily wear as opposed to 1 day wear. The levels of vitronectin on the lens surface were clearly greater over time. A result that correlated with total protein spoilation studies presented in Chapter 6, which show a similar time dependent trend. An initial level of spoilation at one day was shown to rise greatly at 7 days and upwards.

The second point of interest was the significant difference between the cell count on the extended wear lens and the daily wear lens which differ essentially in the fact that the extended wear lenses were kept in overnight. Realising that the daily wear lenses will have undergone a more efficient cleaning process, the ReNu experiment proved the difference was not the key factor in question. To enhance standardization the storage of both lenses was kept the same. Vitronectin levels were known to increase in the closed

eye environment and this was validated by this assay where the levels of vitronectin for similar wear duration are greatly augmented on extended wear lenses. This was most likely to be due to exposure to greater quantities of vitronectin in the closed eye state.

3.7.9. Investigation into Preferential Adsorption of Vitronectin onto Contact Lens Surfaces: Centre versus Periphery

All the previous studies presented the results as a mean and standard deviation of all fields counted on the contact lens, without considering the precise position of the cell/vitronectin adhesion on the lens. The following assay was performed in order to determine and take note if there was preferential absorption of vitronectin *in vivo* onto particular areas of the lens, or if the absorption was unaffected by the position in the eye. The location of vitronectin absorption was analysed on both daily and extended wear lenses, for Group II (Precision UV) and Group IV (Acuvue) lenses. The areas on the lenses were compared as the centre of the lens versus the periphery of the lens.

Initially when counting the cells at the centre of the lens, contained in the 24-well plate, some difficulties were encountered, i.e., in discerning the difference between the cells attached to the lens and those unbound cells with a tendency to settle on the bottom of the well. Defining the difference down a microscope is almost impossible due to the proximity of both fields. To alleviate this problem the lenses were removed from the assay plate and transferred to a new plate, with an intermediate wash step to remove unbound cells.

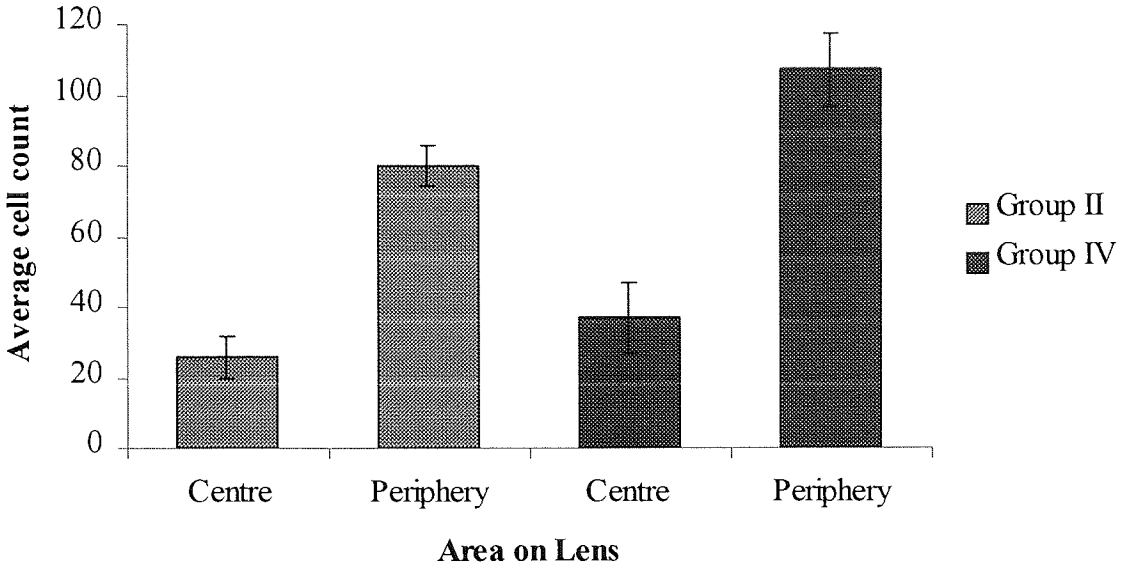


Figure 3.18. Comparative vitronectin-dependent cell counts on daily wear lenses: centre versus periphery

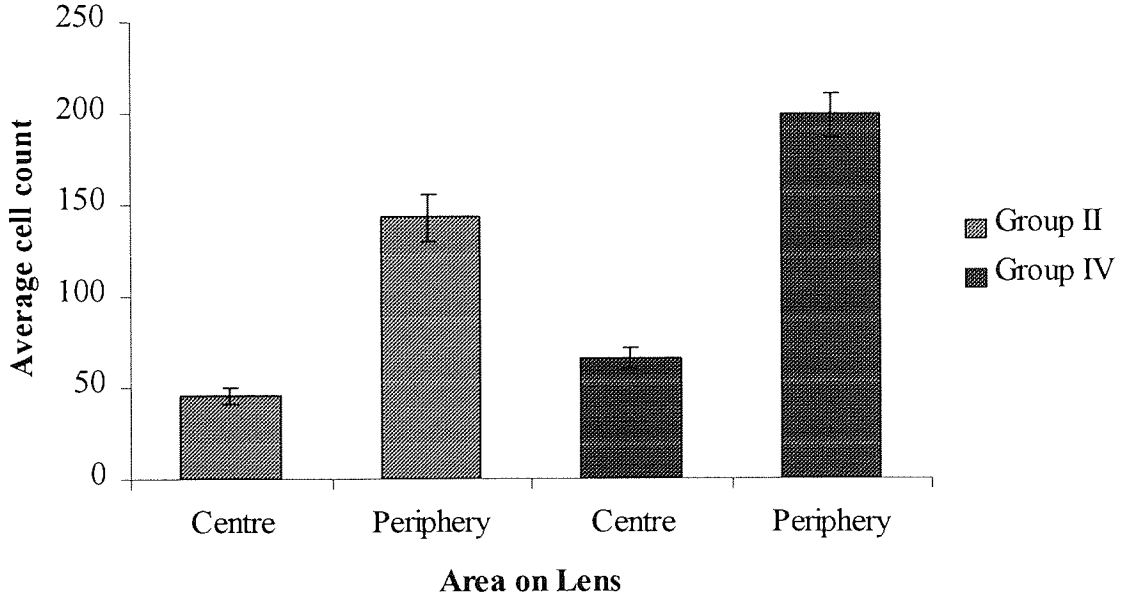


Figure 3.19. Comparative vitronectin-dependent cell counts on extended wear lenses: centre versus periphery

There was a marked increase in vitronectin accumulation at the periphery of the lens in contrast to the centre of the lens. This phenomenon was evident in both Group II and Group IV lenses and for both daily and extended wear regimes. The fact that this preferential absorption was evident for both wear modalities is probably the most significant point of interest. Due to the fact that daily wear lenses are inserted and removed daily, the effect of daily handling of the lens, varying insertion and removal parameters and the affects of daily cleaning and spreading of proteins, would be expected to change the characteristics of vitronectin's deposition. Nevertheless, a similar adsorption trend was observed over all daily wear lenses studied and was significantly similar to those patterns displayed in the extended wear lenses. Both wear modalities appeared to create a vitronectin rich micro-climate at periphery of the lens, which may be the site of localized inflammatory mediation.

3.8. Discussion

The aim of this chapter was to determine the influence of the contact lens on the absorption of vitronectin out of tears onto the lens surface. The initial experiment, performed on vitronectin doped lenses, demonstrated that vitronectin's affinity for glass was also observed for the lens surface. The use of polyclonal anti-vitronectin antibodies as a control in the cell based assay, demonstrated the essential role of vitronectin-mediated 3T3 cell adhesion, and thus validated the use of the cells as a means of detecting vitronectin presence. The polyclonal antibodies blocked the numerous exposed sites of vitronectin, preventing cell attachment.

The *in vitro* doping of contact lenses by vitronectin at varying concentrations showed a direct correlation between an increase in vitronectin concentration and its absorbance onto the contact lens. This increase in vitronectin levels was measurable by the increase in cell-mediated adhesion, further validating the sensitivity of this assay in the detection of vitronectin.

As fibronectin, which shares with vitronectin a role in cell adhesion, was also detected in tears,[112] its possible adsorption out of solution onto the lens surface and mediation of cell attachment could therefore not be ignored. An assay was performed to assess its role as a possible co-adhesive protein in these experiments. At similar *in vitro* doped concentrations of fibronectin, the cell count was very low, similar to those found as background levels in the initial vitronectin validation assay. A small difference in cell count between the control (no antibody) and anti-fibronectin wells was evident, with a lower count on the latter which may suggest a minor influence by fibronectin, but at such low cell counts the results were not significant. Thus, it could be concluded that fibronectin did not adversely interfere with the vitronectin assay.

	Vitronectin	Fibronectin
~ Tear concentration	0.8μg/ml	25ηg/ml
Equivalent cell mediation	5μg/ml	12.5μg/ml

Table 3.4. A comparison of vitronectin and fibronectin mediated cell adhesion

From this table it is clear that a greater concentration of fibronectin was required to equal the cell meditation activity of vitronectin under these *in vitro* doped conditions. Additionally the levels of vitronectin in tears are vastly higher than those detected for fibronectin suggesting a more significant and active role by vitronectin. Looking at the figures in the above table, and those presented in Figure 3.9. in reference to the effect of changing vitronectin concentration *in vitro* on cell adhesion, at levels of vitronectin similar to those suggested for tears - an approximate 5 cell average count was demonstrated. The contrast in approximate tear concentration and the levels required for specific cell counts was significant. The cell counts on the *ex vivo* lenses even the one day worn lenses are far in excess of these doped levels (see Figure 3.17.). This would suggest that vitronectin is not only being absorbed from tears but possibly from the

underlying corneal tissue bed, in order to sustain the attachment of such high cell numbers. The higher levels of vitronectin on the back surface versus the front surface would offer that this is the case (see below). Vascularisation due to lens wear may also increase serum leakage increasing available vitronectin from this source. Fibronectin may also be pulled from the tissue bed but the levels of which would remain insignificant in comparison with vitronectin under these experimental conditions.

The percentage water content directly effects vitronectin adhesion with an increase in water content clearly showing a rise in the adsorbed levels of vitronectin. The ionicity of the lens also greatly influenced the adsorption of vitronectin, with greater accumulation of vitronectin on the ionic versus the non-ionic lenses. The results on the *ex vivo* lenses also suggest that the ionicity rather than the percentage water content is the dominant factor in affecting the adhesion of vitronectin onto the lens surface.

ReNu, one of the leading multipurpose cleaning solution was tested for its effectiveness in the removal of vitronectin from the lens surface. An approximate 30% removal was achieved which would be considered quite low, which mean that ReNu and other similar cleaning solution would allow the accumulation of vitronectin on the lens surface over time. This result was also useful in appreciating the results comparing the extended wear lenses (no cleaning required) and the daily wear lenses (employed ReNu as cleaner). It was reasonable to assume that the affect of daily cleaning with ReNu did not greatly affect the outcome of results over time.

A comparison of the centre versus the periphery of the lens revealed that a greater vitronectin concentration resulted on the periphery. Further work was undertaken by the Aston group in an attempted to investigate the front surface versus the back surface for preferential adsorption. It was found that there were very low levels apparent on the front surface resembling the levels of vitronectin in tears. The back surface presented the greater accumulation of vitronectin, accounting for the majority of vitronectin detected.[101] This accumulation at the back surface may reflect vitronectin moving out of

the corneal tissue bed, as opposed to being simply derived from tears. The aggregation of vitronectin on the back surface of a lens is thought to create a particular micro climate, which due to its multifunctional nature and ability to bind to numerous ligands and proteins, could lead to the initiation of, or assist in, a number of effector systems.

These two points regarding vitronectin's preferential adsorption on the lens surface highlighted the fact that post-lens micro climate, particularly towards the periphery, is rich in vitronectin to a degree that is capable of influencing localised inflammation up regulation.

The results of this build up could be to the well being or alternately to the detriment of the host. The interaction of vitronectin with bacteria as described earlier is an interesting point. Vitronectin may serve to bind and present a potential pathogen to immunocompetent cells, e.g., polymorphonucleocytes, for clearance. On the other hand, the pathogen may utilise and bind to vitronectin as a means of establishing its presence in the ocular surface, which may prevent the immune system from detecting its presence. Whichever theory may prove to be true, the consequence of bacterial adherence to vitronectin in the presence of a contact lens would be further enhanced in the resultant microclimate. During overnight extended wear the consequences of vitronectin mediated bacterial adhesion may prove very important, for example, persistent bacterial presence on the surface of a contact lens may cause an excessive inflammatory response.

The increase in levels of vitronectin from one day wear on the daily disposables to fourteen days daily wear demonstrated its accumulation over time but as stated above other factors including lens wear modality, water content and the ionicity all prove influential. This result reflected those found and presented in Chapter 6, which revealed a significant rise in total protein levels going from one day through to seven-fourteen days wear.

A comparison of daily wear (DW) lenses worn for approximately 198 hours versus extended wear (EW) lens worn for approximately 168 hours demonstrated the significance of overnight wear on vitronectin accumulation. The levels of vitronectin detected on the EW lenses were higher than those observed on the DW lenses, offering the fact that time was not the telling factor here, but rather the wear regime. An influential parameter was the fact that the lenses were worn overnight, in an environment known to experience a state of sub-clinical inflammation; tear vitronectin levels reaching a peak high during this state. During overnight eye closure the eye looks to the cornea for support, for example without the supply of atmospheric oxygen it is the cornea which allows the influx of oxygen. The almost internal nature of the eye during eye closure would suggest that the cornea would assume a more important role and this may be true for vitronectin supply.

By observing the factors that affected vitronectin's adsorption onto the lens surface, lessons can be learned regarding the most suitable modality, material and wear duration in the prevention of contact lens related complications. These results allow us a variety of information on vitronectin adherence, the point of significance is knowing when the levels of vitronectin on the lens surface are dangerous or advantageous. Is the greater accumulation on the surface always beneficial to the host or is vitronectin, for example, assisting bacterial attachment, spreading and growth at certain levels?

Chapter 4

The Development of Immunodiffusion Assays for Tear Analysis and Contact Lens Studies

4.1. Aim

The aim of this chapter was to evaluate the use of immunodiffusion assays in the analysis of tears. Currently there are numerous techniques employed to investigate the concentrations of particular proteins in various tear states. Immunodiffusion assays were utilised in order to take advantage of the small analyte volumes required for analysis; volumes as low as 1µl samples could be investigated.

4.2. Problems in Tear Analysis

Tear analysis has the clear advantage over blood/serum sampling in that it requires non-invasive sampling, and thus can be performed regularly without patient discomfort. The main disadvantage associated with tear analysis lies in the minute volumes available that can be collected without stimulation. Stimulation results in a change in composition of the tears, which can affect the outcome of the investigation. Although difficult to determine, the tear volume of the normal eye has been estimated to be as little as 7.0±2µl,[4] but in practice only about 2-3µl of tears are available for sampling. Evaporation can also reduce the tear volumes extracted.

Techniques requiring small sample volumes therefore are a necessity and the way forward. Assays which require larger volumes of tears - greater than approximately 4µl - are ineffectual and will represent stimulated/reflex tears. For truer basal tear samples 3µl or less is optimum to avoid erroneous results. Stimulated tears demonstrate different overall protein concentrations, for example, tears collected by the Schirmer test (Chapter 2), collect larger volumes of tears through an irritation of the eye, creating excess tearing with demonstrable plasma leakage.

The purpose of the following work was to attempt to develop and employ immunodiffusion assays in tear analysis and integrate them into the multidisciplinary laboratory in conjunction with other analytical techniques. The decision to utilise immunoassays in the examination of tears was made to exploit the property of high specificity, but more importantly to exploit the fact that only small volumes of analyte

were required, which was important in overcoming the limiting nature of the minute volumes of tears available for analysis.

4.3. Immunoassays

Over the past twenty years the immunological laboratory has been developing and optimising many new techniques to analyse the immune response and individual immunocompetent species. More recently, due to the intrinsic specificity of the immunoassay, these methods have been employed in all clinical laboratories, and thus have become a basic tool for a vast array of disciplines. Modern science can not and should not avoid the influence of the immunoassay.

The fundamental principle of the immunoassay is the high specificity reaction between an antibody and its antigen. An antigen is generally a protein, carbohydrate, nucleic acids or any combination of these and is defined as a substance that reacts with and activates, antibodies or T-cell receptors. Antibodies belong to the immunoglobulin supergene family and are glycoproteins that react with the antigen that induced its production. Immunoglobulins, to contend with an almost limitless variety of antigen, can create a huge diversity of antibody molecules. This is due to the unusual structure of the immunoglobulin gene library and the ability of B-cells to arrange and modify their chromosomal DNA, a combination which works to create the specificity and affinity an antibody has for its antigen.

Using antibodies as exceptionally specific tools, a variety of immunoassays are proving important in the detection of other, lessor known, and analysed, proteins in tears and in contact lens wear. Recently, the study of tear protein changes induced during contact lens wear has become of particular interest, with studies, e.g., on the effects of spoilation by tears and extraneous components on the performance of the lens, and in turn the effects the contact lens has on the tear performance.

This study centres on the development and optimisation of immunodiffusion assays, in particular, in the analysis of tear proteins and in contact lens wear. However, the standardisation of tear analysis, extraction and handling needed to be addressed first in

order to be able to present the results uniformly, acknowledging the variables present in tear analysis.

4.4. Standardisation of Analysis

It is important to be able to assay all the main tear proteins with regularity, using similar techniques for all the proteins, to enable correlation of results. The ideal would be to have a simple and standard technique available in every tear analysis laboratory on which more detailed investigations could be based. This end analysis of the tear sample accounts for only a fraction of the variables involved in a complex series of events.

4.4.1. Variables in Tear Analysis

A major problem in the investigation of tears is the lack of standardisation as there exists a vast number of variables, from the method of collection and sampling through to the final stages of analysis and interpretation of results. The following table lists some of the range of variables that can be incurred in order to simply achieve one protein concentration.

Method of Collection	Tear State	Eye State	Collection Characteristics	Sampling Position in eye	Eye Sampled	Storage Conditions	Method of Analysis[1]
Schirmer test	Basal	Normal non-wearer	Flow rate	Meniscus	Left	Below -20°C	HPLC
Cotton thread	Reflex	Compromised non-wearer	Volume (µl)	Medial-canthus	Right	+4°C	Immunodiffusion e.g. RID,CIE, IEP
Sponge	Stimulated	Normal wearer	Contact	Lateral-canthus	Pooled	Room temp.	Immunoasssay e.g. ELISA[2], RIA[3].
Microcapillary	Psychogenic Lacrimation	Compromised wearer	Non-contact			No storage	Isoelectric focusing
Filter paper	Closed eye	AST[4]					SDS-PAGE

Table 4.1. Tear Analysis Classification Variables. A summary of some of the variables encountered in tear analysis from collection through to analysis protocol. 1: Includes a summary of the most widely used techniques in tear analysis, many more available. 2: ELISA, enzymo-linked immunosorbent assay. 3: RIA, radio immunoassay. 4: AST, automatic stimulated tears.

4.5. Tear Handling

In order to simplify and facilitate the standardisation of methodologies of each analysis, a table of parameters, as listed below, would allow ease of interpretation of results presented. These are the parameters used in this research.

Tear state	Basal/closed
Eye state	Normal/AST
Method of collection	Glass microcapillary
Contact with cornea/conjunctiva	No contact
Position in eye	Outer Canthus
Volume/flow rate	~3μl at 1μlmin^{-1}
Eye sampled	L or R (not pooled)
Storage condition	Immediate use - Laboratory volunteers Below -20°C - Clinical patients
Method of analysis	As listed below in immunodiffusion summaries

Table 4.2. An account of the preparation and handling of the investigated tear samples

4.5.1. Tear Sample History

All the tear samples examined in this study were taken from non-contact lens wearers. The samples taken from the volunteers in the laboratory were taken by myself and other samples were kindly donated by Michel Guillion and Lyndon Jones taken from patients during visits to their respective clinical practices.

Tear Sample Number	Type†	Source‡
TS-1	N	Lab
TS-2	AST	Lab
TS-3	N	Clin
TS-4	N	Lab
TS-5	AST	Lab
TS-6	N	Lab
TS-7	AST	Lab
TS-8	N	Clin
TS-9	N	Clin
TS-10	N	Lab
TS-11	N	Clin
TS-12	N	Clin
TS-13	N	Clin
TS-14	N	Clin
TS-15	N	Clin
TS-16	N	Clin

Table 4.3. History of the tear samples investigated in immunodiffusion assays. † N = tear samples taken at a controlled flow rate. AST = automatically stimulated tears. ‡ Lab = Laboratory volunteer. Clin = Clinical

4.6. Assay Development

As can be seen in Table 4.1. there are an array of techniques currently available and employed in the identification and estimation of the main tear proteins. A universal method of analysis which would be easy to perform, be reliable, reproducible, require the minimum of equipment and essentially provide accurate tear analysis-would prove the optimum choice. The area of immunodiffusion assays and the use of macro SDS-PAGE were investigated.

4.6.1. SDS-PAGE

One of the best means of detecting proteins employs electrophoretic methods and polyacrylamide gel electrophoresis has been used extensively in the study of tears. In general, however, this technique is cumbersome, time consuming and somewhat unreliable when using the macro-electrophoretic systems.

Figure 4.1. represents a typical run by SDS-PAGE. This picture shows almost all the bands that were detectable on the gel itself (some of the less well-defined bands were not picked up whilst scanning the gel). The well key reading from left to right is listed in Table 4.4.

Well Number	Protein Sample	Well Number	Protein Sample
1	2mg/ml IgG	8	Molecular weight marker (Sigma-101H9454)
2	TS-9 (L+R)	9	1mg/ml IgA
3	1mg/ml lactoferrin	10	TS-12 (L+R)
4	empty	11	0.25 mg/ml IgG
5	1mg/ml albumin	12	TS-7(L+R)
6	0.25mg/ml IgA	13	0.25mg/ml lysozyme
7	TS-1 (L+R)	14	2mg/ml lysozyme

Table 4.4. SDS-PAGE well key

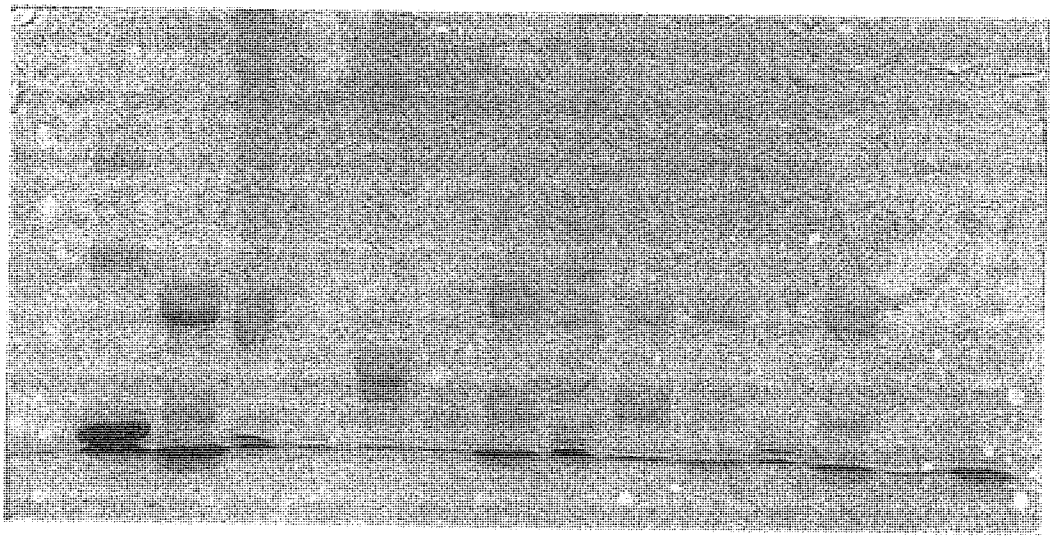


Figure 4.1. Typical run on the macro SDS-PAGE showing tear samples and standards

All the standards, and four tear samples were run under reducing conditions by boiling each sample in an equal volume of treatment buffer, conferring a net negative charge on all the proteins. For tear samples TS-9,1 and 7, four bands were detected at approximately 70kDa, 40kDa, 30kDa and 14kDa. TS-12 showed bands at only 70kDa and 14kDa; these molecular weights were estimated from the molecular weight marker. The 70kDa band in the tears ran parallel to the lactoferrin standard and was assumed to represent this glycoprotein. The 14kDa band could be confidently explained as a lysozyme band. The 30kda band may represent a lysozyme dimer,[116] the 30kDa protein G, or it seems to equate with the light chain band on both of the IgG runs. The 40kDa band seems to run parallel with the light chain band on IgA. No albumin was detected in any of the tear samples.

4.6.1.1. Disadvantages of the Macro-SDS-PAGE System

SDS-PAGE generally demonstrated a certain unpredictability and on occasion important tear samples were lost due to unsuccessful runs. Unpredictability, occurred when making the gels; with no difference between two gels made up on two different days, one gel would polymerise and the other not. Making up the gels is a very time consuming process. The performance of the molecular weight marker also varied and

this being fundamental to the gel run, caused problems and wasted samples and time. The sensitivity of this system combined with Coomassie blue stain is low, as can be seen with the double concentration runs of IgA and lysozyme. Both proteins were run at the two concentration at 2mg/ml and 0.25mg/ml and neither protein was detectable at 0.25mg/ml.

Due to the fact that this macro-system displayed a limited success in the analysis of tears and because of the large sample volumes required (in excess of 7μl), it was not greatly used in this area. The drawbacks found in our laboratory and others encountered by other researchers have recently been tackled and overcome with the introduction of the mini-gel system. This new system requires smaller sample volumes and greatly cuts the time of gel preparation and run. The detection limit of these mini-gel systems combined with silver staining is approximately 0.5ηg protein. This system is currently under investigation and optimisation in our laboratory and should prove an important tool in the study of tears.

4.6.2. Immunodiffusion Assays

The aim of the study was to attempt to quantify the main proteins only by immunodiffusion assays, and to note the results in comparison against concentrations quoted by other researchers by a variety of methods and techniques. Concentrations of tear proteins differ from subject to subject and are greatly influenced by tear collection and the nature of the tear required. The technique of choice must be able to detect the highs and lows in tear concentrations and a broad range is required.

The main drawback in the application of all immunoassays, including immunodiffusion assay, is the requirement for purified antibodies against the analyte under investigation. Where analyte titres are demanded, a purified form of the protein is also required to create standards from which unknown concentrations can be derived.

The obvious example in the case of tear analysis is demonstrated by the lack of antibody or isolated protein for the so called tear specific prealbumin/the tear

lipocalins. At the initial stages the error was to assume its similarity with serum prealbumin in attempting to analyse its presence in tears. Figure 4.2. shows the reaction of a tear sample with minor stimulation (noted by the fact that tear flow rate was in excess of $5\mu lmin^{-1}$) against antibodies for the main tear proteins, with an antibody against human serum prealbumin employed. The absence of a line of reaction between the tear sample and anti-human serum prealbumin further proves the lack of homology between serum prealbumin and the tear lipocalin. With the only recent discovery of the true identity of the lipocalins, there is currently no commercially available antigen or antibody to the tear lipocalins, thus no investigation by immunodiffusion assay could be carried out.

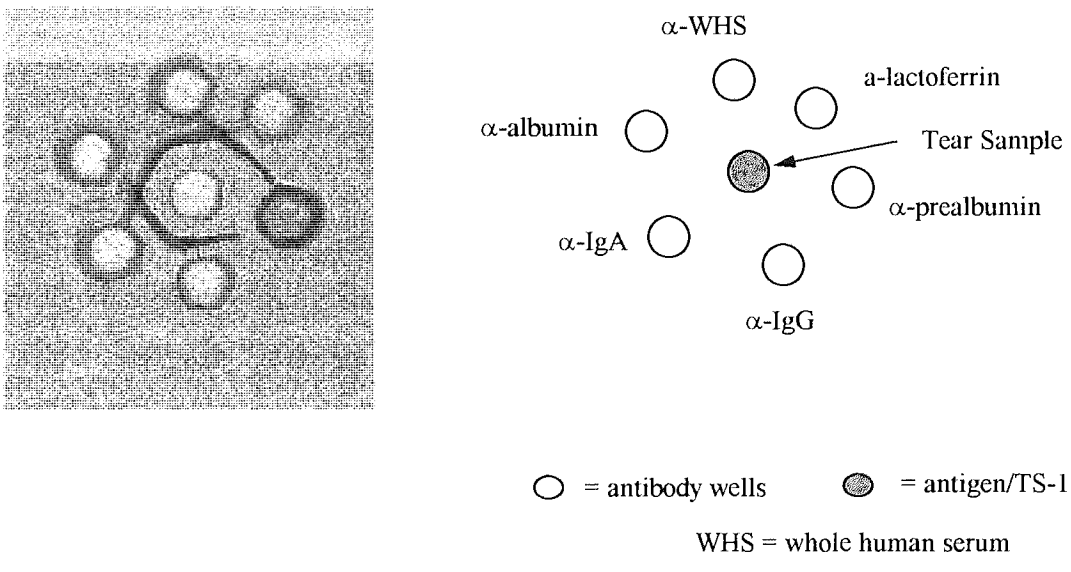


Figure 4.2. Ouchterlony of tear sample versus main tear protein antibodies.

This Ouchterlony resulted in a line of single reaction for all the antibodies (α) against the tear sample, with the exception of serum prealbumin antibody which showed no line of precipitation with the tear sample.

4.6.2.1. Immunodiffusion Assays in Tear Analysis

All the methodologies for the immunodiffusion assays were described in Chapter 2, detailing their principles and mode of use. The following section lists the assays individually with respect to their particular use in tear analysis and in later contact lens studies. Table 4.5., at the end of this section, summarises the assays and applications. Where necessary to enhance visualisation of the gel scans, embossed versions of each gel are shown in Appendix III.

Tear sample-1 was used extensively throughout all the assays in this research as a control and as a means of assessing and comparing the different analytical techniques. Where TS-1 was examined in one assay the result was or could be substantiated by another assay. Further, with regard to the perceived idea of the reality of the automatic stimulated tearers, through nervous reaction during sampling, these AST samples were run continuously against the normal sampled subjects to investigate this idea more thoroughly and to assess their overall significance in the patient population.

4.6.2.2. Lysozyme by Immunodiffusion

Ouchterlony was used as the pre-test for all new antibodies and antigen and was used to provide preliminary information on the performance of a particular protein/analyte. Initial experiments on lysozyme highlighted a poor performance of the antibody against the antigen, no line of precipitation was ever detected. A reaction of the antibody against tear samples, in particular TS-1, shown previously to have a high lysozyme content by SDS-PAGE, displayed no line of precipitation. Due to the fact that lysozyme is possibly the most studied, best understood protein in tears and the fact that there are numerous quantitative and qualitative lysozyme assays already in present use, it was decided not to spend time analysing it considering problems were evident on initial analysis.

4.6.3. Immunoelectrophoresis (IEP)

IEP is used to separate complex mixtures of proteins by means of an electrical field. The individual proteins can be identified by their distinctive electrophoretic movement. The complex mixture, that is tears, was originally analysed by IEP in 1979[10] where approximately sixty proteins were detected. It is an extremely useful detection technique, with the ability to test approximately seven samples on one 7.5cm x 10cm gel. The antigen wells required only a 2µl volume of tear sample.

The following gel was run on the Paragon electrophoresis system. The use of TBE (solution 14, Appendix I), as the gel buffer was found to be optimum as other buffers in the gel did not perform as efficiently. 45mls of barbital buffer was poured into each side of the tank and the gel was electrophoresised for 1 hour. The anti-human tear mixture listed in the gel was a simply a combination of antibodies against IgG, IgA, albumin, lactoferrin and lysozyme all loaded in equal volumes to the antibody trough. The wells in the plate below were placed centrally, but because of the nature of the proteins tested a well placed to the left of the plate would have allowed a greater separation due to the greater anodal movement of the analytes.

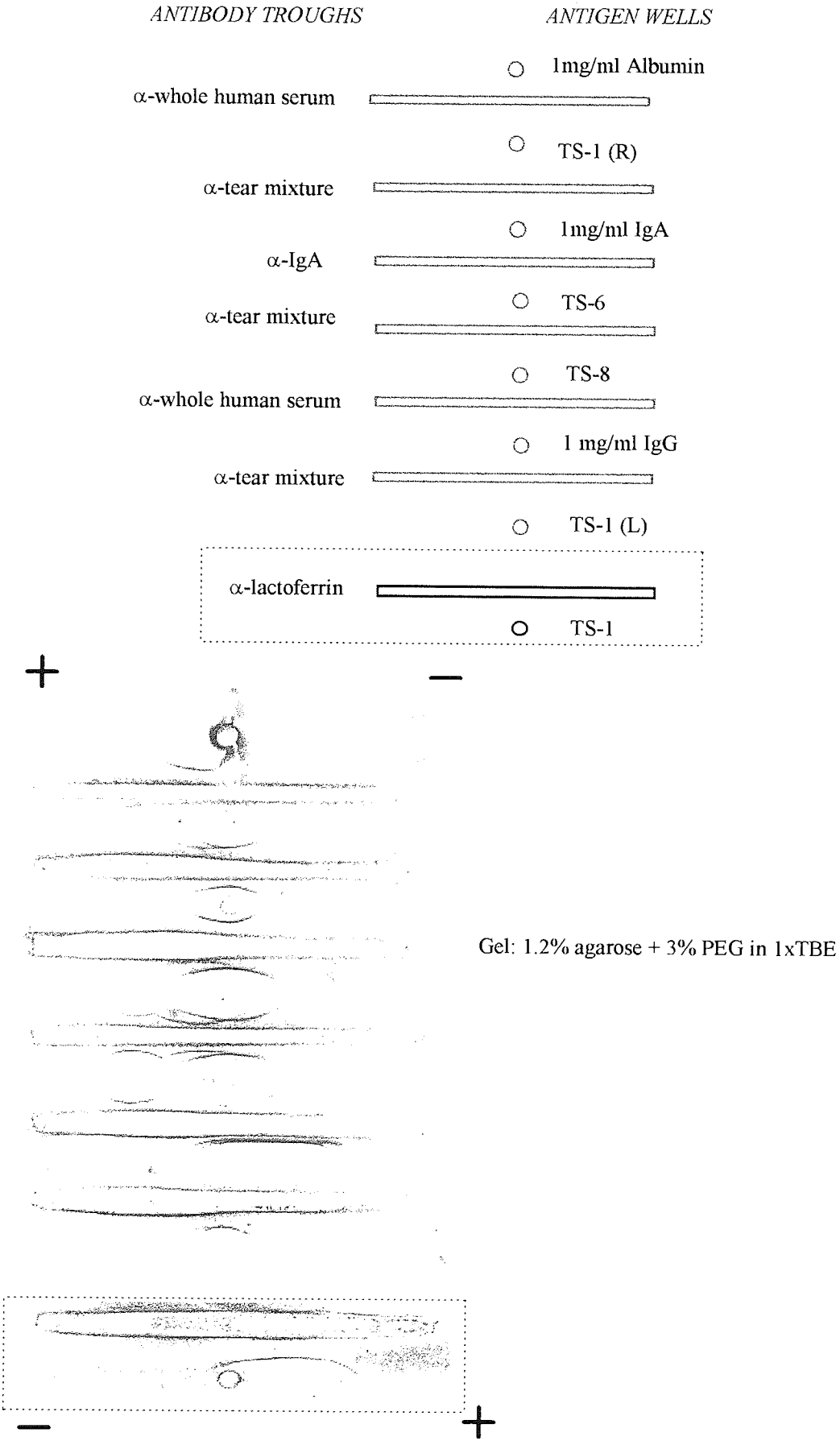


Figure 4.3. Immunoelectrophoresis of tear samples

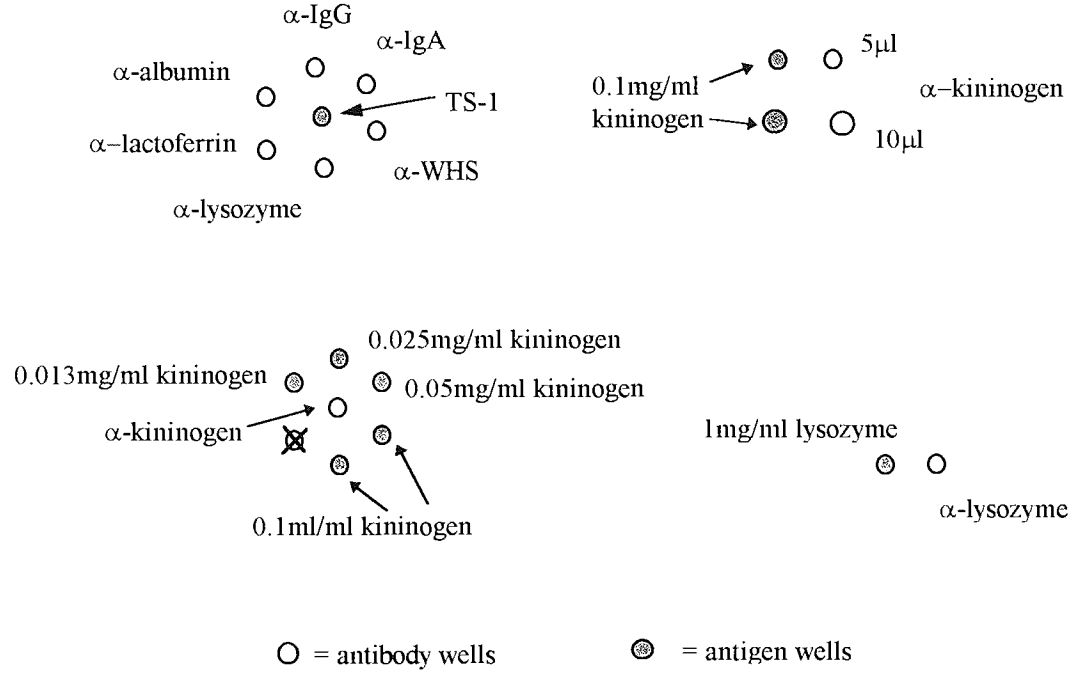
All the tear samples showed definite arcs of precipitate for IgA, lactoferrin and albumin. A trace IgG arc was seen on TS-8 against anti-human serum, but not against the anti-tear mixture. The other tear samples did not show an arc of precipitate with IgG. TS-1 was the only other tear sample run against the antiserum but the IgG arc was not evident. The lysozyme antibody was run in the mixture, but again failed to show an arc of identity with its tear component. IgA, IgG, albumin and lactoferrin standards were run to identify and demonstrate their movement by IEP.

The first point of note to be addressed is the comparison of this electrophoresis system over the above macro SDS-PAGE system. IEP is a non-reducing system that does not rely on the effective running of standards and markers, and that can identify many of the components of tears. The proteins by IEP can be sufficiently separated and identified through a prior understanding of the individual movement of the analyte(s). The main advantage of IEP was its requirement for only 2μl tear sample volumes; the 10μl tear sample required for SDS-PAGE would allow the tear sample to be run against five different antibodies or whole antibody systems. The macro SDS-PAGE system was found to be useful in the detection and quantification of lysozyme in tears and was deemed to be important in this area, due to the lack of precipitate seen with lysozyme by this and other immunodiffusion methods. The main drawback of IEP is the fact that the arcs of precipitate are only semi-quantitative, the intensity of the arc is related to the concentration of antigen present.

4.6.4. Ouchterlony

This is another detection method like IEP, but less sensitive due to the fact that diffusion between the antigen and antibodies takes place without the aid of electrophoresis. As stated earlier it was a useful technique in assessing various protein performances and highlighted the lack of reaction between the commercially available purified lysozyme and the antibody. The principle of Ouchterlony relies on the diffusion of the two reactants until a point of equivalence is reached and an immune precipitate is formed.

Well Key:



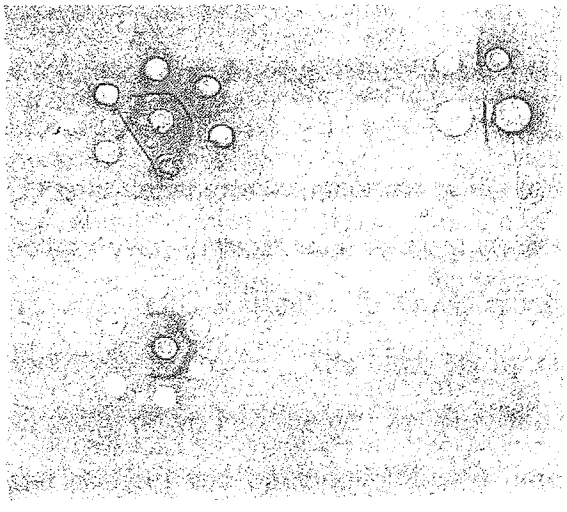


Figure 4.4. Ouchterlony of tear sample-1, serially diluted kininogen and assessment of commercially available lysozyme

This gel served and demonstrates two purposes. The first was the non-reaction of lysozyme, as discussed: no line of precipitation was evident against the purified form or against tears (against which the other proteins clearly showed line of identity). The second point was the reaction of kininogen; the plasma protein important in inflammation. Kininogen was not detectable below 0.025mg/ml by this method, but the reaction of identity was clear against its respective antibody and suggested its detection by immunodiffusion methods was feasible. Open eye tear samples were tested for kininogen by Ouchterlony but were not detectable as would be expected. The levels of kininogen, if present at all in basal tears would be beyond the limits of this assay.

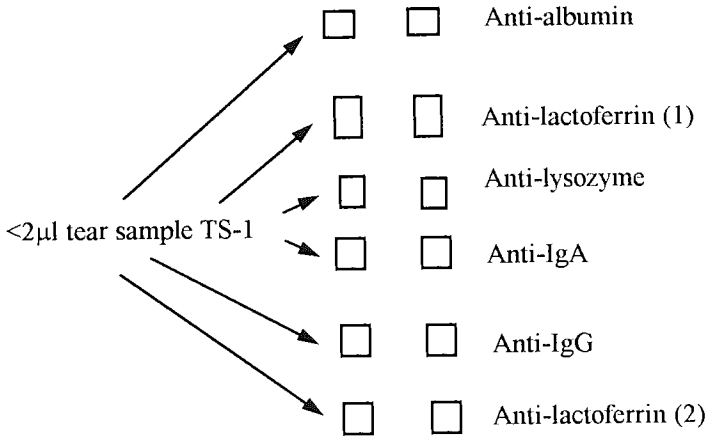
Ouchterlony presented an excellent visual pattern of the main proteins in tears with only a 2-4µl tear sample required. Although not a quantitative assay, it is clear from the line of precipitation indicating the immune complexes formed, that a low level of albumin is present in this tear sample. This volunteer's tear sample (TS-1) was tested by every method and the low levels of albumin found here was backed up by the other assays, for example, no albumin was detected by SDS-PAGE for TS-1. Thus, Ouchterlony was a beneficial, basis, assay which can be used to ascertain a tear profile, which can then be confirmed by other assays.

4.6.5. Counter Immunoelectrophoresis

Counter immunoelectrophoresis (CIE) involves an antigen and its respective antibody being driven towards each other in an electrical field. It is a very sensitive and rapid method. CIE is similar to Ouchterlony in that is it a detection method, but its sensitivity is greater. The basic set-up requires the diffusion of the antigen and the antibody down into the gel; the diffusion times were found to vary between samples and analytes and optimum diffusion times were defined. The diffused antigen and antibodies were then run on the Paragon electrophoresis system for 30-60 minutes, depending on analytes under investigation. Barbital buffer was used as the tank buffer and TBE was combined with the gel.

After electrophoresis most of the precipitation lines of identity were identifiable but to enhance visualisation Coomassie blue stain was used. The detection sensitivity is increased here over its use in SDS-PAGE, for example, due to the fact that the antigen and antibodies combine to form immune complexes in the gel and the stain detects a complex and not just the antigen. An example of CIE in tear analysis is shown in the figure below.

Well Key:



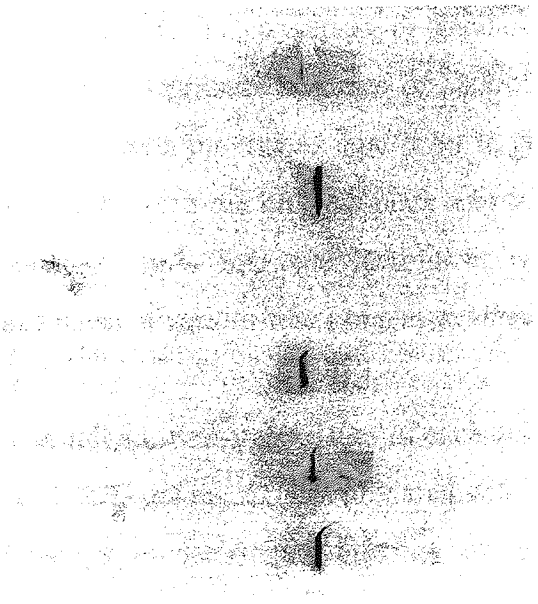


Figure 4.5. Counter immunoelectrophoresis of a normal unstimulated tear sample for the main tear proteins

The anti-human lysozyme antibody did not react with the lysozyme in tears. The second anti-lactoferrin antibody was run, simply to test this newly purchased antibody. 10µl of tears from the same TS-1 was used to characterise the profile of this volunteer. A less defined and intense line was seen for albumin, which was consistent with the results shown previously by Ouchterlony

CIE was optimised successfully for tear analysis of the main tear proteins. Kininogen, a protein of particular interest with respect to contact lens wear and currently undetected in tears, was assessed by this sensitive assay in tears. The relevance and interest in this protein was introduced in Chapter 1 and will be further discussed in Chapter 6. A CIE of kininogen in open eye basal tears was unable to detect this protein in a number of tear samples.

4.6.6. Radial Immunodiffusion (Mancini's Assay)

Radial immunodiffusion (RID) is an accurate quantitative method that was used successfully in the quantification of the main tear protein. It is already an important assay in the measurement of serum proteins. This method differs from those mentioned above in that one of the reactants, in this case the antibody, remains fixed and the other, the tear sample or standard, is allowed to move and complexes with the antibody. Optimum volumes of antibody were predetermined for each analyte and mixed in with the molten agar prior to pouring the gel. Sets of known concentration standards were run on each plate simultaneously in order to calculate the unknowns. Albumin, IgA, IgG and lactoferrin were routinely measured by this assay and examples of various tear samples examined are shown below.

The individual characteristics of each gel presented are listed with the respective plate. Each RID gel matrix was prepared with 1.2% agarose and 3% polyethylene glycol, to which a predetermined quantity of respective antibody was added. Each plate required the addition of specific antibody volumes to the gel, in order to keep the PEG to a 3% concentration, a proportion volume was also added. For example, when 100µl of antibody was added, 10µl of a 33% PEG solution was also added.

4.6.7. Rocket Electrophoresis (Laurell's Assay)

Rocket electrophoresis (RE) functions on the same principle as RID, that is, one reactant is fixed with the other free to diffuse. The difference between the assays lies in the fact that RE is aided by the addition of an electrical field to the system. This serves to speed up the reaction, but also increases the sensitivity. The important disadvantage of RE is the necessity for the antigen under scrutiny to have anodal movement. Immunoglobulins possess only a weak negative charge which prevents their electrophoretic mobility in this system.

RE was performed also on the Paragon system using barbital buffer in the tank. A 1.2% agarose and 3% PEG in 1xTBE gel composition was proven optimum for the reliable an clear running of the RE gels. The individual parameters of each gel are listed as appropriate with each plate presented. Albumin was the only protein run by RE; it was chosen as a marker of the integrity of the blood/tear barrier in response to and in conjunction with the work done on the closed eye environment. RE was the method of choice to assess albumin concentrations in open versus closed eye tear samples, details of which as are presented in Chapter 5. The open eye albumin RE gels are shown below.

These final two immunodiffusion assays were employed in the quantitative analysis of the main tear proteins, the concentration results of which are shown in detail below.

4.6.8. A Summary of the Applications of the Immunodiffusion Assays

The table below highlights the principle assays employed in the analysis of tears and in contact lens studies.

Assay	Primary Application	Proteins Analysed
Ouchterlony	Detection	Albumin, IgA, IgG, Lactoferrin, Kininogen‡
RID	Quantification	Albumin, IgA, IgG, Lactoferrin
Laurell's Assay	Quantification	Albumin
IEP	Detection	Main tear proteins
CIE	Detection	Albumin, IgA, IgG, Lactoferrin, Kininogen

Table 4.5. Immunodiffusion assays in tear and contact lens studies. ‡ Kininogen not detected in open eye basal tears.

4.7. Calculation of Quantitative Immunodiffusion Results

As stated above, when quantitative gels were performed, calibration standards were run simultaneously in the gels in order to calculate the titres of the unknowns in tears. For each gel, a calibration curve was derived from a set of serially diluted standards, from which, the unknown antigen concentrations were calculated. The relationship between ring diameter or length of arc and antigen concentration is described by the line/curve drawn from the known amounts of antigen. The equation of each curve is listed with its respective graph, which represents the logarithm of the antigen concentration in proportion to the diameter of the ring.

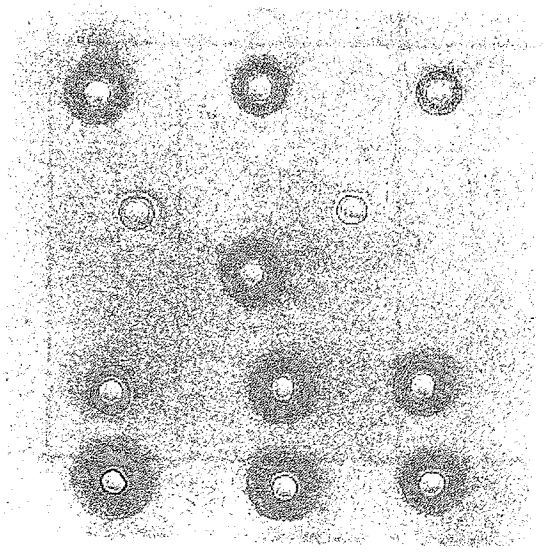
$$y = mLn(x) + c$$

From this equation the value of x (analyte concentration) can be calculated with y determining the ring diameter value, m is the slope of the line and c signifies the intercept with ordinate.

$$Ln(x) = \frac{y-c}{m}$$

4.7.1. Measurement of IgA Concentration in Open Eye Tear Samples by Radial Immunodiffusion

The following gel typifies the results found by numerous RID runs in the quantification of IgA in tears. The main points of interest in this gel are the comparisons drawn between the concentrations resultant between AST and non-AST samples and the differences found between the right (R) and left (L) eye of a single subject. 50µl of anti-human IgA was incorporated into the gel matrix. Diffusion was allowed to proceed for 24 hours before the reaction was stopped.



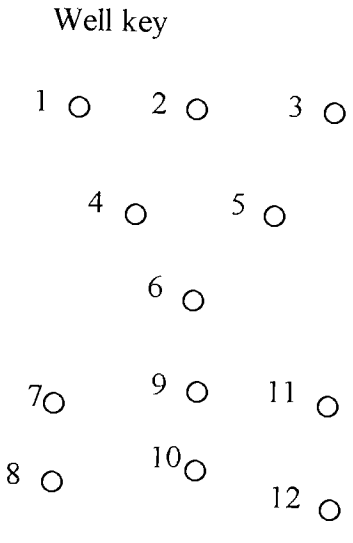


Figure 4.6. Analysis of open eye tear samples for IgA by RID. 1-5 = IgA standards, 6 = TS-1, 7 = TS-2, 8 = TS-3, 9 = TS-4 (R), 10 = TS-4 (L), 11 = TS-6 (R), 12 = TS-6 (L)

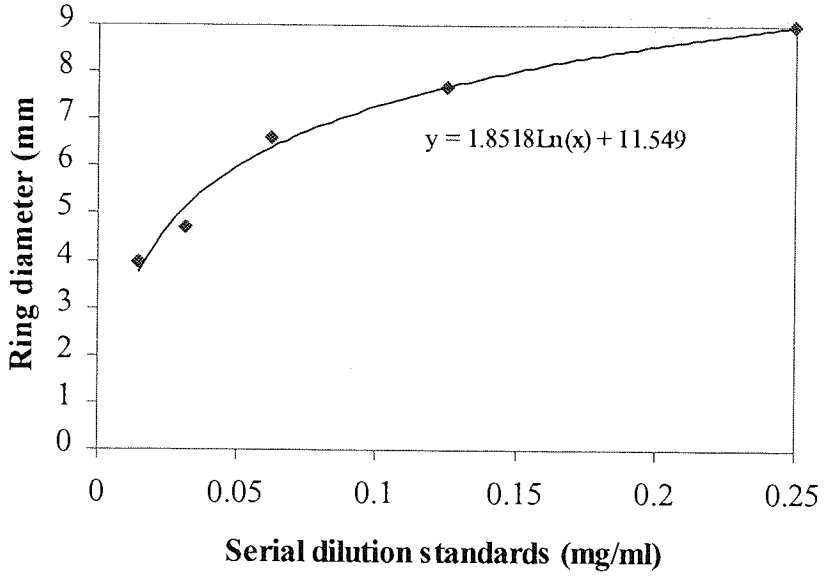


Figure 4.7. IgA calibration curve derived by RID

IgA Standards			IgA Unknowns		
Name	Concentration (mg/ml)	Diameter (mm)	Tear Sample	Diameter (mm)	Concentration (mg/ml)
1	0.25	9.0	TS-1	8.3	0.17
2	0.125	7.7	TS-2 (AST)	5.6	0.04
3	0.063	6.6	TS-3	10.4	0.52
4	0.031	4.7	TS-4 (R)	8.8	0.22
5	0.015	4.0	TS-4 (L)	9.4	0.3
			TS-6 (R)	7.7	0.12
			TS-6 (L)	8.4	0.18

Table 4.6. Results of the calculation of IgA concentration in open eye tear samples

Sample Number	Mean	StDev (±)
7	0.22	0.154

Table 4.7. Mean IgA concentrations in seven tear samples

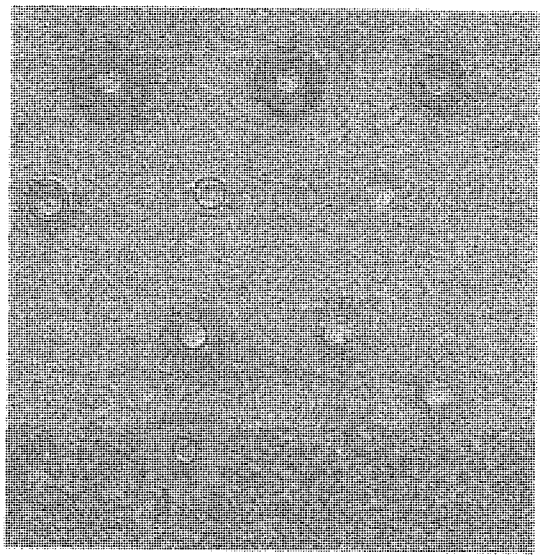
The average concentration of IgA in the open eye has been quoted as 0.29mg/ml[12] with a range between 0.1-0.6mg/ml.[26, 36] The results shown here agree with these figure albeit with a small sample number, but it would suggest the efficiency of this assay in the calculation of IgA levels in tears. It is very interesting to note, in such a small sample number, the range of values obtained. The lowest IgA concentration value (TS-2) can be explained by the fact that this tear sample was designated an AST sample.

In Chapter 2, two tear samples were compared by CIE, a normal tear sample (TS-1) and an AST sample (TS-2) and the result highlighted a reduction in concentration of all the proteins tested in the AST sample in comparison against the non-stimulated TS-1 sample. Both these tear samples were run on this plate and the same outcome was observed. The AST sample (TS-2) displayed a degree of dilution five time smaller than the mean.

Additionally, a right and left eye tear sample was taken from both TS-4 and TS-6 volunteers. The differences between the left and right eyes in two samples was quite substantial, as has been highlighted in many previous reports in the analysis of a variety of tear components.

4.7.2. Measurement of Lactoferrin Concentration by Radial Immunodiffusion in Open Eye Tear Samples

This lactoferrin gel investigated the tear samples of five subjects, including two who were designated as presenting AST samples. 100µl of anti-human lactoferrin was added to the molten gel. Diffusion was stopped after 24 hours.



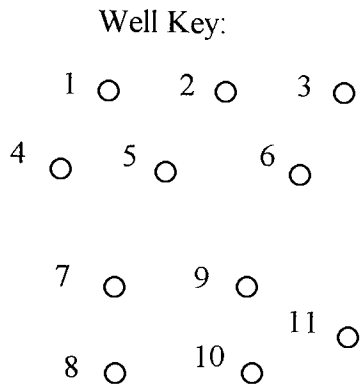


Figure 4.8. Analysis of open eye tear samples for lactoferrin by RID. 1-5 = lactoferrin standards, 6 = empty, 7 = TS-2, 8 = TS-1, 9 = TS-5, 10 = TS-4, 11 = TS-9

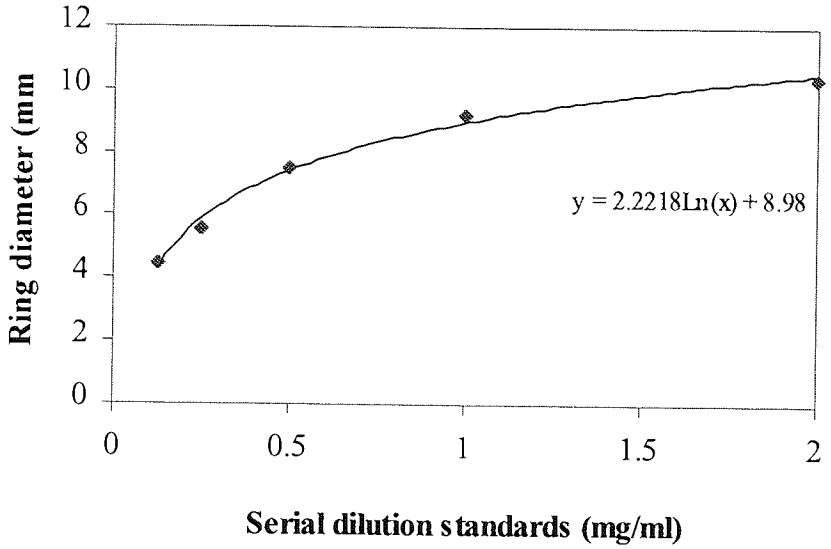


Figure 4.9. Lactoferrin open eye tear samples calibration curve derived by RID

Lactoferrin Standards			Lactoferrin Unknowns		
Name	Concentration (mg/ml)	Diameter (mm)	Tear Sample	Diameter (mm)	Concentration (mg/ml)
1	2	10.4	TS-2 (AST)	8.7	0.74
2	1	9.2	TS-1	10.3	1.89
3	0.5	7.5	TS-5 (AST)	8.8	0.92
4	0.25	5.6	TS-4	10.2	1.7
5	0.125	4.9	TS-9	7.9	0.61

Table 4.8. Results of the calculation of lactoferrin concentration in open eye tear samples

Sample Number	Mean	StDev (±)
5	1.172	0.583

Table 4.9. Mean lactoferrin concentrations in five tear samples

The mean lactoferrin levels that have been cited in various papers have been quoted at approximately 1.4mg/ml[17] but with the values mainly occurring between 1-3mg/ml.[18] These workers also discovered a few patients presenting values as high as 6.3mg/ml. The 1.4mg/ml mean measured by Janssen *et al* was also performed by RID. The mean calculated in the five samples on this plate adhere to these figures. In keeping with the trends displayed in previous assays the AST samples demonstrated lower concentrations than seen in the non-stimulated samples.

4.7.2. Measurement of IgG Concentration by Radial Immunodiffusion in Open Eye Tear Samples: Plate-1

100µl of anti-human IgG was added to the gel and diffusion time allowed was 24 hours. A left and right eye tear sample was analysed and compared against the AST sample on this plate. The TS-1 versus TS-2 comparison was run as a continuation of the on-going analysis of the AST versus normal tear sample analysis that was identified by CIE in Chapter 2.

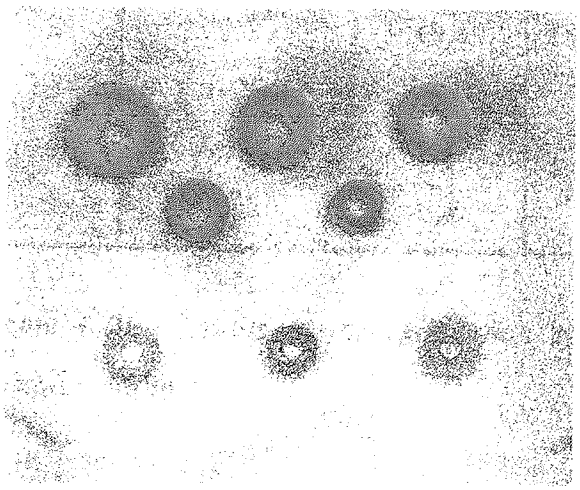


Figure 4.10. Analysis of open eye tear samples for IgG by RID. 1-5 = IgG standards, 6 = TS-1(L), 7 = TS-2, 8 = TS-1(R): Plate-1

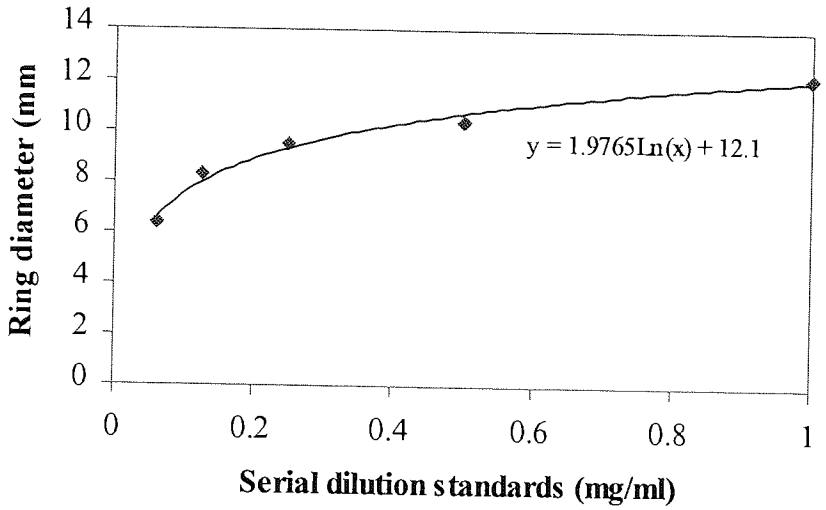


Figure 4.11. IgG open eye tear samples calibration curve derived by RID: Plate-1

IgG Standards			IgG Unknowns		
Name	Concentration (mg/ml)	Diameter (mm)	Tear Sample	Diameter (mm)	Concentration (mg/ml)
1	1	12.2	TS-1(L)	7.6	0.10
2	0.5	10.4	TS-2 (AST)	6.7	0.07
3	0.25	9.5	TS-1(R)	8.8	0.18
4	0.125	8.3			
5	0.0625	6.4			

Table 4.10. Results of the calculation of IgG concentration in open eye tear samples: Plate-1

4.7.2.1. Measurement of IgG Concentration by Radial Immunodiffusion in Open Eye Tear Samples: Plate 2

100µl of anti-human IgG was added to the gel. Diffusion was stopped after 48 hours. Two different AST samples were run and compared against two normal samples.

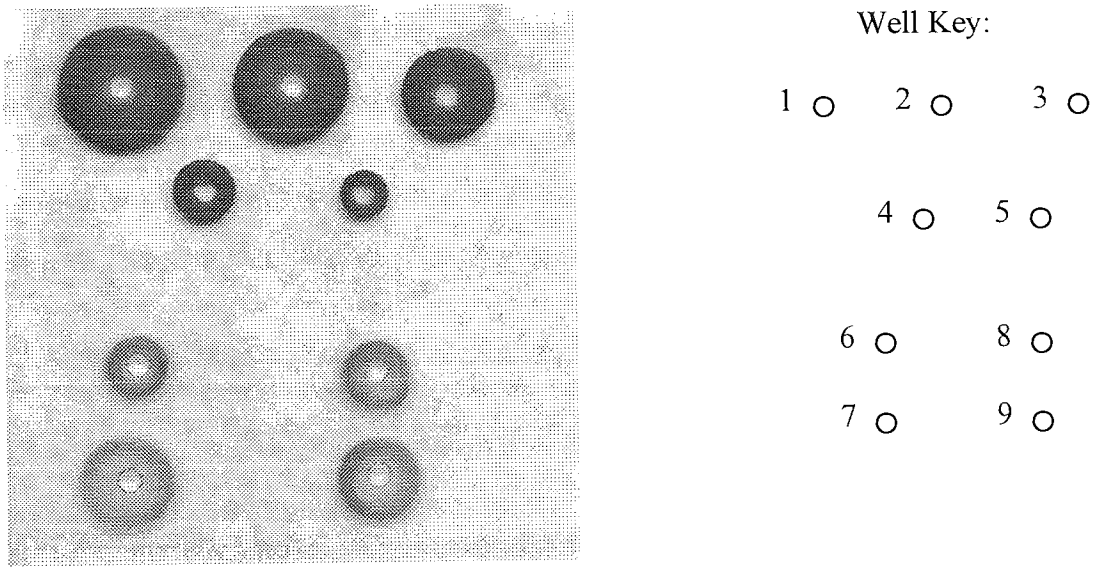


Figure 4.12. Analysis of open eye tear samples for IgG by RID. 1-5 = IgG standards, 6 = TS-5, 7 = TS-6, 8 = TS-7, 9 = TS-8: Plate-2

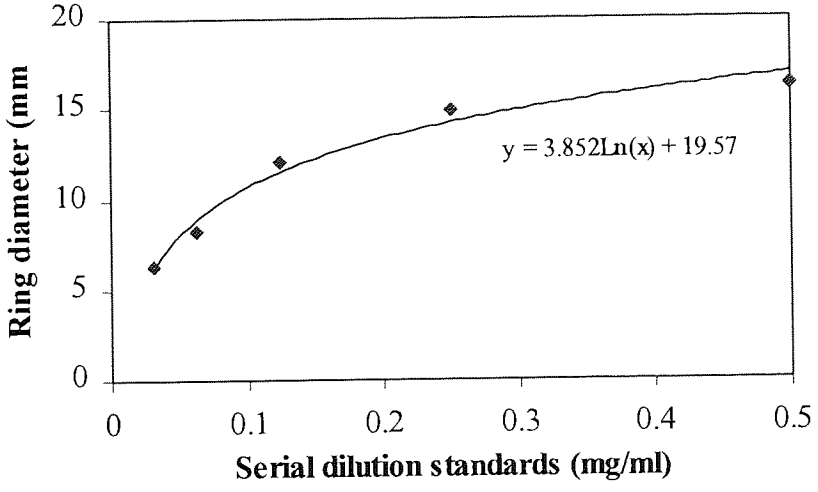


Figure 4.13. IgG open eye tear samples calibration curve derived by RID: Plate-2

IgG Standards			IgG Unknowns		
Name	Concentration (mg/ml)	Diameter (mm)	Tear Sample	Diameter (mm)	Concentration (mg/ml)
1	0.5	16.3	TS-5 (AST)	7.8	0.05
2	0.25	14.9	TS-6	12.3	0.15
3	0.125	12.1	TS-7 (AST)	9.0	0.06
4	0.0625	8.2	TS-8	10.9	0.11
5	0.03125	6.3			

Table 4.11. Results of the calculation of IgG concentration in open eye tear samples: Plate-2

Sample Number	Mean	StDev (±)
7	0.10	0.048

Table 4.12. Mean IgG concentrations in seven tear samples

The average concentration of IgG in tears has been quoted at 0.13mg/ml,[12] but this value has been shown to vary greatly depending on the mode of tear collection, method of analysis and tear state. The AST samples again illustrated the dilution factor that occurs affecting the concentration. Additionally a left and right eye variation was observed.

4.7.3. Measurement of Albumin Concentration by Rocket Electrophoresis in Open Eye Tear Samples

50µl of anti-human albumin was added to the molten agarose before pouring. Three open eye tear samples were run, TS-1 which signified the control and consistency among all gels and two other normal samples. No AST samples were run as previous assays displayed no detectable albumin levels in these tears. Electrophoresis time was one hour.

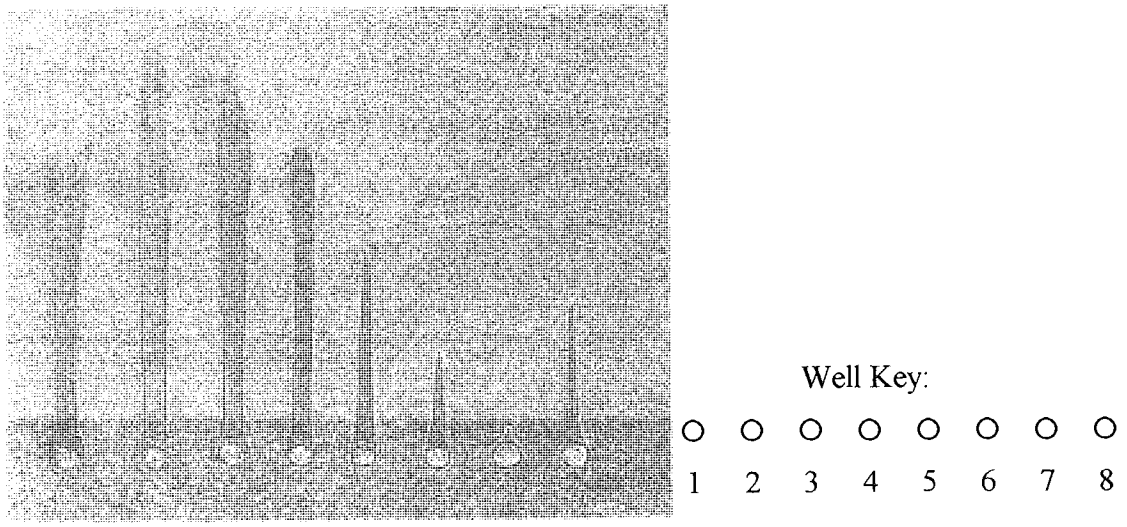


Figure 4.14. Analysis of open eye tear samples for albumin by rocket electrophoresis. 1-5 = Albumin standards, 6 = TS-9, 7 = TS-1, 8 = TS-10

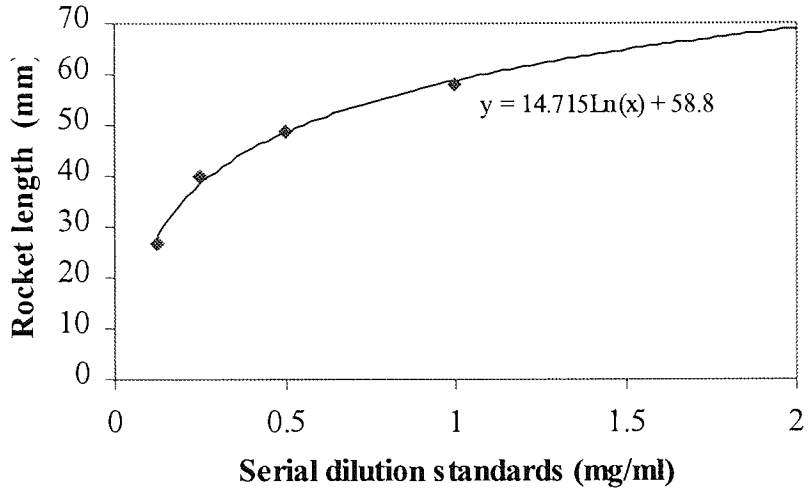


Figure 4.15. Albumin open eye tear samples calibration curve derived by rocket electrophoresis

Albumin Standards			Albumin Unknowns		
Name	Concentration (mg/ml)	Rocket Length (mm)	Tear Sample	Rocket Length (mm)	Concentration (mg/ml)
1	2	---	TS-9	14	0.05
2	1	58	TS-1	3.5	N/A
3	0.5	49	TS-10	20	0.07
4	0.25	40			
5	0.125	27			

Table 4.13. Results of calculation of albumin concentration in open eye tear samples

The albumin levels described here were much lower as compared with the 1.3mg/ml levels quoted as the mean. Other plates (not shown here) and those presented in Chapter 5 substantiate these results that display low albumin concentrations detected in open eye tear samples. The Ouchterlony shown in Figure 4.4. was fundamental in

presenting the initial findings of low albumin levels in TS-1. As stated above, previous assays with AST samples could not quantify the albumin levels even with lower antibody volumes and lower standards only millimetre movements were observed that could not be calculated accurately.

4.8. Conclusions

- The immunodiffusion assays were useful in the detection and quantification of main tear proteins; the sensitivity levels were suitable for the proteins under analysis. Ouchterlony, IEP and CIE were utilised in tear protein detection and RE and RID were applied in tear protein quantification.

- The immunodiffusion assays combine clear, reliable and reproducible results, with ease of performance.

- The following mean values of proteins were observed.

Protein	Mean Concentration (mg/ml)
IgA	0.22
IgG	0.10
Lactoferrin	1.172
Albumin	0.06

Table 4.14. Mean concentrations of IgA, IgG, lactoferrin, and albumin in open eye tears

- The results highlighted the left and right eye differences that occur.

- The importance of recognising the presence of AST subjects in a population was addressed, noting their property for presenting diluted levels of proteins.

- Kininogen, an important inflammatory mediator, was not detected in open eye tears under these condition.

- The assays were developed for further application in the analysis of other tear states, e.g., closed eye tears, and for the study of tears and their interactions with contact lenses.

Chapter 5

Application of Immunodiffusion Assays in Closed Eye Tear Studies

5.1. Aim

At the outset of this section of work, the aim of this of chapter was to continue the pioneering work performed, mainly by a group of researchers at the University of New South Wales, Sydney, Australia, and their collaborators, on the closed eye environment (CEE). The CEE has been defined by this group as a state in which the ocular surface sustains an environment of sub-clinical inflammation, which differs greatly in protein composition from the open eye basal tear. As only small volumes are available (less than those evident in the open eye), the aim was to take advantage of the small volume requirement (1-3μl) of the immunodiffusion assays, in the analysis of closed eye tear samples. As the work progressed the difficulties in this area of research became very apparent. The main problem encountered derived from the fact that it is virtually impossible to extract a true closed eye tear sample, relying on 'on-waking' samples which creates collection variables.

5.2. Introduction

Recently the tear film has been shown to be biphasic. A diurnal cycle between the open eye and the closed eye has been proposed,[43,49,83,117] with a change in tear composition, source and, possibly function to some extent, evident. The open eye displays a lacrimal gland derived secretion rich in lysozyme, lactoferrin and tear lipocalin, with eye closure moving towards a more plasma derived secretion rich in a variety of proteins including, plasmin, complement proteins, vitronectin, albumin, and with high levels of sIgA. It is an environment that has given rise to the definition of a state of sub-clinical inflammation.[43,49,65,69,83,117]

The following is a list of the differences identified in the closed eye:

- Decrease in oxygen availability
- Temperature increase
- Tear secretion decrease
- Loss of blink/flushing mechanics

- Reduction in influx and outflow of tears
- Change in nervous stimulus
- Increase in vascular permeability
- Decrease in lacrimal gland secretion

Resultant consequences:

- Decease in lacrimal derived proteins
- Increase in plasma derived proteins
- Overall shift in tear composition

The dramatic change in protein composition from the open to the closed eye, due to a shift from a dynamic, mainly lacrimal gland derived tear, to a sIgA rich layer, stagnant in nature, is summarised in the table below.

Analyte	Protein Shift
Total protein	Gradual increase
Albumin	Gradual increase
sIgA	Gradual increase
IgG	Gradual increase
Vitronectin	Gradual increase
Complement proteins	Gradual increase
C3c conversion	Gradual increase
Fibronectin	Gradual increase
Plasmin	Rapid increase
Lysozyme	Decrease
Tear lipocalin/TPA	Decrease
Lactoferrin	Decrease

Table 5.1. Effects of overnight eye closure on tear protein composition

The main physical changes that are evident in the closed eye, as mentioned above, involves a lack of blink mechanics with a dramatic reduction in tear influx and outflow. This results in an almost stagnant environment, which possibly must be compensated through other mechanisms to maintain ocular stability and health and may be achieved by the change in tear composition.

The mechanisms of change between the open eye environment (OEE) and the CEE are not known, but the effects are seen in the dramatic change in tear composition. Whether the composition shift is, in conjunction with, or as a consequence of the physical changes incurred in the CEE remains to be seen. However, it is this difference in the tear make up, presenting a sub-clinical state, which is of particular interest and which may function to protect the eye from potential pathogens, in this almost stagnant environment which lacks the tear blink and flush properties.

5.2.1. Problems in the Collection of Closed Eye Tear Samples

There were many factors to be taken into consideration before an attempt could be made to analyse closed eye tears. The first point addressed was to outline the definition of an achievable closed eye tear sample. The main problem in the extraction of closed eye tear samples is an obvious one, i.e. a sample can not be taken until the eye is open and the speed with which the eye reverts to an open eye status is largely undetermined. A tear sample taken 10 minutes post eye opening demonstrates a definite open eye state, the 0-10 minutes time gap remains unexplored.

Possibly the best methodology, which was attempted by the Australian group, involved the requirement for subjects to sleep in a controlled environment and to be then woken up by a technician who would, immediately on their waking, take a tear sample. Unfortunately, this is an expensive and generally impractical proposal and has not been utilised to any great extent.

The majority of all the research on closed eye tears has been done by the Australian group, and the main tear collection method that they performed involved a method of self-collection. The volunteer/patient was required, on waking, to keep their eyes

shut, reach for a glass microcapillary and mirror and immediately on eye opening take a tear sample. In some cases samples were taken from one eye and then the other, the second eye closed while taking the first sample. The time between waking and taking the tear sample is a critical period, and the question needs to be asked how the dynamics of the tear state are changed in these few minutes. The state of the tears taken by this method of extraction must be dependant on the collection time and efficiency. This collection may affect the outcome of results, due to the certain lack of control and extraction variability.

The significantly lower tear volumes available on eye opening also create problems. From a personal observation, on immediate eye opening the eye is quite dry and no extractable tear volume is available. To take an adequate tear sample volume immediately would possibly take some form of stimulation. In fact extracting 1μl of unstimulated open eye basal tears takes approximately 1 minute and to take samples over this mark (therefore longer than 1 minute) must question the validity of defining and identifying this tear sample as a representation of the closed eye environment. The longer it took to collect the tears, the closer the tear must revert to and resemble the open eye tear.

5.2.2. Defining the Closed Eye Tear Sample

The physical and mechanical changes are very important in defining and characterising the CEE, but nervous stimulus is a factor in the closed eye which can not be dismissed. Consciousness, or the lack of, by some patients during the closed eye time period or for a duration prior to sampling must bear some relevance on the nature of the resultant tears obtained. This point was addressed by the Australian group, who examined eye closure versus a loss of consciousness (sleep).[118] They compared the results of a group of patients over a set period of eye closure while awake and then over the same period after sleep. This experiment was also used to assess the definition of closed eye versus sleep, acknowledging the fact that the mechanisms of change between the open and closed eye environment are unknown.

Using IgA as the marker, no significant difference was found between the closed eye with sleep and the closed eye without sleep, which would suggest that eye closure rather than a loss of consciousness is the key. The problem again in the determination of this, consciousness/lack of consciousness, conclusion lies in the method of the sample collection, and unfortunately no collection method was described in the abstract. If the usual self-collection method was performed, then those tear samples collected as 'sleep' samples would inevitably involve a period of time when the subject was conscious, the effect of which, may equate both subject groups.

More recently one paper resorted to referring to the tear samples taken on waking as, "samples representative of the fluid present under the lid after overnight eye closure."[119] This representation of closed eye tear fluid remains the closest sample available presently and is probably the most accurate description and definition of the tear state obtained, as opposed to employing the slightly inaccurate, closed eye term.

However, for simplification in this chapter, the tear samples obtained on waking were referred to as closed eye samples, bearing in mind the ambiguity of this term.

5.3. Assessment of Closed Eye Tear Samples

The aim of the following set of experiments was to assess the application of the immunodiffusion assays in the analysis of closed eye tears and to make use of the low volume prerequisite of these assays. No tear samples had to be pooled which meant there was no requirement for both left and right eye collection, thus eliminating this time variable on waking. Radial immunodiffusion was employed to investigate IgA and IgG, and rocket immunoelectrophoresis was used to study albumin in closed versus open eye tears.

5.3.1. Closed Eye Tear Collection

The method of self-collection originally described by the Australian group was the only method feasible in this study. Volunteers from the laboratory were asked to take glass microcapillary tubes home and to take a sample at night before going to bed and again in the morning immediately on waking. All the volunteers were non-contact lens wearers. The problems in sampling erroneous tear samples were explained to each volunteer, for example, noting a delay between opening the eye and taking the tear sample. The microcapillary tubes were sealed with foil and sticky tape and taken into the laboratory as soon as possible. In some cases the samples were kept in the volunteer's freezer prior to analysing them in the laboratory. No more than 3μl of an individual sample was ever taken. Other closed eye tear samples were kindly taken and supplied by Contact Lens Research Consultancy, London, and the same collection parameters were adhered to. Unfortunately, due to the nature of the tear collection set-up, extensive control could not be achieved and some lack of uniformity was unavoidable. The collection time period was inevitably going to vary to a small degree.

As some volunteers found that they were unable to collect any tears on waking, this created a section of people who could not be studied, which ultimately lead to results possibly not representative of the population as a whole. As a natural progression from the already notable dry eye environment that is created during sleep, it would follow that dry eye subjects would create further problems in closed eye tear collection. The fact that samples are relatively difficult to obtain from these subjects further limits the population from which the closed eye environment can be investigated, thus biasing and narrowing the range of results. The time period required to take 1μl of open eye unstimulated tears is approximately 1 minute for non-dry eye subjects and increases for dry eye subjects. This collection time is extended when taking a closed eye sample as the eye is initially quite dry, consequently in dry eye patients this problem may be exaggerated.

5.3.1.1. Personal Observation

From a personal viewpoint, the self-sampling of tears on waking was not possible, with a dry tear state constantly presented. The period of time required to be able to collect an extractable quantity of tears was in excess of 10 minutes, which would then have represented an open eye tear sample. The control TS-1, therefore could not be used in these closed eye tear studies and only open eye samples are given as a comparison and control.

5.3.1.2. Sample Supply

Allowing for the reality that there were problems encountered in the definition and reproducibility of collection of closed eye tears, it was the fact that the eye presents an almost dry eye state on waking for most people, that proved the foremost hinderance in obtaining tear samples. This fact alone limited the availability of tears and consequently only a minimal number of assays could be performed. As a result no samples were available for kininogen analysis in the closed eye. Alternate schools of action were taken as detailed later.

5.3.2. Application of Immunodiffusion Assays

To analyse the CEE, quantitative techniques were required and thus only RID and RE were utilised. RID was used for IgA and IgG estimation and RE was used for albumin estimation. The RID and RE experimental parameters were optimised and developed in the previous chapter for the individual proteins. Obviously due to the limited sample supply, trial and development of these assays on CEE samples could not be performed. This highlights the importance of the previous open eye investigations. Consequently, these predetermined parameters including diffusion times, antibody volumes and standard ranges were applied in this investigation of the CEE. The lack of closed eye samples obtained and supplied limited the research to these three proteins. Further work on other tear protein components was desired but was curtailed due to this insufficient supply.

5.4. Results

The calculation of the mg/ml of the individual tear protein concentrations were performed as described in the previous chapter according to each calibration curve derived from a set of known standards run concurrently on each plate

5.4.1. Comparison of IgA Concentrations in Open versus Closed Eye Tear Samples by Radial Immunodiffusion: Plate-1

A more thorough investigation of the IgA protein was performed in order to instil a degree of consistency in the estimation of the closed eye protein concentrations and to provide a baseline on which other results could be compared. IgA was chosen due to the nature of the CEE which has been described as being a stagnant layer rich in IgA. Two plates of IgA quantification were thus run. 50µl of anti-human IgA was added to the molten gel before pouring. The standards and unknowns were allowed to diffuse for 24 hours before the reaction was stopped. Only normal subjects were used, and no AST samples were added to the plate.

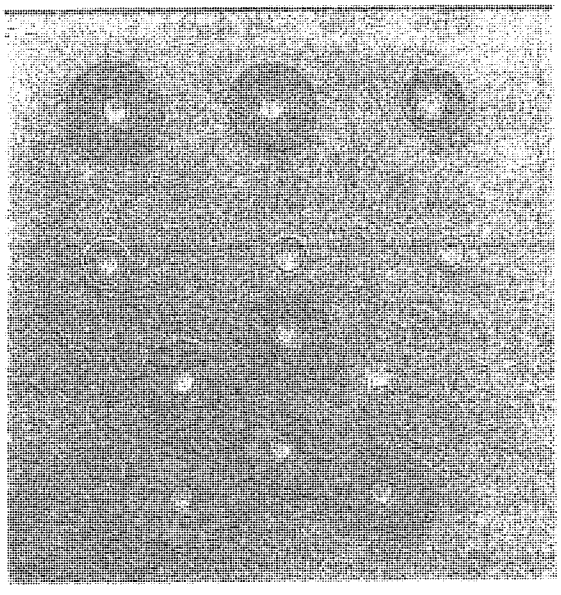


Figure 5.1. Analysis of closed versus open eye tear samples for IgA by RID: Plate-1. 1-5 = IgA standards, 6 = empty, 7 = TS-11 (0), 8 = TS-12 (0), 9 = TS-12 (C), 10 = TS-13 (O), 11 = TS-13 (C), 12 = empty.

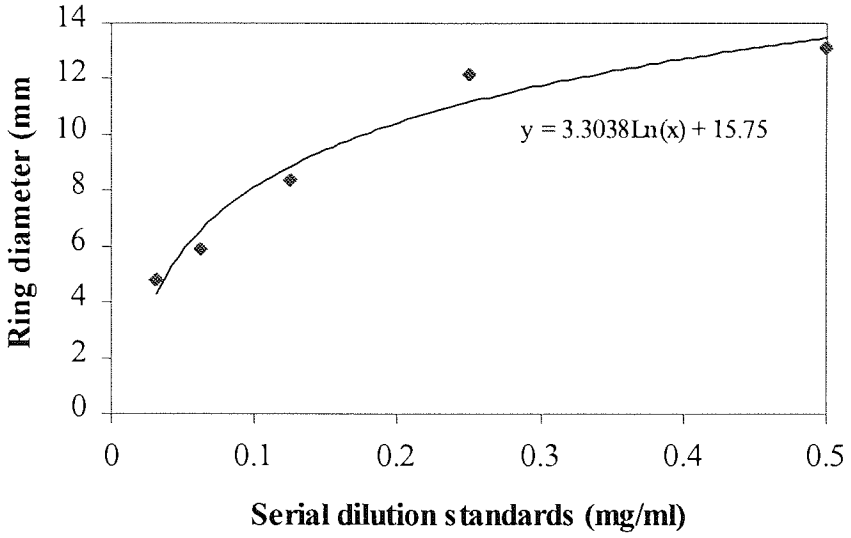


Figure 5.2. IgA calibration curve derived by RID: Plate-1

IgA Standards			IgA Unknowns		
Name	Concentration (mg/ml)	Diameter (mm)	Tear Sample	Diameter (mm)	Concentration (mg/ml)
1	0.5	13.1	TS-11 (O)	11.6	0.28
2	0.25	12.0	TS-12 (O)	10.9	0.23
3	0.125	8.4	TS-12 (C)	14.2	0.63
4	0.0625	5.9	TS-13 (O)	10.5	0.20
5	0.03125	4.8	TS-13 (C)	12.4	0.36

Table 5.2. Results of the calculation of IgA concentration in open versus closed eye tear samples: Plate-1

TS-11 was used as a control and demonstrated levels similar to those found in the open eye as estimated in the previous chapter. The other two patients demonstrated a clear increase in IgA levels going from the open eye to the closed eye tear samples. These results substantiated the previous reports of the increase in IgA levels overnight and verified the use and value of RID in the analysis of comparative IgA concentrations.

5.4.2. Comparison of IgA Concentrations in Open versus Closed Eye Tear Samples by Radial Immunodiffusion: Plate-2

A second IgA plate was carried out to corroborate the results of the previous assay. The experimental conditions of this assay were as described for Plate-1. No AST samples were employed.

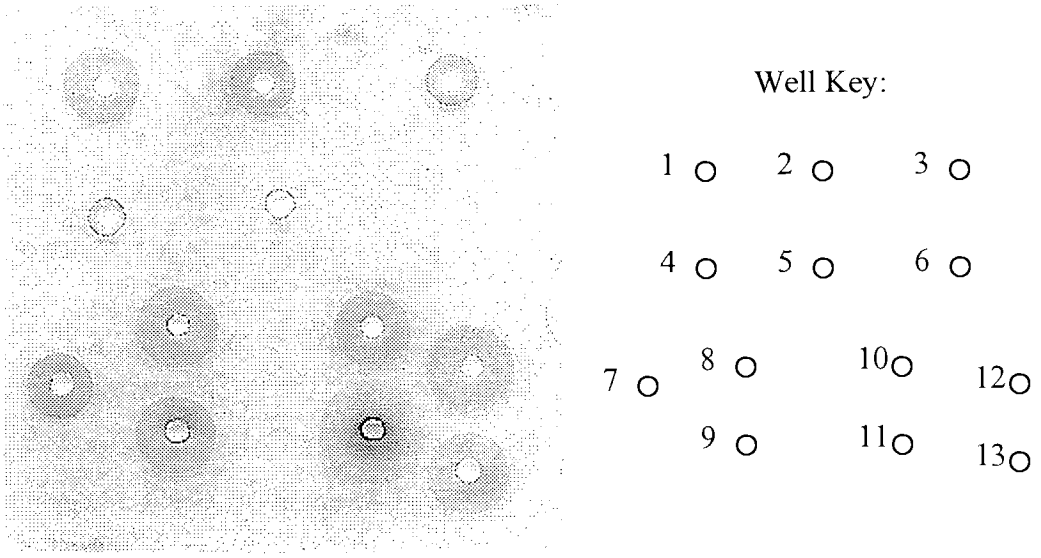


Figure 5.3. Analysis of closed versus open eye tear samples for IgA by RID: Plate-2. 1-5 = IgA standards, 6 = empty, 7 = TS-1 (0), 8 = TS-14 (C), 9 = TS-14 (O), 10 = TS-4 (O), 11 = TS-4 (C), 12 = TS-15 (C), 13 = TS-15 (O)

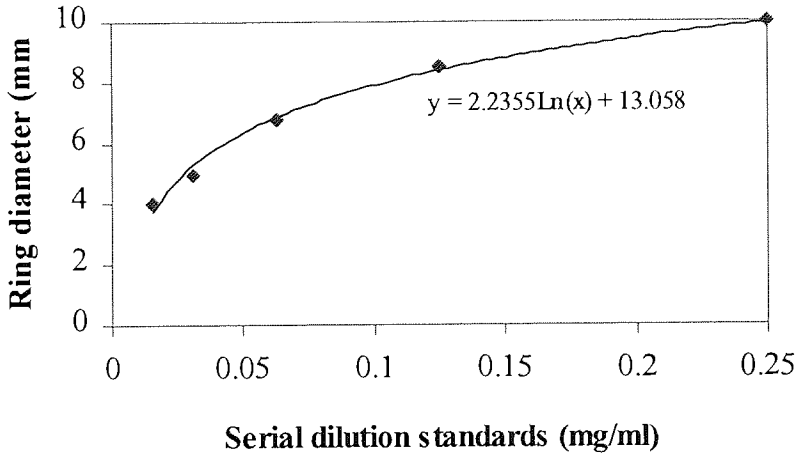


Figure 5.4. IgA calibration curve derived by RID: Plate-2

IgA Standards			IgA Unknowns		
Name	Concentration (mg/ml)	Diameter (mm)	Tear Sample	Diameter (mm)	Concentration (mg/ml)
1	0.25	10.0	TS-1 (O)	8.5	0.13
2	0.125	8.5	TS-14 (C)	10.5	0.31
3	0.0625	6.8	TS-14 (O)	10.3	0.29
4	0.0313	5.0	TS-4 (O)	9.8	0.23
5	0.0156	4	TS-4 (C)	13.8	1.37
			TS-15 (C)	11.3	0.45
			TS-15 (O)	10.8	0.36

Table 5.3. Results of the calculation of IgA concentration in open versus closed eye tear samples: Plate-2

TS-4 and TS-15 both showed clear increases going from the open to closed eye tear samples, but TS-14 exhibited only a minor rise in IgA levels. Of particular note was the range of degree of increase among even this small three sample group. The differences demonstrable were: TS-14 - 0.02mg/ml increase, TS-4 - 1.14mg/ml increase and TS-15 - 0.09mg/ml increase. This range of values may be due to patient variation, but the tear collection and the duration of sampling may be the influencing factor. As previously mentioned, precise control of the tear sampling on waking was relatively impossible and standardisation was wholly unattainable due to the patient handling variables. It is quite feasible to assume that different tear collection times were incurred during sampling and that each sample represented a progression of the eye recovering from closed eye sleep to open eye consciousness. Unfortunately, the nature of individual home patient collection and use of clinically derived samples did not allow accurate sampling records. However, in conclusion it was found that IgA did show clear levels of increase post sleep.

5.4.3. Comparison of IgG Concentrations in Open versus Closed Eye Tear Samples by Radial Immunodiffusion

Unfortunately only one IgG closed eye tear sample was performed as presented below. 100µl of anti-human IgG was added to the molten agarose prior to pouring. The reactants were allowed to diffuse for 48 hours before the reaction was stopped. A normal, non-AST sample was analysed.

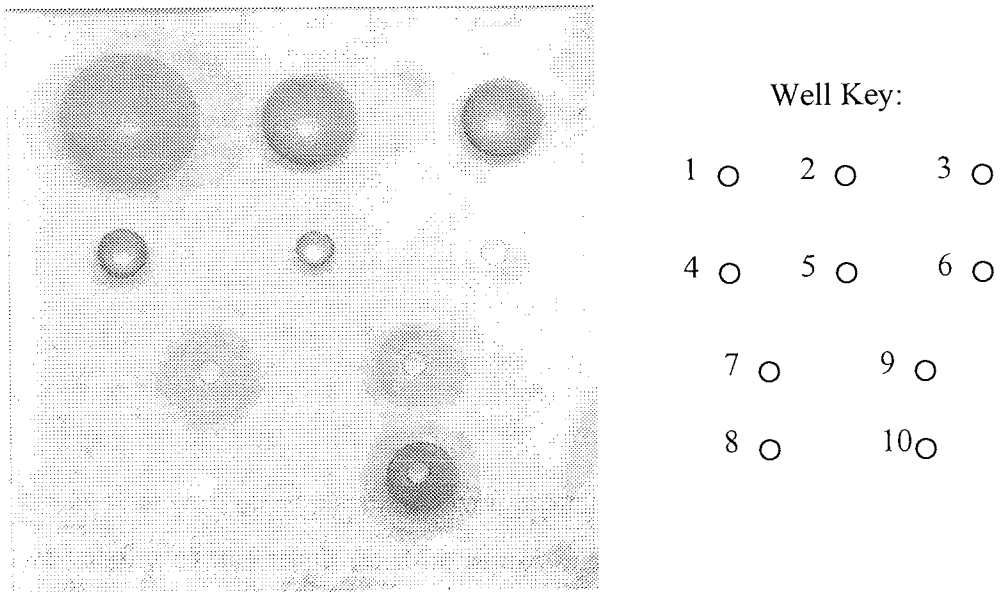


Figure 5.5. Analysis of closed versus open eye tear samples for IgG by RID. 1-5 = IgG standards, 6 = empty, 7 = TS-1 (0), 8 = empty, 9 = TS-4 (O), 10 = TS-4 (C)

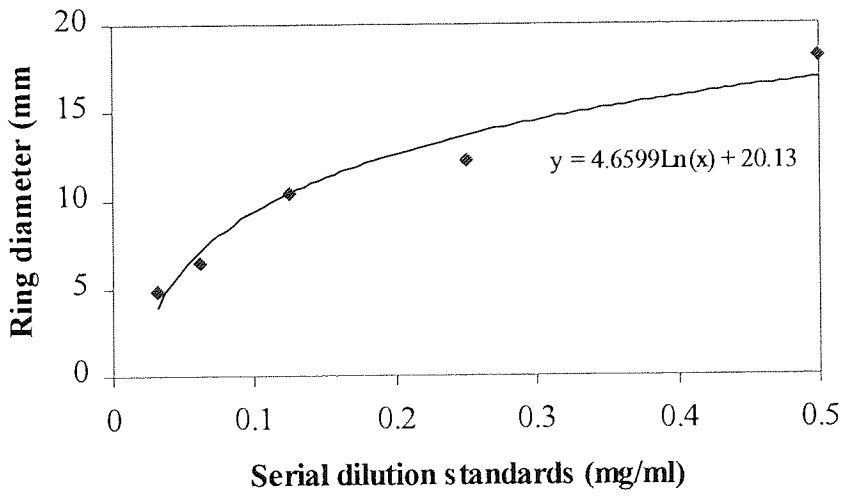


Figure 5.6. IgG calibration curve derived by RID

IgG Standards			IgG Unknowns		
Name	Concentration (mg/ml)	Diameter (mm)	Tear Sample	Diameter (mm)	Concentration (mg/ml)
1	0.5	18.2	TS-1 (O)	12.5	0.19
2	0.25	12.2	TS-4 (O)	11.5	0.16
3	0.125	10.4	TS-4 (C)	15	0.33
4	0.0625	6.5			
5	0.03125	4.9			

Table 5.4. Results of the calculation of IgG concentration in open versus closed eye tear samples

Both the control (open eye TS-1) and the open eye test sample exhibited similar IgG levels as observed in the previous chapter. Unfortunately, as stated above only one IgG sample was run; this sample did follow the same pattern of increase in the closed eye as seen with other plasma derived proteins, but with only a one sample population the validity of this result must be accepted with caution. The ongoing difficulties in obtaining closed eye tear samples was the reason why only one plate was performed.

5.4.4. A Comparison of Albumin Concentrations in Open versus Closed Eye Tear Samples by Rocket Electrophoresis: Plate-1

50µl of anti-human albumin was added to the molten agarose. The gel was electophoresised for 1 hour on the Beckman Paragon electrophoresis system after which the rockets of precipitation were clearly visible.

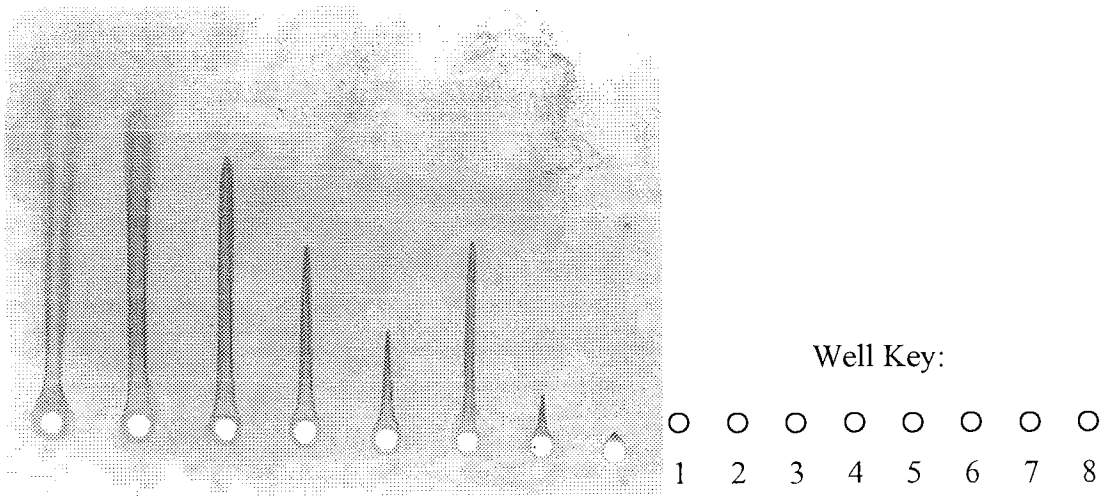


Figure 5.7. Analysis of closed versus open eye tear samples for albumin by RE: Plate-1. 1-5 = albumin standards, 6 = TS-16 (C), 7 = TS-16 (O), 8 = TS-1 (O),

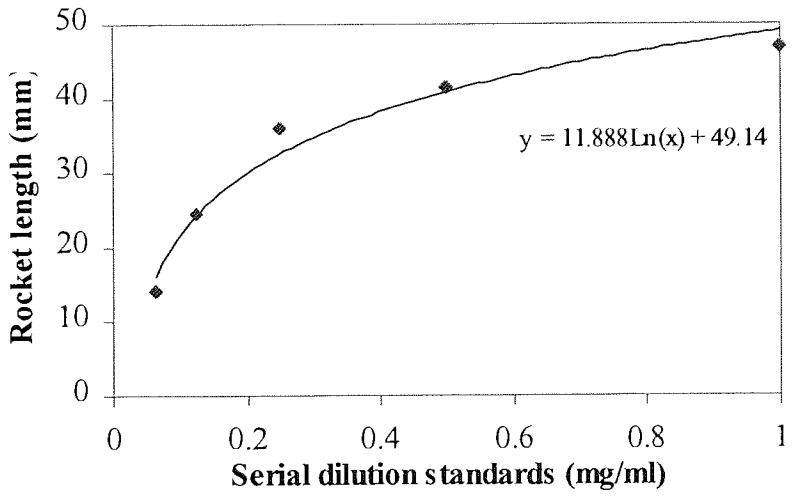


Figure 5.8. Albumin calibration curve derived by RE: Plate-1

Albumin Standards			Albumin Unknowns		
Name	Concentration (mg/ml)	Rocket Length (mm)	Tear Sample	Rocket Length (mm)	Concentration (mg/ml)
1	1	47.0	TS-16 (C)	26.0	0.14
2	0.5	41.5	TS-16 (O)	7.5	0.03
3	0.25	36.0	TS-1 (O)	3.0	N/A
4	0.125	24.5			
5	0.0625	14.3			

Table 5.5. Results of the calculation of albumin concentration in open versus closed eye tear samples: Plate-1

The low levels observed in the analysis of open eye albumin concentrations were once again evident in this assay. The control TS-1 albumin level was extremely low here and not calculable. A comparison of the open to closed eye concentrations demonstrated a 0.11mg/ml increase, confirming the increase in serum albumin levels observed overnight, and suggesting the decreased integrity of the blood/tear barrier and the influx of plasma proteins.

5.4.5. A Comparison of Albumin Concentrations in Open versus Closed Eye Tear Samples by Rocket Electrophoresis: Plate-2

The parameters of this assay are as described previously for Plate-1 analysis of albumin.

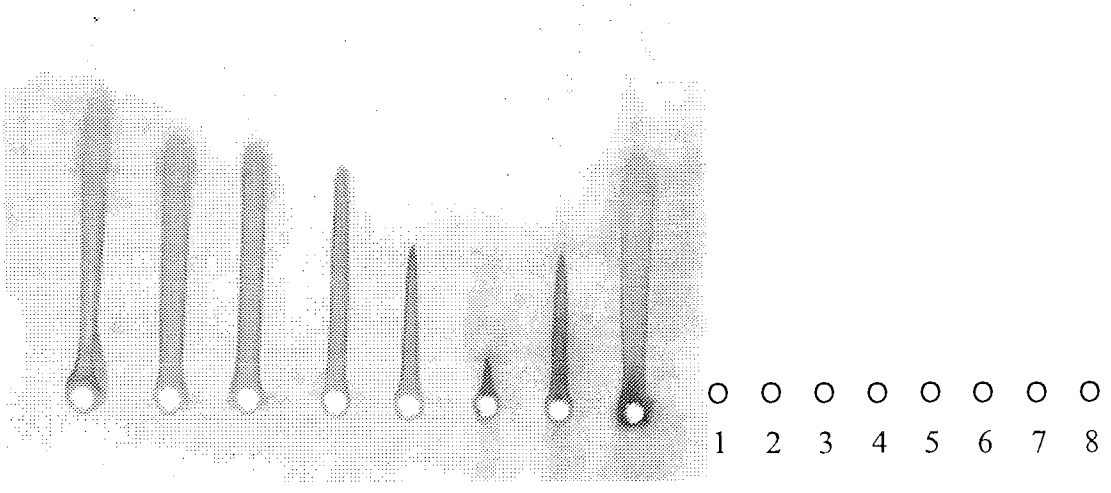


Figure 5.9. Analysis of closed versus open eye tear samples for albumin by RE: Plate-2. 1-5 = Albumin standards, 6 = TS-1 (O), 7 = TS-4 (O), 8 = TS-4 (C)

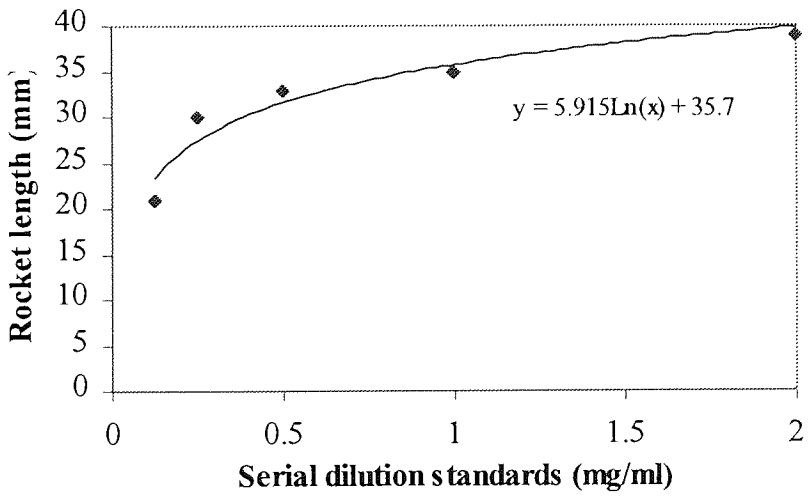


Figure 5.10. Albumin calibration curve derived by RE: Plate-2

Albumin Standards			Albumin Unknowns		
Name	Concentration (mg/ml)	Rocket Length (mm)	Tear Sample	Rocket Length (mm)	Concentration (mg/ml)
1	2	39	TS-1 (O)	6.5	0.01
2	1	35	TS-4 (O)	21	0.09
3	0.5	33	TS-4 (C)	34	0.75
4	0.25	30			
5	0.125	21			

Table 5.6. Results of the calculation of albumin concentration in open versus closed eye tear samples: Plate-2

The nature of the development of the standard rocket precipitate made the accurate calculation of albumin concentrations difficult, but mere visual analysis showed a clear increase in albumin levels going from the open to closed eye tear states. TS-1 again displayed a minimal albumin concentration.

5.4.6. Summary of Results

Protein	Assay	Open Eye Concentration (mg/ml)	Closed Eye Concentration (mg/ml)
IgA	RID	0.23	0.63
IgA	RID	0.20	0.36
IgA	RID	0.29	0.31
IgA	RID	0.23	1.37
IgA	RID	0.36	0.45
IgG	RID	0.16	0.33
Albumin	RE	0.03	0.14
Albumin	RE	0.09	0.75

Table 5.7. A summary of the results comparing IgA, IgG and albumin concentrations in open versus closed eye tear samples

The protein levels determined by RID and RE for IgG, IgA and albumin correlate with those presented by Sack et al,[43] who highlighted an increase in plasma derived proteins overnight. These results confirm the change in tear composition between open and closed eye that may be working to protect the eye from potential pathogens trapped during eye closure.

The levels of albumin in the open eye were again found to be well below the mean generally quoted, but the important fact of note was the significant influx of plasma albumin levels that were observed in the closed eye. The change in albumin levels in the closed eye have been used to assess the integrity of the blood/tear barrier which under normal circumstances allows only the diffusion of water and crystalloids. Increased vascularisation disrupts the blood/tear barrier and plasma protein can move more freely between into the tissues. Plasma albumin levels in tears have become a suitable marker of the estimation of vascular permeability.

IgG, found in low quantities in open eye tears, was also shown to increase in concentration in the closed eye confirming the trend observed with plasma derived albumin into the closed eye environment. Due to the short supply of closed eye tear samples, only a few gels could actually be performed. The initial aim was to assess both albumin and IgA, a plasma derived protein and a tear derived protein, respectively, in the tears of open versus closed eye patients as a marker of the tear versus plasma derived protein ratios. The next step was to investigate other lessor proteins, but unfortunately only one IgG plate was completed due to the problems in sample supply. This one IgG result was conclusive, with a clear increase in its levels in the closed eye tears going from 0.16 to 0.33 mg/ml, but no other assay was able to be performed to substantiate this result.

IgA was used, as stated earlier, as a means to validate and ascertain the application of the immunodiffusion assays in the identification of tear proteins in the closed eye environment. The five tear samples analysed for IgA all exhibited relatively similar open eye concentrations but demonstrated a wide variability in the levels of increase in the closed eye tears. At the low end of the scale a mere 0.02mg/ml increase was presented, but at the other extreme a 1.14mg/ml rise in IgA was observed. This range may be due to patient influence or may highlight the extremely sensitive temperament of CEE tear sampling and the importance of controlling and clarifying the sampling method and parameters. The time delay encountered during collection may be the key to the differences incurred.

5.5. Discussion

One of the central issues in the area of CEE tear analysis was the lack of sampling control and availability. However, these problems, naturally only became apparent during the course of the work but rather than concede to these obstacles work was done in an attempt to assess the field of CEE and understand this phenomenon. Motivation behind the investigation into the CEE was also driven by the idea that there are some similarities between the CEE and contact lens wear.

An understanding of the closed eye environment, a natural phenomenon, may prove fundamental in our understanding of contact lens wear and may provide a basis on which to improve contact lens biocompatibility. The CEE and contact lens wear present some similar physical and mechanical properties that contrast those displayed in the open eye. The main aspects include a decrease in the blink mechanics, a creation of a more stagnant environment, a reduction in atmospheric oxygen availability and a change from the open eye in tear protein profile. Additionally, contact lens wear was shown to cause an ocular susceptibility due to the inhibition of tear resurfacing, an increase in epithelial microtrauma, altered cell desquamation and altered tear proteins.[120] The CEE creates a pro-inflammatory environment to a minor degree, as described earlier. This highlights the fact that both situations have the presumed potential to create an immune reaction.

Increased vascularisation is another potential factor common to both situations. The walls of the small blood vessels in most tissues are fully permeable to water and crystalloids, but only very slightly permeable to plasma proteins. In contrast, during inflammation these walls increase in permeability. The majority of patients with long term wear display, to some degree, peripheral vascularisation over time, the extent of which differs from patient to patient. Vascular permeability is also indicative of the CEE in the presence of the sub-clinical inflammation. Thus, a comparable plasma protein profile may occur, for example, vitronectin levels have been shown to increase in both contact lens wear and in the CEE.[83]

Some of the problems inherent in extended wear lenses have been attributable on the reduction in oxygen availability and resulting hypoxia created during overnight wear,[121] but this can not be wholly responsible for the high rate of corneal infection caused by wearing lenses while sleeping. The answer may be explained by the surge of plasma proteins, a change in nervous stimulus and the almost internal nature of the eye, in conjunction with hypoxia, which is seen in the CEE. Obviously there are differences between contact lens wear and CEE, but in understanding the CEE we may learn valuable information that may improve the way we treat and use the hydrogel contact lens.

In summary, the tears investigated under the title of closed eye tear samples, as pointed out were not technically indicative of such, but in reality, were possibly 'diluted' versions of the true close eye tear. Nevertheless, the results found for these samples, representative of the closed eye tear, displayed clear increases in the plasma derived/leaked proteins and also substantiated the IgA rich tears previously observed during the closed eye phenomenon. It is quite possible that whenever 'true' closed eye tear samples can be obtained the protein levels detected may show similar trends at different higher concentrations.

Due to the said state of sub-clinical inflammation that occurs during overnight sleep, it was speculated here that the kinin family of inflammatory mediators may also be present in the plasma influx. Therefore, work was to be done on some closed eye tear samples but owing to the lack of closed eye samples, kininogen in the closed eye was not able to be investigated. To overcome this drawback it was decided to take advantage of the certain similarities that exist between the CEE and contact lens wear in an attempt to assess the presence of the kinins in relation to lens wear. Certain members of the kinin family have an affinity for, and can be activated by, contact with negatively charged surfaces, i.e., contact lenses. Lens wear may induce some vascular permeability but more importantly provide may a medium for their accumulation, facilitating easier detection. The results of this investigation are presented in Chapter 6 where CIE proved important in the analysis of the contact lens eluate.

Chapter 6

Application of Immunodiffusion and Biochemical Assays to the Study of Tear : Lens Interactions in Daily Wear

6.1. Aim

The aim of the work described in this chapter was to look at some of the factors affecting the spoilation of contact lenses *in vivo*. Deposition analysis was undertaken in two ways. The first approach involved the measurement of the total protein deposited on and in the matrix of the lens by ultra violet spectrophotometry. The second necessitated the use of an efficient extraction method in order to look at the individual nature of the proteins involved. Once an optimum extraction method was selected immunodiffusion assays were employed to aid in the detection of particular proteins released into the eluate.

6.2. Introduction

Soft hydrogel contact lenses, based on polyHEMA, which have provided an alternative to the hard PMMA lenses, increase oxygen permeability and provide a more comfortable lens. However, as a general rule in life, when we solve one problem another is created, and that which the soft contact lens has created was that of greater deposition and spoilation. The obvious advantage that soft contact lenses offer is comfort and thus a more compatible fit. A more comfortable lens means more use, longer wear periods and ultimately a greater demand on the lens, resulting in a larger accumulation of deposition. Cleaning of the smoother hard lenses is also more readily achieved in comparison with the soft flexible lenses.

Foreign polymers, once inserted into the body, come into contact with a layer of biological fluid, for example, blood, synovial fluid or tears. Various components from the contacting fluid can be adsorbed out of solution onto the polymer surface or into its matrix. This host-polymer interaction can alter the polymer performance and acceptability, there is no exception when it comes to the insertion of a contact lens into the ocular environment. On a contact lens, the interaction of tear film components with its surface and matrix can effect the visual performance of the lens and therefore shorten the life span of the lens especially if the deposited substances are not easily removed. On the other hand, not only can the lens become spoilt with tear

constituents, including lipids and proteins, but this absorption may drain the tears of important elements and/or upset the normal balance.

For most people contact lens wear is trouble free and provides a preferable alternative to wearing spectacles. Unfortunately, there is a limited section of the population for whom contact lens wear causes problems, in either adaptation to the new lens or over longer periods of time. It would be the ideal if this group of patients could be predetermined but this is only going to be achieved with further tear analysis and a greater understanding of the interactions between the surrounding tear fluid and contact lens. Thus it is important to assess the changes in composition and levels of proteins in the tear fluid that arise during contact lens wear.

Deposits on contact lenses have long been implicated in limiting successful lens wear. Blurry vision, irritation and giant papillary conjunctivitis are often cited as adverse reactions arising as a result of deposition. Spoilation of the lens can be affected by a number of factors including, surface topography, chemical structure, and degree of hydration of the lens and also the nature of the spoilant, in this case the tear film.

Contact lens deposits consist primarily of proteins, mucins and lipids from tears, with extraneous substances like make up and skin lipids, for example, also having a contributing role. In this study, it is the role of the proteins which remains at the fore of attention in spoilation, due to their assumed potential for stimulating, mediating and/or producing immunological reactions, be they to the detriment or well-being of the host.

As stated, many of the adverse effects of contact lens wear can be attributed to the deposits formed on and in the lens, but the true mechanisms of this deposition mediated complication remains largely unknown. The changes in the properties of the lenses brought about by the absorbed host substances and the changes in protein conformation/function must be the basis of many contact lens wear complications presented.

6.3. Materials

Lens Name	Manufacturer	FDA Classifications	% Water Content	Ionicity	Composition ‡
Acuvue	Vistakon	Group IV	58	Ionic	HEMA, MA
Surevue	Vistakon	Group IV	58	Ionic	HEMA, MA
Focus	Ciba	Group IV	55	Ionic	HEMA, PVP, MA
Medalist 66	Bausch and Lomb	Group II	66	Non-Ionic	HEMA, VP
CSI	Pilkington Barnes-Hind	Group I	41	Non-Ionic	MMA, GMA
Frequency 55	Aspect	Group IV	55	Ionic	HEMA, MA

Table 6.1. Description and characterisation of the soft contact lenses employed in the deposition and extraction studies. ‡ HEMA = 2-hydroxyethyl methacrylate, MA = methacrylic acid, GMA = glyceryl methacrylate, MMA = methyl methacrylate, PVP = poly (vinyl pyrrolidine)

6.4. Ultra Violet Spectrophotometry

The analysis of tear protein accumulation on a contact lens can be conveniently accomplished by means of ultra violet (UV) spectrophotometry. Spectroscopy involves the absorption, emission or scattering of electromagnetic radiation by atoms or molecules. All biochemical compounds absorb energy from particular regions of the electromagnetic radiation spectrum; proteins can be measured spectrophotometrically by means of intrinsic chromophores in the UV region, that absorb around 280nm. UV spectrophotometry, therefore, has provided us with a means of analysing surface and bulk protein deposition on a contact lens, as previously described in Chapter 2 by providing the individual absorbance of each lens.

Each absorbance measurement is related to the concentration of protein deposited and protein levels can be calculated by creating an absorbance/concentration calibration curve. A protein mixture was made up to represent the tear film protein composition in their approximate milligrams per millilitre concentrations. The curve was created by setting up a series of diluted standard concentrations of the 'protein pseudotear' and an absorbance to concentration relationship was obtained. This created a means of translating the absorbance results into milligrams of protein. The calibration curve was stored in the instrument. Ultimately, this pseudotear solution was not going to imitate the actual dynamics of tears and different on-lens deposition characteristics are inevitable, but it was thought to be the simplest method of relating absorbance to milligrams of protein. Single protein calibration curves could also be set up for the *in vitro* analysis of individual spoilation characteristics of particular proteins. The conversion of mg/ml in solution from the standards to mg protein on the lens could be calculated by multiplying the results by πr^2. An absorbance to concentration conversion table is provided in Appendix II from which any absorbance given in the results could be easily converted to milligrams of protein.

6.4.1. Reproducibility of UV Spectrophotometry

The U2000 spectrophotometer (Hitachi) was used to study protein spoilation on a variety of different lenses, but an initial investigation into the reproducibility of this method was performed. Repeated analysis of the absorbance of a single lens, under the same experimental conditions, demonstrated a slight but important variance in readings, thus an experiment was performed to assess the standard deviation in absorbance over a series of measurements. To demonstrate the variability involved, the absorbance readings for eight blank lenses were taken and each lens was read 10 times. Four commercially available lenses were analysed, three Group IV and one Group II lens, using a positive powered and negative powered lens for each lens type, except in the case of the Medalist 66 lens where no positive powered lens was available. All lenses had a visibility handling tint. Each lens was measured ten times at 280nm. The results are shown in Table 6.2. and depicted graphically in Figure 6.1.

Lens Name / Power / Reading	Focus 2.00	Focus -2.00	Medalist 66 -4.00	Medalist 66 -2.00	Surevue 2.00	Surevue -2.00	Acuvue 2.00	Acuvue -2.00
1	0.264	0.223	0.143	0.117	0.081	0.073	0.062	0.050
2	0.262	0.221	0.143	0.116	0.077	0.071	0.061	0.049
3	0.261	0.220	0.143	0.117	0.073	0.069	0.060	0.049
4	0.260	0.219	0.143	0.117	0.072	0.069	0.060	0.049
5	0.259	0.219	0.143	0.117	0.072	0.068	0.057	0.049
6	0.258	0.218	0.138	0.118	0.071	0.068	0.057	0.049
7	0.257	0.217	0.137	0.119	0.071	0.060	0.057	0.048
8	0.255	0.217	0.137	0.117	0.068	0.059	0.057	0.044
9	0.243	0.216	0.137	0.116	0.068	0.058	0.057	0.044
10	0.242	0.215	0.137	0.117	0.069	0.058	0.057	0.044
Mean	0.2561	0.2185	0.1401	0.1171	0.0722	0.0653	0.0585	0.0475
StDev	0.0076	0.0024	0.0031	0.0009	0.0039	0.0059	0.0021	0.0025

Table 6.2. UV reproducibility over 10 absorbance measurements

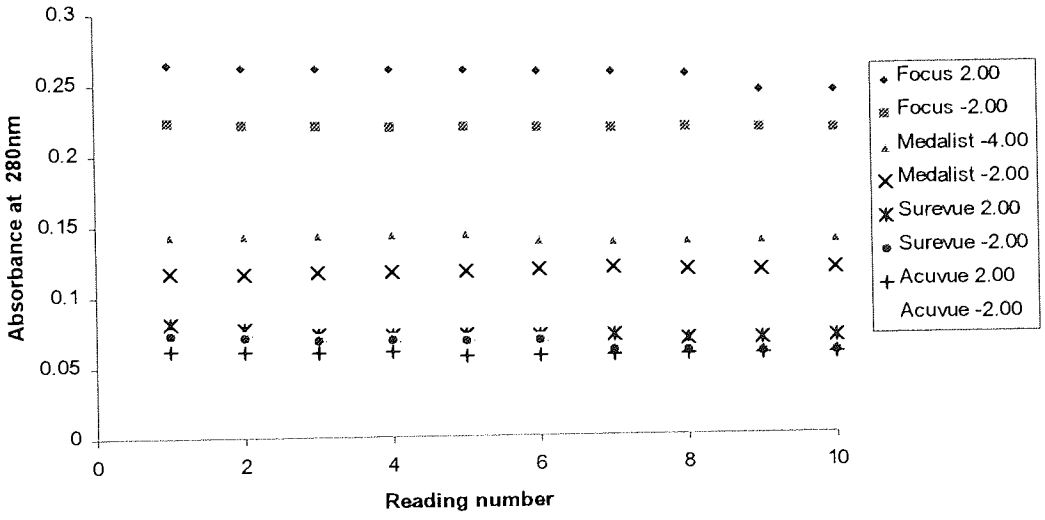


Figure 6.1. UV reproducibility over 10 absorbance measurements

This experiment demonstrated that statistically the reproducibility over ten measurements was stable, the highest standard deviation presented on these blank lenses was ±0.0075 which in terms of concentration is below the 0.000mg of protein range employed in this assay and did not register a concentration equivalent. Thus the reproducibility of the instrument was considered satisfactory. Importantly this study highlighted the variances encountered in the measurement of the absorbance, over the different lens types and powers. The background for the Focus +2.00 lenses (the highest background noted), for example, was 0.256±0.00076 in absorbance, which would give a mean concentration of 0.104±0.030. The minimum value, as shown by the Acuvue -2.00, with an absorbance of 0.0475±0.0025 giving a mean background concentration of 0.015±0.005. The next procedural step was to further ascertain the effect of lens power/prescription on the background absorbance of the lenses investigated in this study. The results from the reproducibility study were put into practice in all studies performed.

6.4.2. Blank (Background) Analysis

The U2000 contains two absorbance cells, an analyte/sample cell and a reference cell. The reference cell was used in order that background interference can be subtracted, for example, background lens or containing solution absorbance influence. When measuring contact lens deposition, an identical blank contact lens to the one under examination may be placed in the reference cell to subtract the background absorbance. This did not prove ideal for these studies as problems arose when analysing the thinner lenses, which made reproducibility more difficult to achieve. The problem was most evident with Acuvue lenses which have the tendency to curl up when inserted into the cuvette, which in a reference cell for long periods of time, would create erroneous background results, due to movement in the cell. The purpose of this experiment was to avoid the need to use the reference cell for background blank lens subtraction and thus eliminate this error. The reference cell could then be used to subtract the containing solution, e.g. distilled water. To alleviate the problem, blank lenses, from a range of materials and powers, analysed in

the clinical studies, were measured in the analyte cell against distilled water in the reference cell.

The analysis of the four lens types at two powers in the reproducibility study highlighted two important properties that can ultimately affect the outcome of deposition results, the influence of lens type and the lens power/prescription. Therefore, it was deemed necessary to obtain blank values over a range of powers for all lenses under investigation in order to subtract background absorbance levels from analyte measurements.

A summary of the pattern of results for the blank lens absorbances for the six lens types are shown in Figure 6.2. It illustrates a lack of pattern, i.e., the power and absorbance have no direct correlation and hence the need to assess all powers and not assume regularity. This figure emphasises the huge variations incurred over a range of lens powers and highlights the need to consider the background of each individual lens power, type and group. Only one lens of each power was analysed, thus the variability assessed below did not take into account the differences incurred during the manufacturing process. All the lenses had a handling visibility tint with the exception of the 1 day Acuvue lenses.

The mean absorbance readings for these lenses used in the studies are shown in Appendix II.

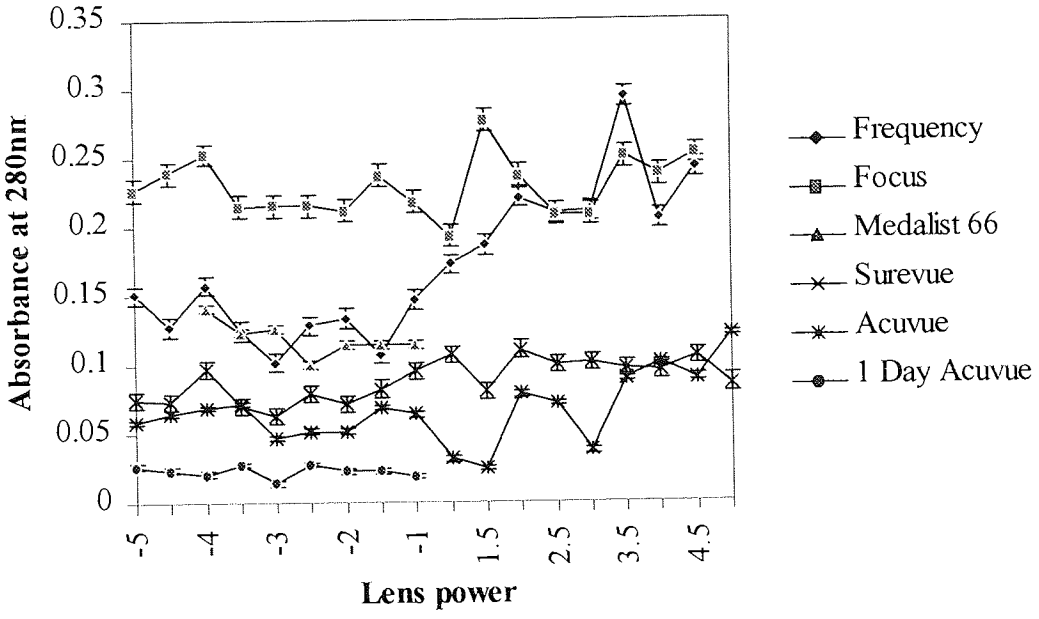


Figure 6.2. Effect of lens power on background UV absorbance measurements for five Group IV and one Group II lenses.

Figure 6.2. demonstrates the small, but important, influence of the lens power on the background absorbance for any given lens type. As a general rule, the Medalist 66, Surevue, Focus, Acuvue and 1 day Acuvue lenses all displayed a somewhat regular pattern. Demonstrating a slight change in absorbance from power to power, but over a range of powers which vary in lens thickness, the measurements do not differ vastly A mean value of all lens powers was calculated and subtracted as background absorbances, these values are listed in Appendix II. Frequency 55 lenses on the other hand require more attention as they showed a more exaggerated difference from power to power. The negatively powered lenses presented similar trends and patterns as observed in the above mentioned lenses. The positively powered lenses, on the other hand, displayed an almost steady increase in absorbance with increase in power, possibly relating to the handling tint in these somewhat darkly tinted lenses. Thus, the individual powers of this lens type would have to be considered separately.

6.5. Analysis of *ex vivo* Lenses

This section of work employed the use of the UV spectrophotometry assay in order to determine the levels of deposition on a variety of lenses under different wear regimes and worn by a diversity of patients. Taking the reproducibility variables and the effect of background lens absorbances into consideration, the lenses could be measured for total protein deposition at 280nm. The lenses tested in the following studies were kindly supplied by Lyndon Jones.

6.5.1. The Analysis of Protein Deposition on Three, Monthly Disposable Group IV Contact Lenses *ex vivo*

The following deposition experiment was designed to assess intra-group differences, apparent in the Group IV lens group. This study also allowed us to estimate the affect of the patient on lens spoilation and observe the differences apparent between the subject's left and right eye. Overall, this work highlighted the many factors involved in influencing or affecting the rate, concentration and possibly the nature of the protein accumulated. Acuvue, Surevue and Focus lenses were studied, the first two with a 58% EWC, and the latter with a 55% EWC, all three composed of HEMA and MA with Focus also consisting of PVP. The aim was to show the differences evident under the same FDA grouping. The Acuvue and Surevue lenses differ only in lens thickness, which presented the opportunity of assessing the affect of the lens thickness on the deposition profile. The Focus versus Acuvue/Surevue comparison was beneficial in estimating the affect of a PVP plus MA/HEMA composition as opposed to an MA/HEMA lens.

All the lenses were worn for 1 month, under the standard, controlled, randomised, masked and cross over parameters. The same patients were used for the Focus and Surevue lenses but the Acuvue lens were worn by a separate set of patients.

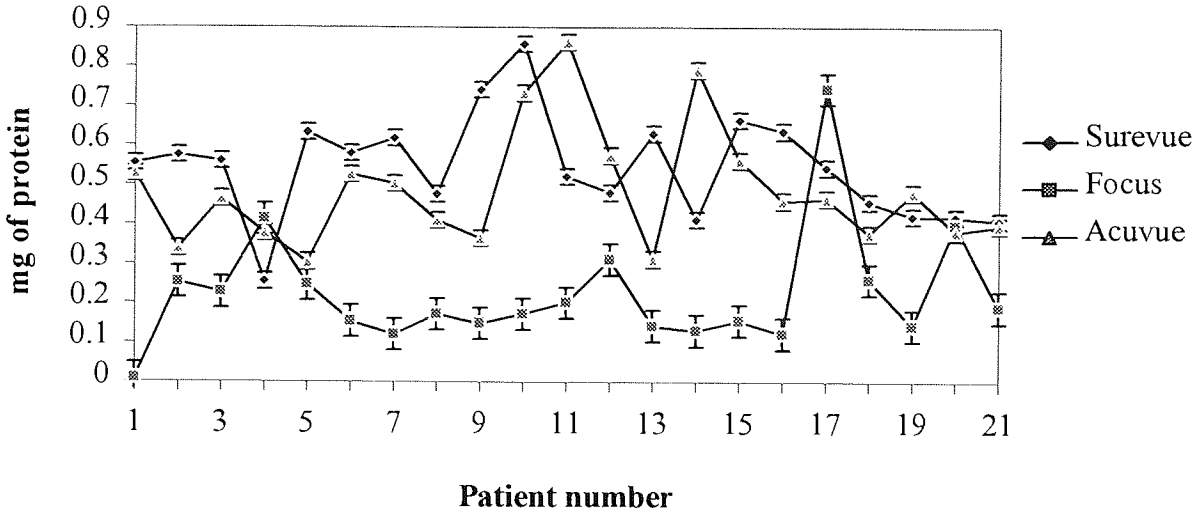


Figure 6.3. A comparison of the mean total protein deposition on three Group IV lenses measured by UV spectrophotometry

Lens Name	Focus	Acuvue	Surevue
Mean	0.225	0.487	0.545
Max	0.744	0.862	0.857
Min	0.009	0.305	0.256
StDev	0.151	0.148	0.132

Table 6.3. The mean, maximum, minimum and standard deviation of results in a population of 21 patient studying the total protein deposition on three Group IV lenses

As a general summary, it would appear that the levels of total protein deposition are greater in Surevue lenses followed by Acuvue and then Focus. A comparison of the Surevue and Acuvue lenses would argue that the thicker lens allows the greater levels of spoilation, as this was primarily the only difference between the two lens types, which would suggest a build up of protein in the matrix.

The Focus lenses displayed a lower level of deposition after 1 month wear compared with the HEMA/MA only compositions, with the exception of one patient who presented a well above average deposition. These results would suggest that the Focus lens, with the least total protein accumulation, would prove the most superior lens. When these patients were asked to make a preference between the Focus and the Surevue lenses, 62% chose the former, which substantiates the above result. It follows that the greater the level of lens spoilation, the greater the lens depreciation, but the nature of the spoilation must not be ignored. However, as a total protein assay, the individual nature of the deposition could not be ascertained by this method.

6.5.2. A Study of the Total Protein Deposition Characteristics of Two Monthly Disposable Contact Lenses over Different Time Periods.

The previous study illustrated the effects of patient variability, lens thickness, intra-eye variations and the lens material on lens deposition. This experiment was designed to investigate the rate and extent of total protein deposition over a 1 day, 1 week, 2 weeks, 3 weeks and 4 weeks time period and to attempt to observe any patterns of deposition. The maximum and minimum values are given to remind us of the extent of the above noted variability than can be observed even in small sample groups. A set of Group II (Medalist 66) and Group IV (Acuvue) lenses were compared, with a 66% and a 58% equilibrium water content (EWC) respectively, differing also in monomer composition, Acuvue with HEMA and MA and Medalist 66 with HEMA and VP. 22 patients were investigated in this cross over, randomised study. The results presented below are given taking the average of the left and right eye of each patient into consideration. The error bars shown in Figures 6.4. and 6.5. were drawn from the previous reproducibility and blank lens background results. The bars drawn in the Acuvue versus Medalist 66, Figure 6.6. highlights the standard deviation about the mean in the patient population.

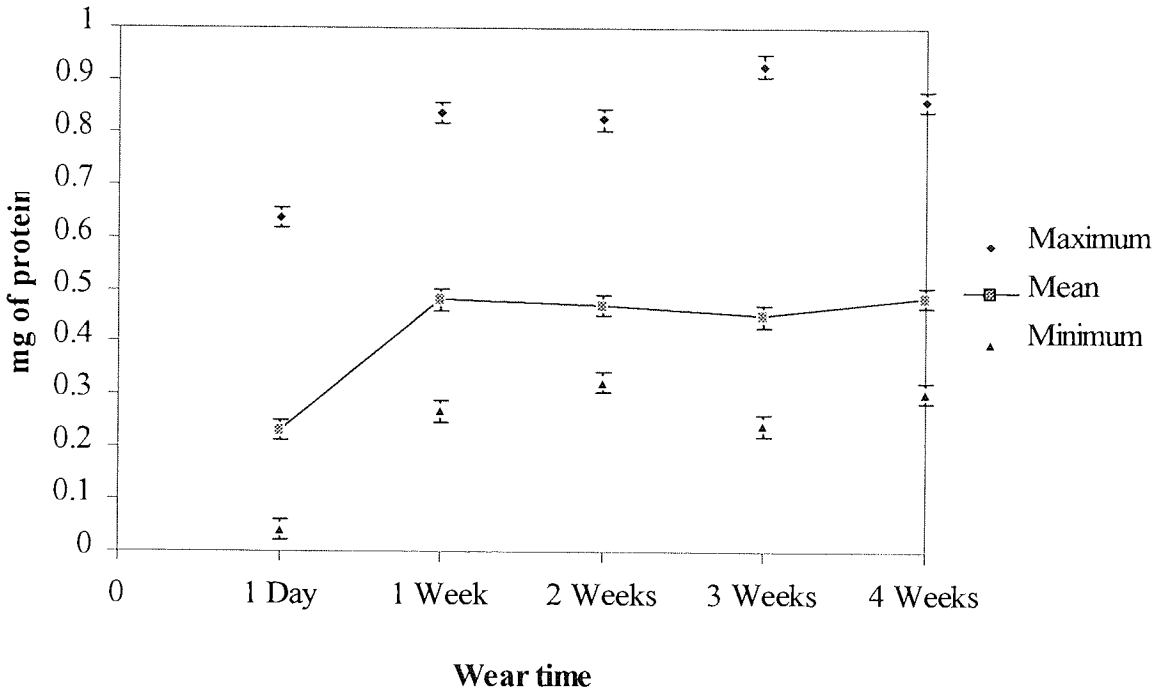


Figure 6.4. Mean total protein deposition on Group IV Acuvue lenses ex vivo over time. The maximum and minimum values over the 22 patient sample group are also given for each time period

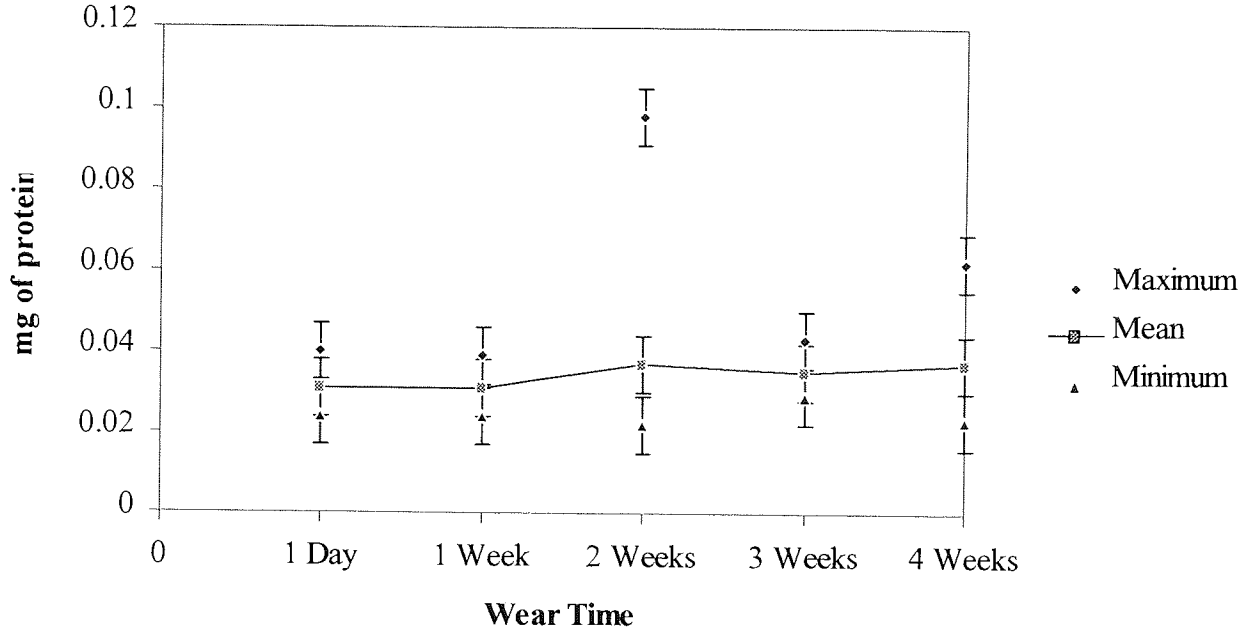


Figure 6.5. Mean total protein deposition on Group II Medalist 66 lenses ex vivo over time. The maximum and minimum values over the 22 patient sample group are also given for each time period

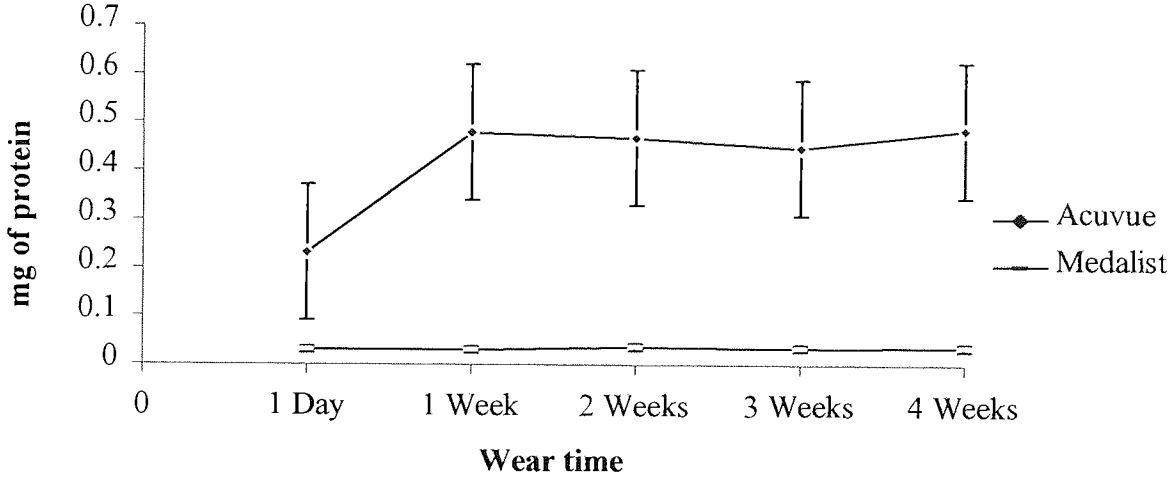


Figure 6.6. A comparison of the mean total protein deposition and standard deviation in the patient population for ex vivo Medalist 66 versus Acuvue lenses over time

The Group IV lens materials deposited significantly more protein after all the wear times than the Group II lenses. The most notable result was the trend seen on the Group IV lenses after 1 day of wear, after which there was a dramatic increase in deposition, reaching quite a stable plateau from one week through to the four week time period. The Group II lenses demonstrated an initial protein build-up that did not change over all the wear periods. There was no significant difference in the total protein, the deposition levels remained quite static and low. Deposition, therefore, is greatly influenced by the lens material as shown by the difference in the total protein levels deposited on the Group II versus Group IV lenses. There is an approximate 10-fold difference in the protein deposition levels between the two lens types.

On the Group IV lenses the protein deposition occurs rapidly between 1 and 7 days, after which there is no dramatic increase. Further work done by the Aston group, on this study, has shown that the protein accumulation at the surface of the lens, measured by fluorescence spectroscopy, remains regular and steady from 1 day through to 28 days. This suggests that the difference in measured protein levels occurs in the lens matrix spoilation as opposed to at the surface. This idea was identified and substantiated in the previous experiment in the comparison of the Surevue lenses against the thinner Acuvue lenses. The thicker lens allowed a greater quantity of protein deposition in these otherwise similar lens types. This build up on Group IV lenses has generally been perceived as being the rapid accumulation of small, positively charged proteins, namely lysozyme, an entity which has been demonstrated to move in an out of the lens matrix. However, lysozyme accumulation was possibly not wholly responsible for the dramatic rise.

Results by UV spectrophotometry could not provide further information regarding the protein accumulated; it is not clear whether there is an initial protein build up that remains permanent throughout or whether there is a dynamic of protein exchange. Consequently, further analysis of the lens accumulates was performed on both lens materials as detailed later in this chapter, in association with the lens extraction studies. In conclusion, the influence of the lens material was important. The effect of VP in the lens polymer as opposed to MA, results in a lens composition which is more protein resistant.

6.5.3. Importance of Patient Influence

These deposition studies as a whole provided informative results regarding the overall performance of individual lens types but, in particular, they emphasised the influence of the patient and the mistake in assuming particular lens related trends irrespective of patient variability. This patient variance, emphasised the need for large patient groups when performing deposition studies. For example, patient seventeen wearing Focus lenses displayed a huge deviance from the mean, which could greatly affect the mean in small sample groups, consequently presenting false high or low concentration measurements. It was important to gain an insight into the individual protein spoilation profile of each particular patient in comparison with sample group. Later in this chapter work was done on a select sample number of patients; the use of this total protein assay provided a means of identifying the potential of the subjects employed and their position in the population as a whole.

6.5.4. The Influence of the Eye

This title bears reference to the right versus left eye variance which has been noted in individuals. Figure 6.7. below presents the results of the deposition studies on the Surevue lenses worn for 1 month and demonstrates an example of the differences apparent between the left and right eyes of a various patients.

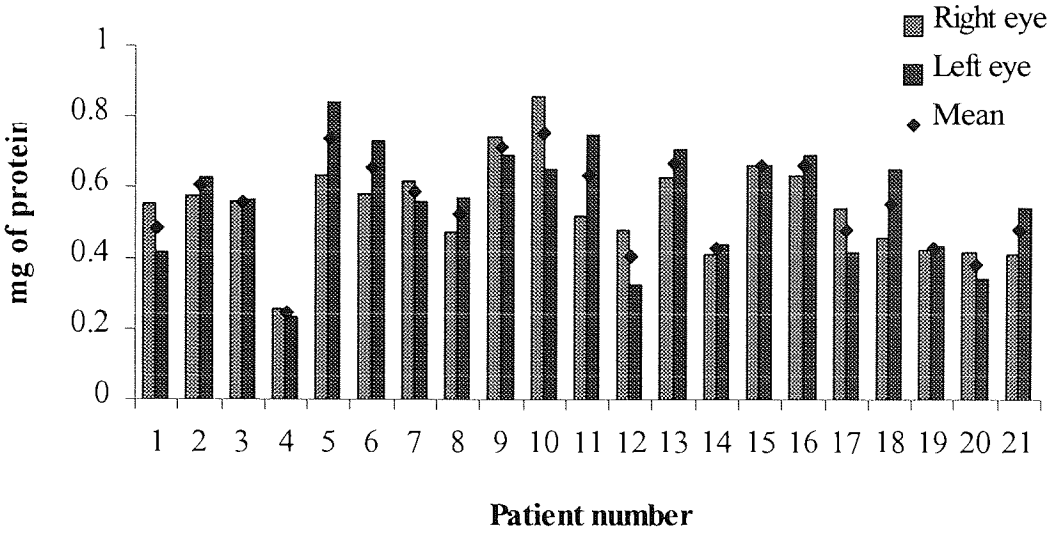


Figure 6.7. Demonstration of the right versus left eye variability

The deposition studies highlighted the diversity of results that can be presented by a group of patients and the left versus right eye figures show that moreover the individual can display notable variation characteristics. This was shown by the change in right eye versus left eye total protein values. Table 6.4. summarises the differences in concentration measured in both eyes. There was a mean concentration variance of 0.110±0.08, which was quite a significant difference and highlights the influence of the eye sampled.

Px No.	Difference in mg	Px No.	Difference in mg	Px No.	Difference in mg
1	0.139	8	0.094	15	0.000
2	0.054	9	0.053	16	0.056
3	0.002	10	0.205	17	0.124
4	0.022	11	0.277	18	0.198
5	0.209	12	0.156	19	0.013
6	0.148	13	0.081	20	0.078
7	0.060	14	0.028	21	0.135

Table 6.4. Concentration (mg) differences between the left and right eye of a 21 patient population sampled

6.6. Extraction Studies

The purpose of this part of the spoilation study was to test the efficacy of a series of extraction methods in the removal of deposited proteins from contact lenses and then with a view to investigating the feasibility of analysing individual proteins in the resulting eluate. Currently, there is no combined extraction and analysis methodologies which provide sufficient detail on the profile of the protein spoilation on a contact lens. This next section addresses this point.

6.6.1 Introduction

The on-lens UV spectrophotometric assay has proven an invaluable method in calculating the concentration of total protein deposition, providing us with valuable comparative information for various lens classifications and type, and it has also demonstrated the patient variability inherent in all studies. The main drawback encountered by this method was that it did not provide information on the nature of the spoilant; only a broad picture of protein spoilation was presented. There were two obvious solutions to this problem, the first was the use of the on-lens assays as employed in Chapter 3 for the detection of the adhesive glycoprotein, i.e., vitronectin. The second solution involved the removal and extraction of the protein from the lens, with the resultant eluate becoming the source of investigation.

The use of the on-lens assay proved successful for the detection of vitronectin, due to vitronectin's advantageous receptors for both the contact lens and its visual probe, the cell. Unfortunately, this type of assay could not be applied in the assessment of the main proteins (lacking the appropriate cell receptors) in lens spoilation, thus the second option of extraction was chosen and attempted.

The second option changes the parameters of the investigation, the analysis switches from the lens itself onto the eluate solution. To complement Chapter 4, which detailed the development of immunodiffusion assays in tear analysis, the obvious progression was to apply the immunodiffusion assays in the analysis of the resulting eluate and evaluate their use in this investigation.

6.6.2. Determination of Optimum Extraction Conditions

The initial step in procedures was to determine the most efficient and reproducible extraction method(s) available. No technique was expected to be 100% efficient but the one which best fulfilled the criteria was chosen. The criteria were:

- High % of protein removed
- Low extraction solution volumes sufficient
- Proteins of interest detectable in extract
- Reproducible

Many different extraction systems were attempted, some of which were cited in a variety of papers, some were modified versions of these techniques and others were new methods. A short list of five methods, from all the extraction techniques performed, was chosen, which encompassed the range in current use and demonstrated a broad overview of results.

Method (a) represented the preferred multi-purpose care system,[115] method (b) is a common method quoted, methods (c) and (d) highlighted the most efficient methods cited in a range of papers and method (e) introduced the idea of a physical/mechanical procedure.

6.6.3. Short List of Extraction Methods

a) ReNu. ReNu is an all in one, multi-purpose solution which contains 0.00005% Dymed (polyaminopropyl biguanide). Each lens to be extracted by this method was emersed in a bijou which contained 300µl of ReNu multi-purpose cleaning solution; the bijous were then placed on a shaker for 24 hours at room temperature. ReNu was previously shown to be the most effective multi-purpose commercial cleaning system in the removal of proteins[115] and thus was used to represent these care systems.

b) 10% Sodium Dodecyl Sulphate (SDS). Each lens was immersed in a bijou which contained 300µl of 10% SDS solution and put on a shaker for 24 hours at room temperature.

c) Tris[hydroxymethyl]aminomethane (Tris)/SDS/Glycerol/Dithiothreitol (DTT) solution. A 0.5M Tris, 2.5% SDS, 10% glycerol and 5% DTT solution (pH 9.5) was prepared. Each lens for extraction was heated to 95^0C (on a heated stirrer) for 1 hour in 300µl of solution, in a 1ml eppendorf, and allowed to cool gradually overnight in a waterbath to 25^0C.

d) Urea/SDS/DTT/Tris solution. A 40% urea, 1% SDS, 1mM DTT and 100mM Tris solution (pH 8.0) was prepared. Each lens to be extracted was heated at 90^OC (on a heated stirrer) for 3 hours, in a 1ml eppendorf and allowed to cool.

e) Polynet method. Polynetting (Merck 222/0669/10) is used ordinarily as a protective mesh sleeving over glassware in case of breakage. In this instance it was used as lens support during centrifugation which allowed the free flow of extracted liquid through to the bottom of the centrifuge tube. There were no mechanical methods described in the extraction of proteins from lenses and thus a method was designed to assess whether physical/mechanical forces could be used in the extraction process. This method illustrated in Figure 6.8. involved the insertion of a cut section of polynetting into the bottom of a 10ml centrifuge tube.

The contact lens was then carefully placed on the polynet and centrifuged at 2000rpm from 5 min to 1 hour.

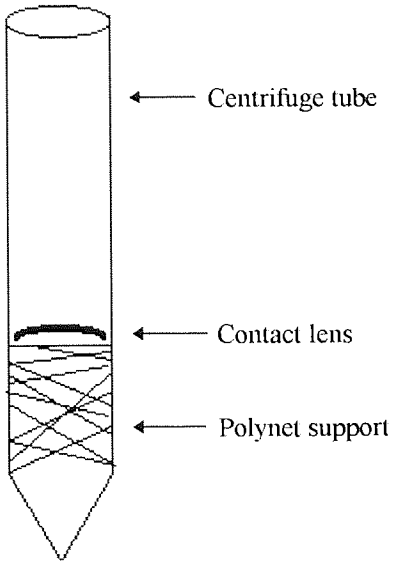


Figure 6.8. The polynet extraction method

6.6.4. *In vitro* Doping of Acuvue Lenses for Extraction Experiments

All the Group IV Acuvue lenses, used to investigate the most efficient extraction conditions, were spoilt *in vitro* in one of the two solutions prepared, one containing lysozyme and the other without lysozyme. The lysozyme positive solution was made with the main tear protein at levels approximating those quoted in tears and the lysozyme negative solution was prepared with slightly higher levels of the other tear protein to equate the final concentration of both solutions. All the proteins used were of human origin except the use of chicken lysozyme. The reasons for using chicken lysozyme were both economical (human lysozyme is vastly more expensive than chicken lysozyme) and for standardisation (due to the initial use of chicken lysozyme in this laboratory in certain spoilation studies).[115] To avoid confusion with the use of the word solution in relation to the extraction methods above, these two solutions will be referred to as pseudotear solutions (PTS-1 and PTS-2). The lenses analysed in all the extraction studies were Acuvue daily wear lenses.

PTS-1	PTS-2
2mg/ml Lysozyme	---
2mg/ml Lactoferrin	2.5mg/ml Lactoferrin
1.5mg/ml Albumin	2.0mg/ml Albumin
0.3mg/ml IgA	0.9mg/ml IgA
0.1mg/ml IgG	0.5mg/ml IgG

Table 6.5. Artificial tear solutions used in in vitro extraction studies.

The purpose of using two PTS was to draw a comparison between a lysozyme-containing and a lysozyme-free solution. The main spoilant of Group IV lenses is known to be lysozyme and in order to avoid masking by lysozyme a second PTS was prepared without lysozyme to analyse the spoilation profile of the other proteins, and for the purpose of assessing the extraction characteristics of the other main proteins.

As the total protein concentration for both solutions was kept constant, slightly higher quantities of lactoferrin, albumin, IgA and IgG levels than would be detected in tears, were utilised. Analysis of previous extracts by UV spectrophotometry always demonstrated a decrease in absorbance from the initial absorbance to the extracted absorbance, but the nature of the protein removed analysed by SDS-PAGE demonstrated only the presence of lysozyme in the eluate. This evidence would suggest that it was only lysozyme that was removed, thus a non-lysozyme solution (PTS-2) was employed to investigate if this 14.6kDa protein was the only protein being removed or whether the other main tear protein constituents were also reversibly bound.

6.6.5. Extraction Protocol

The extraction studies were carried out on *in vitro* doped Group IV Acuvue lenses and *ex vivo* Acuvue and Group II Medalist 66 lenses, the *ex vivo* lenses were worn on a daily wear regime. All lenses were doped in either 1ml of PTS-1 (with lysozyme) or PTS-2 (without lysozyme) in a bijou for 24 hours on a gentle shaker. The lenses were then run under UV spectrophotometry at 280nm and an absorbance and concentration for each lens was obtained and taken as the initial pre-extraction reading (i).

The protein extraction on each lens was then performed by means of one of the five methods listed above (a-e). For all procedures where applicable, the eluate, into which the protein was extracted, was kept to a minimum volume to prevent excess dilution of already low levels of protein, thus enabling easier detection of the proteins by further assays.

On completion of each extraction, each lens was again measured by UV spectrophotometry at 280nm in order to calculate the concentration of protein removed. The second absorption reading on the lens demonstrated the non-extracted bound protein and was designated (x).

6.6.6. Calculation of Extraction Results

The results given for both the PTS-1 and PTS-2 extraction studies are given presenting the maximum and minimum % removal values that were recorded. The % protein removed from each lens was calculated by the following equation.

$$\frac{i-x}{i} \times 100 = \% \text{ protein extracted/removed}$$

The measurements comparing the absorbance (i) against absorbance (x) are given without background blank lens subtractions due to the double readings involved, the resulting % protein removal thus remains unaffected. This reduces measurement error.

6.6.6.1. Results of Extraction on PTS-1 Spoilt Group IV Acuvue Lenses

Extraction Method	Pre-Extraction Absorbance (i)	Post-Extraction Absorbance (x)	% removed
a	0.462	0.345	25
a	0.437	0.357	18
b	0.432	0.103	76
b	0.421	0.085	80
c	0.393	0.345	12
c	0.388	0.269	31
d	0.441	0.075	82
d	0.371	0.040	89
e	0.428	0.401	N/A
e	0.406	0.391	N/A

Table 6.6. On lens absorbances pre and post extraction on PTS-1 spoilt lenses

The % removal figures were not calculated for extraction method (e) due to the fact that the differences between the pre- and post-extraction absorbances were minimal and in terms of concentration these figures represented only approximately 0.007mg/ml of protein. The distinction between the pre- and post-extraction figures border the standard deviation values of reproducibility.

6.6.6.2. Results of Extraction on PTS-2 Spoilt Group IV Acuvue Lenses

Extraction Method	Pre-Extraction Absorbance (i)	Post-Extraction Absorbance (x)	% removed
a	0.161	0.127	21
a	0.142	0.101	29
b	0.181	0.149	18
b	0.127	0.042	67
c	0.138	0.142	N/A
c	0.077	0.162	N/A
d	0.136	0.052	61
d	0.071	0.039	45
e	0.184	---	---
e	0.172	---	---

Table 6.7. On lens absorbances pre and post extraction on PTS-2 spoilt lenses

No post-extraction absorbances were obtained for method (e). This was as a consequence of attempting to obtain a significant reduction in post extraction absorbance. After 10 minutes of centrifugation the post absorbance reduction was minimal, thus prolonged centrifugation intervals were undertaken. However, after 1 hour of centrifugation the measure of reduction remained small and spinning for longer periods of time caused further problems i.e., at this point the lenses became very dry and sticky, making it difficult to remove the lens from the centrifuge tube without it breaking up and falling apart.

Consequently, it was decided that this method of extraction was not proficient in the removal of proteins in lens spoilation and that its capabilities were better employed in the extraction of the tear film from freshly removed lenses.

6.6.6.3. A Summary of the Extraction Efficiency Study on Group IV Acuvue Lenses

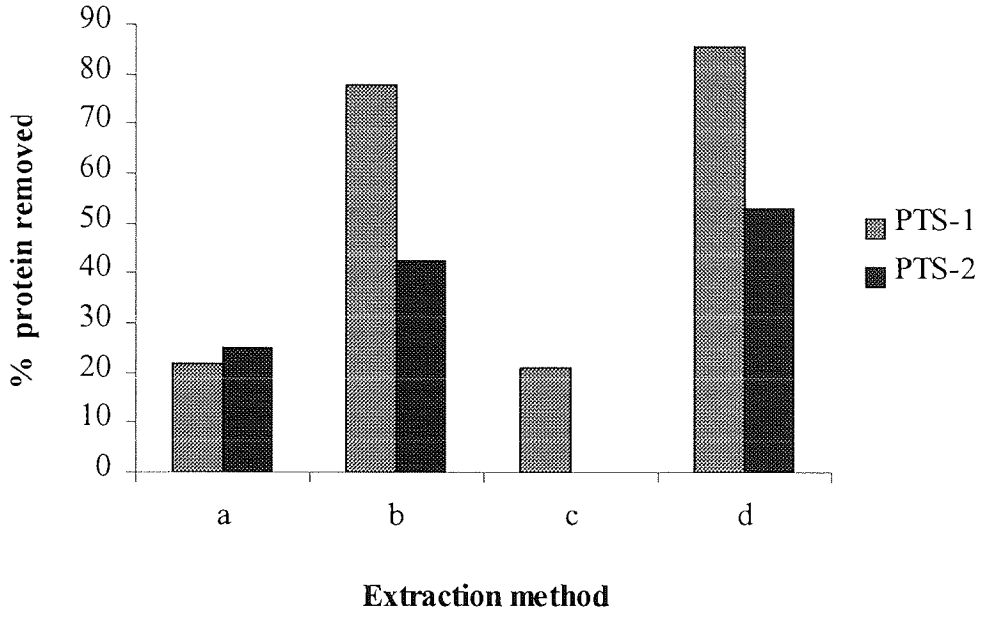


Figure 6.9. Results of efficacy of extraction methods on in vitro doped Acuvue lenses

The results of this experiment clearly showed that extraction method (d) which was made up with a combination of urea, SDS, DTT and Tris was the most efficient and effective method in the removal of protein from the lens surface under both PTS-1 (~85%) and PTS-2 (~53%) conditions. Overall method (b) results mirrored those of method (d), but to a lessor degree. However, the PTS-2 results were inconsistent with the maximum percentage removal at 67% and the minimum at 18%, and thus was deemed somewhat changeable. Both solutions contained SDS, DTT and Tris, but (b) was also made up with glycerol as opposed to urea in solution (d). The urea containing solution was the most effective. Therefore, from these results it was decided that extraction solution (d) would be the formula and method employed in the extraction of the study of *ex vivo* lenses.

Erroneous results were uniformly demonstrated by the application of method (c) with the PTS-2 solution, the reason for this remains unknown. The efficiency of this method was relatively inadequate in the removal of proteins from the lens on the PTS-1 doped lenses, added with the erratic behaviour of this method, this procedure was discontinued. Method (a) gave similarly poor, yet reproducible, extraction proficiency results and was also rejected.

For the two most effective extraction procedures (b) and (d) it is quite clear that the percentage removal was higher for the lysozyme containing solution, PTS-1 doped lenses. In PTS-1, lysozyme constitutes one third of the protein present with an approximate 85% removal efficiency. The non-lysozyme, PTS-2, doped lenses demonstrated only a 50% reduction. These results suggest that almost all of the lysozyme could be removed from the lens, leaving behind mainly the other main tear proteins in undefined quantities.

It was important to note the fact that the extraction method, although efficient, left behind a small percentage of bound protein. The following assays were designed only to investigate the protein eluted into solution, thus the nature of the irreversibly bound or trapped protein was left unidentified. In order to complete the picture, an on-lens immunofluorescence or cell based assay, for example, would provide an optimum means of assessing this group of non-eluting protein.

6.6.7. Extraction of Lysozyme Doped Group IV and Group II Lenses *in vitro*

As a precursor to the *ex vivo* studies and to correlate the above PTS-1 and 2 results, an assay was performed on *in vitro* lysozyme doped Acuvue and Medalist 66 lenses. The purpose of this experiment was to estimate the performance of lysozyme singly in the extraction studies. The above PTS-1 and PTS-2 comparison suggests that it was mainly lysozyme that was removed by method (d) and that only approximately 50% of the other proteins were removed. Additionally lysozyme is known to penetrate the lens matrix in Group IV lenses, a longer lens-in-solution incubation time was therefore allowed in order to assess the efficacy of method (d) in the extraction of matrix bound lysozyme. A comparison between Group II and Group IV lenses was performed.

Six Acuvue and Six Medalist 66 lenses were doped, on a shaker, in a 2mg/ml chicken lysozyme solution for 96 hours. On completion of the *in vitro* spoilation, the lenses were read by UV for the initial absorbance (i). The lenses were then extracted by method (d) and a further absorbance reading by UV was taken as the post-extraction measurement (x). The maximum and minimum (percentage removed) of the six lenses tested for both lens types are shown in Table 6.8.

Lens Name	Pre-Extraction Absorbance (i)	Post-Extraction Absorbance (x)	% removed
Medalist 66 - max	0.645	0.063	90
Medalist 66 - min	0.656	0.094	86
Acuvue - max	2.057	0.248	88
Acuvue - min	1.847	0.286	85

Table 6.8. Results of the extraction efficiencies on lysozyme doped Group IV and II lenses in vitro by method (d)

A noteworthy outcome of this assay was the greater accumulation of lysozyme on the Acuvue lenses after 96 hours of spoilation as opposed to 24 hours of spoilation, shown in the previous experiment. This result was reciprocated in the *ex vivo* clinical analysis earlier in this chapter with respect to overall protein accumulation, with a rapid increase in protein spoilation between 1 day and 1 weeks wear.

In the case of the Acuvue lenses, as stated earlier, lysozyme may penetrate the matrix, but from these results it would seem that most of the lysozyme can be extracted. This extraction method performed efficiently in the removal of lysozyme irrespective of the quantity of the protein deposited. In fact for both the Group II and IV lenses the extraction efficiency was very high, highlighting the ease of lysozyme removal from lenses by method (d).

6.6.8. Extraction of *ex vivo* Time Dependant Acuvue Lenses

The aim of this section was to apply extraction method (d) to *ex vivo* lenses and assess its efficacy on *in vivo* spoilt lenses. As a progression from the *ex vivo* time dependant spoilation studies, which only allowed us a total protein picture, the aim, was to employ this extraction method in order to be able to look at the individual protein spoilation characteristics. The spoilation studies presented an overall profile of deposition and spoilation characteristics, demonstrating the influence of the ionicity and water content of the contact lens and unpredictability of the patient involved. The next step was to take a closer look at the nature of the proteins on the contact lens over various wear regimes.

Initially the lenses were read by UV at 280nm (i) and then each lens was placed in 200µl of the extraction solution (d) in an eppendorf and heated to 90°C for 3 hours. The lenses were rinsed in distilled water, read again (x) by UV and then stored in distilled water. The eluate was thus ready for further analysis. The eluate itself could not be read by UV for total protein content due to the low volumes involved. A 200µl sample could not be read in the cuvettes, thus to calculate the percentage removal a lens absorbance (i) versus absorbance (x) was performed.

Sets of lenses from three patients were chosen for the extraction studies from the time dependency study, as dictated by the previous total protein clinical studies to best represent the sample group as a whole.

Patient 1 Acuvue Lenses			
Wear Time	Pre-Extraction Absorbance (i)	Post-Extraction Absorbance (x)	% removed
1 Day	0.443	0.095	78
1 Week	0.920	0.277	70
2 Weeks	0.923	0.144	84
3 Weeks	0.931	0.215	77
4 Weeks	1.139	0.211	81

Table 6.9. Method (d) extraction on patient 1 ex vivo Acuvue lenses

Patient 1 Medalist 66 Lenses			
Wear Time	Pre-Extraction Absorbance (i)	Post-Extraction Absorbance (x)	% removed
1 Day	0.084	0.067	N/A
1 Week	0.086	0.067	N/A
2 Weeks	0.086	0.071	N/A
3 Weeks	0.094	0.081	N/A
4 Weeks	0.095	0.077	N/A

Table 6.10. Method (d) extraction on patient 1 ex vivo Medalist 66 lenses

Patient 2 Acuvue Lenses			
Wear Time	Pre-Extraction Absorbance (i)	Post-Extraction Absorbance (x)	% removed
1 Day	0.631	0.163	74
1 Week	0.898	0.363	60
2 Weeks	0.994	0.222	78
3 Weeks	0.933	0.087	91
4 Weeks	0.773	0.145	81

Table 6.11. Method (d) extraction on patient 2 ex vivo Acuvue lenses

Patient 2 Medalist 66 Lenses			
Wear Time	Pre-Extraction Absorbance (i)	Post-Extraction Absorbance (x)	% removed
1 Day	0.079	0.063	N/A
1 Week	0.078	0.073	N/A
2 Weeks	0.111	0.083	N/A
3 Weeks	0.088	0.081	N/A
4 Weeks	0.103	0.098	N/A

Table 6.12. Method (d) extraction on patient 2 ex vivo Medalist 66 lenses

Patient 3 Acuvue Lenses			
Wear Time	Pre-Extraction Absorbance (i)	Post-Extraction Absorbance (x)	% removed
1 Day	0.452	0.078	83
1 Week	1.025	0.190	81
2 Weeks	1.049	0.088	92
3 Weeks	1.007	0.144	86
4 Weeks	1.184	0.172	85

Table 6.13. Method (d) extraction on patient 3 ex vivo Acuvue lenses

Patient 3 Medalist 66 Lenses			
Wear Time	Pre-Extraction Absorbance (i)	Post-Extraction Absorbance (x)	% removed
1 Day	0.074	0.069	N/A
1 Week	0.068	0.060	N/A
2 Weeks	0.081	0.070	N/A
3 Weeks	0.087	0.080	N/A
4 Weeks	0.080	0.078	N/A

Table 6.14. Method (d) extraction on patient 3 ex vivo Medalist 66 lenses

The extraction procedure (d) proved very effective in the extraction of the *ex vivo* Group IV Acuvue lenses, a similarly high percentage removal was displayed as was seen for the *in vitro* lenses. The percentage removal efficiency was irrespective of time or total protein quantity. On the other hand, there was very little extraction influence on the deposited protein on the Group II lenses, as very little if any protein was removed. The already low levels of protein deposition on the Medalist 66 lenses made the extraction of these Group II lenses difficult. The percentage removal calculations were not achieved on these lenses due to the low absorbances and the resultant low pre- and post-absorbance differences, which would be of poor statistical significance.

6.6.9. Investigation into *ex vivo* Group II (Medalist 66) and Group IV (Acuvue) Lenses Eluates

Studies were thus undertaken on the eluates of the lens extracts to investigate a more precise nature of the proteins removed. It should be noted at this point that while the method (d) extraction showed a high percentage of efficacy in the extraction of proteins from the lenses, a small percentage of protein remained bound. Thus, the eluate would not be wholly representative of the deposited protein but only of the reversibly bound protein.

6.6.9.1. Analysis by SDS-PAGE

The initial analysis of the lenses eluates was done on SDS-PAGE. The gel shown in Figure 6.10. represents a single patient's data, but was indicative of the other results displayed by the other patients.

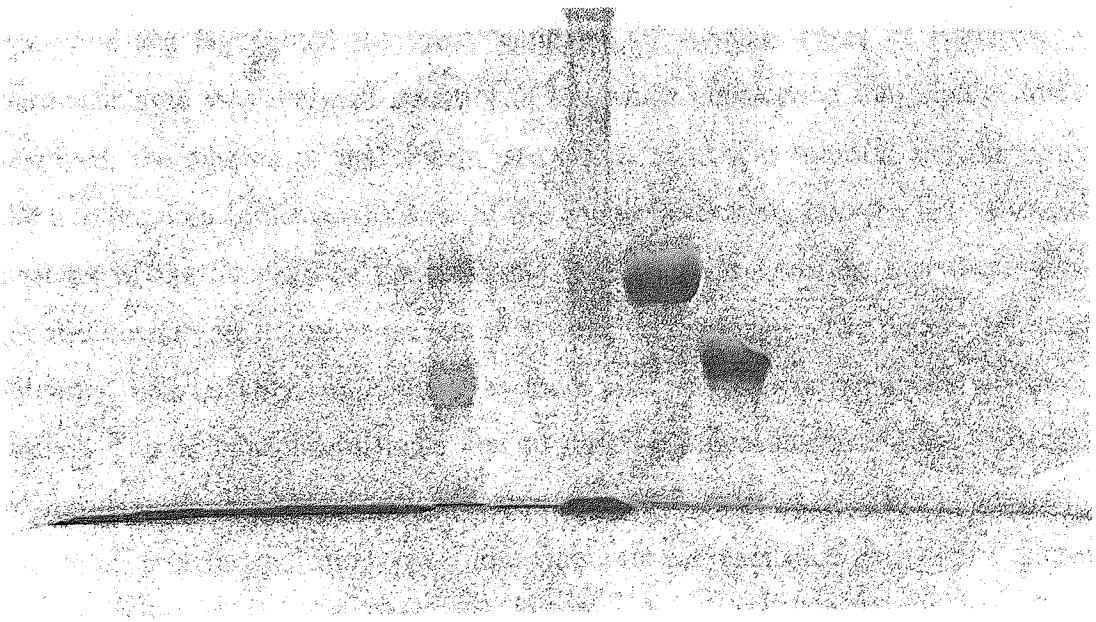


Figure 6.10. SDS-PAGE of Group II and IV lens eluates

Well Number	Protein Analyte	Well Number	Protein Analyte
1	1 Day Acuvue	9	2.0mg/ml Lactoferrin
2	1 Week Acuvue	10	1.5mg/ml Albumin
3	2 Weeks Acuvue	11	1 Day Medalist 66
4	3 Weeks Acuvue	12	1 Week Medalist 66
5	4 Weeks Acuvue	13	2 Weeks Medalist 66
6	1mg/ml IgA	14	3 Weeks Medalist 66
7	0.5mg/ml Lysozyme	15	4 Weeks Medalist 66
8	Standards Marker		

Table 6.15. SDS-PAGE well key for Group II and Group IV lens eluates extracted by method (d)

No protein removal was detected at all for the Medalist 66 lenses, the Acuvue lenses on the other hand demonstrated high quantities of lysozyme in the eluate, but this was the only protein detected. The SDS-PAGE gel again proved useful in the detection of lysozyme and highlighted the gross spoilation of Acuvue lenses by lysozyme. Of particular note was the high intensity of the bands considering the dilution factors involved, the dilution of the protein into 200µl of eluting solution and the double dilution required in the preparation of the eluate for loading into the gel. By visual analysis of the intensity of the lysozyme bands, it was apparent that there was a greater quantity of lysozyme present in 1 week versus 1 day. The pattern of greater intensity, i.e., quantity, stops there, as there was no trend in the spoilation after 1 week. This is most likely to be due to different removal efficacy as opposed to representing the actual deposition characteristics, but as was seen in the analysis of the total protein spoilation over time the rate of spoilation reached a plateau somewhat after the 1 week wear.

The SDS gels provided limited information on the spoilation characteristics of each lens and, consequently, further examination was required. Immunodiffusion assays and, in particular, the detection assay counter immunoelectrophoresis was employed to attempt to give a more in depth analysis of the eluates.

6.6.9.2. Analysis by Counter Immunoelectrophoresis (CIE)

Due to the relatively low detection limits of SDS-PAGE, which revealed only lysozyme from the Group IV lenses, the obvious step was to exploit the greater sensitivity and specificity of the immunodiffusion assays. CIE was used as a detection assay to ascertain the individual nature of the proteins involved in the spoilation process, and to observe any patterns of deposition over time. Patient 3 extracts were used to illustrate the outcome of this set of experiments and was representative of the overall results. Investigation into the eluates of all the Medalist 66 lenses yielded poor results, and are not shown. This was to be expected due to the low levels of extracted protein that were achieved.

The following assays describe results found in the eluates of the Group IV Acuvue lenses. Each gel analysed all five of the timed intervals against the antibody specific to the protein under investigation. As a large quantity of eluate was available, 5µl of the eluate was added to each well according to the described template, and allowed to diffuse downward for 1½ hours. Each gel underwent electrophoresis for 1 hour on the Paragon system. The gels were then washed, dried, stained and destained as usual.

The main disadvantage of CIE is the lack of quantitative results, concentrations of proteins detected are simply relative on each gel. The intensity of each band on a particular gel against the same antibody can be compared against each other and a relative concentration can be obtained. Embossed versions of each gel scan are shown in Appendix III, in order to enhance visual analysis of each band. Unfortunately, there was no means of measuring the relative intensity the bands on either the embossed or stained versions of the gels. The use of densitometry was not feasible, the parameters of error would be too great, with difficulties in subtracting background absorbance and the variation of band intensity across the, somewhat curved, line of precipitation. CIE was not designed to calculate the concentration, but rather to detect particular proteins in complex mixtures, e.g. tears and serum. A control of the antibody against a specific concentration of the antigen analyte was run

with each gel in order to provide an approximate comparison and indication of the intensity of a known concentration. In order to aid the interpretation of the lines of precipitation an arbitrary scale of band assessment on the CIEs was devised, although a crude method of analysis, it provided a means of comparing and correlating the results. By visual analysis and interpretation of the band intensity a scale was set up assessing each line of precipitation. The scale chosen was defined by 0-20, 0 signifying no band traceable up to 20 of very high control intensity. The scale was designed to be interchangeable between each gel, but it must be noted that a direct comparison of one gel against another is not valid due to the differences in antibodies utilised in each gel. The results of this visual analysis on all four CIEs is detailed, after the individual gels are presented, in Table 6.14.

6.6.9.2.1. Analysis of Patient 3 Acuvue Eluate for Albumin Content by CIE

Polyclonal goat anti-human albumin antibody was used to identify albumin in the lens eluates.

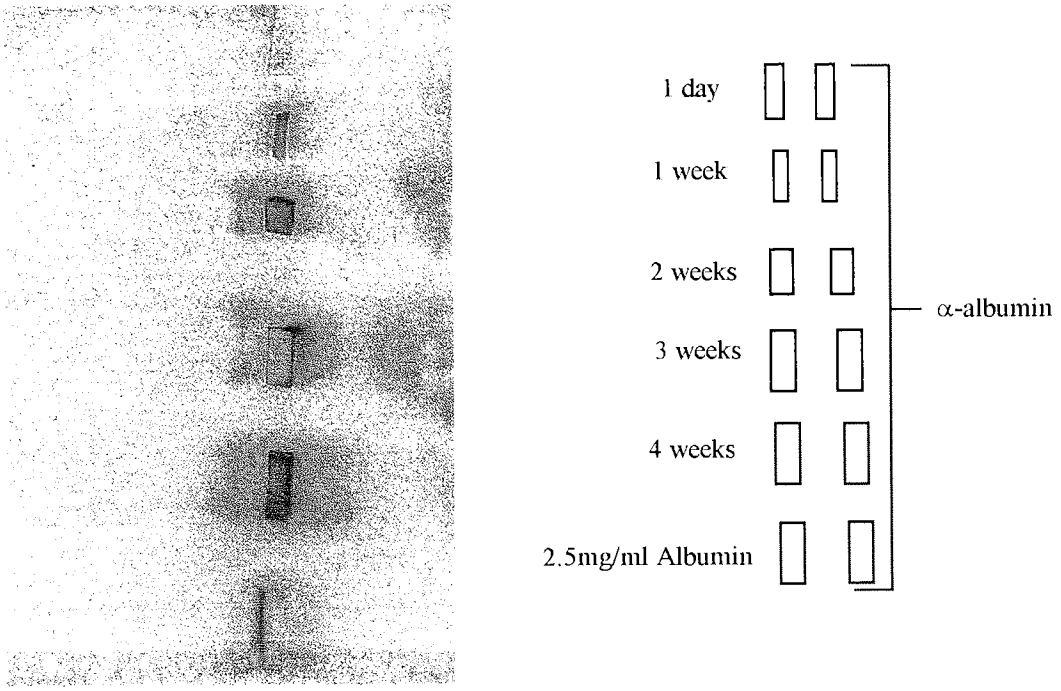


Figure 6.11. CIE of eluate comparing albumin levels on 1 day - 4 weeks wear Acuvue

This gel was representative of all three patients tested, with a line of antibody:antigen immunocomplex precipitate demonstrable at 2, 3 and 4 weeks wear, and with no line evident at 1 day or 1 week. The intensity of the band against the 4 weeks eluate appeared slightly greater than the other two bands, which indicated that albumin even after 4 weeks wear was still adsorbing onto the contact lens. The result of this analysis demonstrated the fact that albumin in the eluate was detectable only after 2 weeks wear and was present thereafter in the eluate of longer wear times. This suggests an accumulation of albumin over time, a build up which may indicate a non-changing bound layer of protein which is added to over time, and is not in constant variance.

6.6.9.2.2. Analysis of Patient 3 Acuvue Eluate for Lactoferrin Content by CIE

Polyclonal rabbit anti-human lactoferrin antibody was used to identify lactoferrin in the lens eluates.

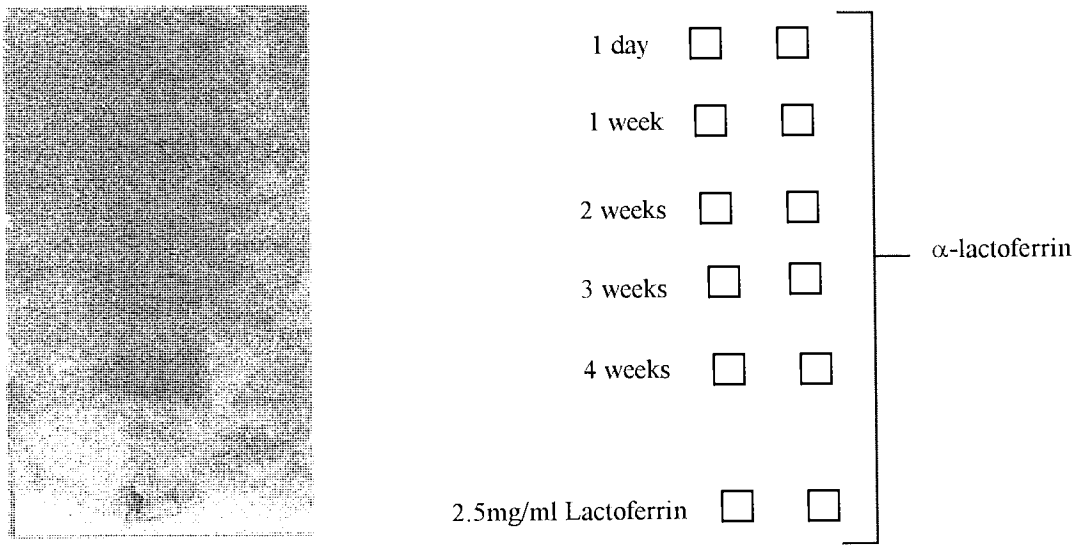


Figure 6.12. CIE of eluate comparing lactoferrin levels on 1 day - 4 weeks wear Acuvue

This gel demonstrated a reaction of identity by the anti-lactoferrin antibody against all the eluates with the exception of the 1 day eluate. The initial time of detection of lactoferrin was on 1 week wear, with similar bands of intensity visible at 2 and 3 weeks. The band intensity of the 4 week eluate appeared a little more intense than those seen in the other band eluates, with the possible evidence for the continued deposition of lactoferrin over time.

6.6.9.2.3. Analysis of Patient 3 Acuvue Eluate for IgA Content by CIE

Polyclonal goat anti-human IgA antibody was used to identify IgA in the lens eluates.

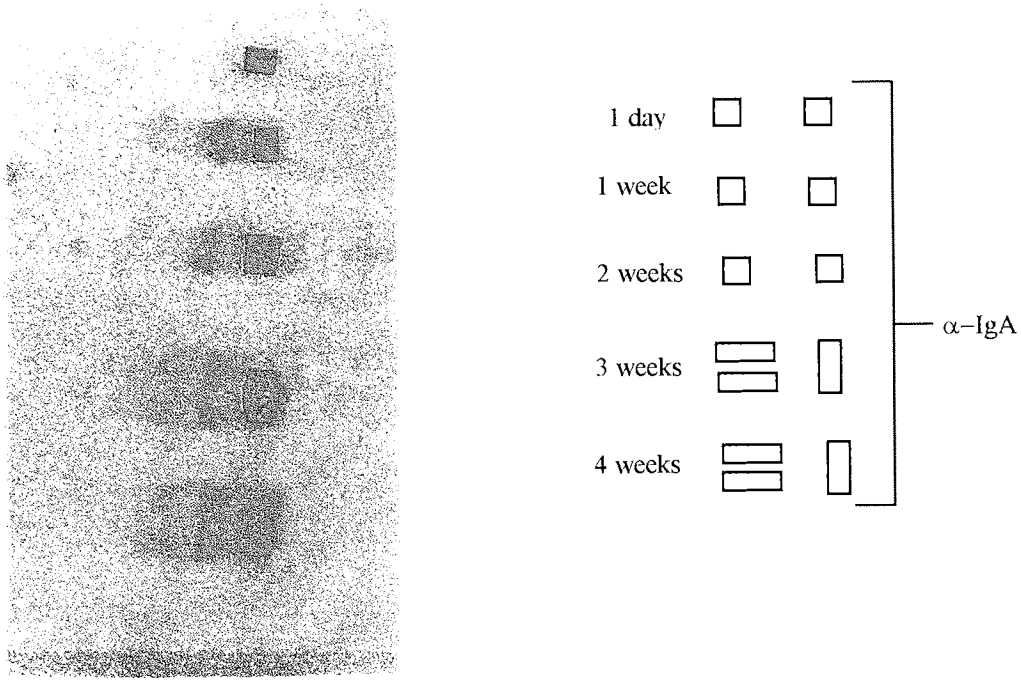


Figure 6.13. CIE of eluate comparing IgA levels on 1 day - 4 weeks wear Acuvue

The results of this gel were similar to those seen on the lactoferrin CIE run with a reaction of identity observed for the 1 week through to 4 weeks, with no IgA detected after 1 day wear. However, the intensity of all four bands appear to be of a relatively similar intensity offering the possibility that the levels of IgA seen at 1 week reach a plateau and there is no further increase in the IgA levels after this time. There also exists the possibility that the extraction method employed was efficient in removing only a certain unchanging quantity of IgA and further bound IgA may remain irreversible bound onto the lens.

6.6.9.2.4. Analysis of Patient 3 Acuvue Eluate for IgG Content by CIE

Polyclonal goat anti-human IgG antibody was used to identify IgG in the lens eluates.

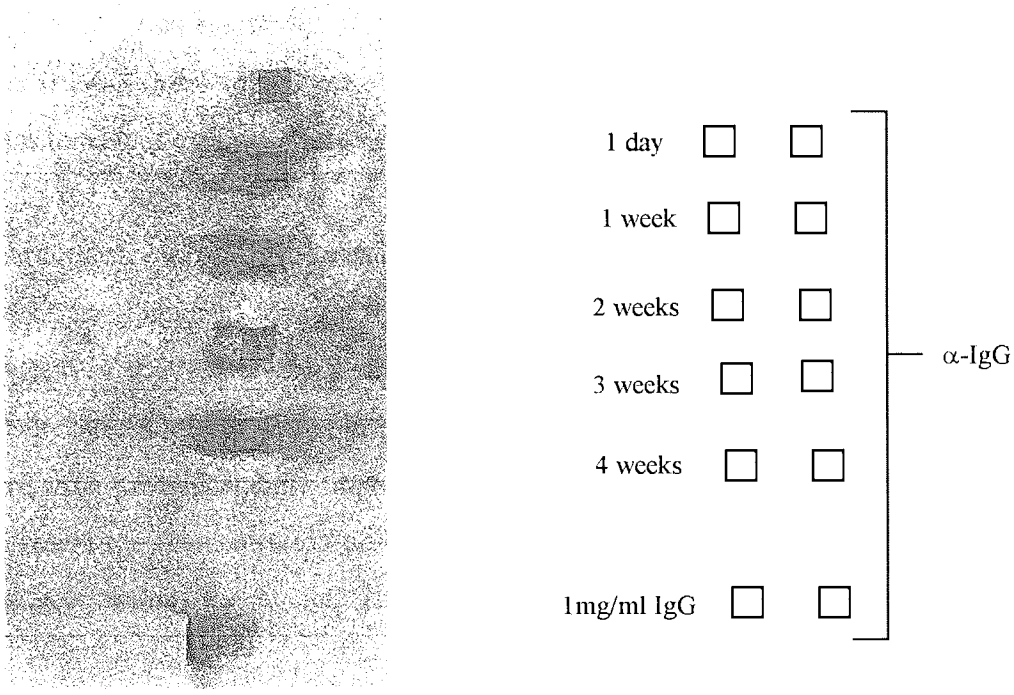


Figure 6.14. CIE of eluate comparing IgG levels on 1 day - 4 weeks wear Acuvue

A reaction of identity can definitely be seen between the IgG antibody and the 1 week through to 4 weeks wear eluates, and although not particularly clear in the scan of the gel above, the original gel demonstrated a faint line of precipitate between the IgG antibody and 1 day wear eluate. As was observed in previous gels, the line of precipitation against the 4 week eluate was more intense than the shorter wear periods.

6.6.9.2.5. Conclusions

An important point to understand when discussing and interpreting the results of the CIE assays was that the detection or non-detection of proteins in certain eluates was defined by the sensitivity of the assay. The sensitivity levels of CIE are approximated at 0.1-0.3μg/ml of antigen, thus defining the limit of the results, below which the proteins in the eluate could not be detected.

In order to correlate the results of each gel an arbitrary scale of the visual analysis of each band intensity was set up, the results of which are shown in the table below. A graph of the results was also drawn but it must be noted that over the scale of 1-12 calculated the differences in band intensity appear more exaggerated. A comparison of each protein against the scale relates to the concentration detected but for each protein the concentration non-comparable, with the 1-12 scale representing different concentrations for the individual protein.

	Albumin	Lactoferrin	IgA	IgG
1 Day	0	0	0	2
1 Week	2?	7	9	9
2 Weeks	10	10	10	10
3 Weeks	8	7	8	7
4 Weeks	10	13	10	12
Control	18	20		20

Table 6.16. Results of Acuvue lens extracts on CIEs for albumin, lactoferrin, IgA and IgG calculated visually on a 0-20 scale of precipitation intensity

These results demonstrated that as a general rule both the plasma and tear derived proteins were deposited and extracted quite similarly. With the possible exception of IgG, where a faint, questionable line of precipitation was evident, there was no protein detectable after 1 day of wear for each of the proteins. All the 1 week wear periods, with the exception of albumin, displayed a band of precipitation, but all four proteins clearly demonstrated a line of identity with 2 and 3 weeks wear. For 4 weeks wear a further increase in intensity from the 2 and 3 week bands was observed for both lactoferrin and IgG, while the albumin and IgA retained equal intensity as observed for the 2 and 3 weeks wear times.

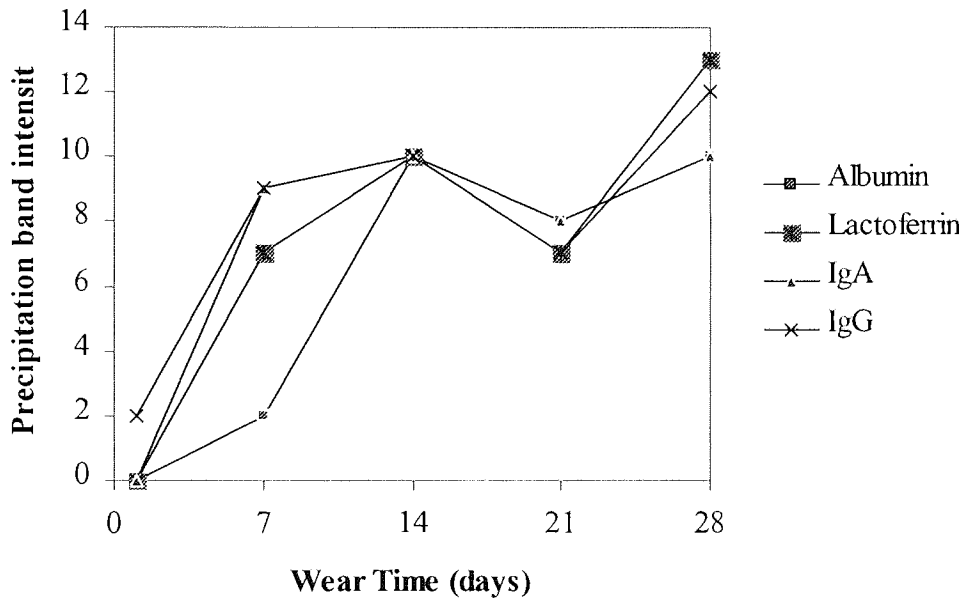


Figure 6.15. Results of Acuvue lens extracts on CIEs for albumin, lactoferrin, IgA and IgG calculated visually on a 0-20 scale of precipitation intensity

6.7. Kinins in Contact Lens Wear

The aim of this section of work was to consider the presence, or lack of, of the kinins in the ocular environment and in particular in relation to contact lens wear. To date no work has been done on kinins in this area but due to the increased awareness of plasma protein leakage into tears, the overall change in tear protein composition and the modification of the physical parameters experienced during sleep and contact lens wear, its presence was assessed. The kinins may prove to be present in parallel/conjunction with the derivation of vitronectin in the sub-clinical state of the closed eye environment. A commonality may be seen between vitronectin and the kinins with their potential to regulate inflammatory responses, both are plasma derived and both have an affinity for glass. Additionally, and more importantly the recent discoveries of the kinins in allergic reactions make the investigation into their detection or non-detection enticing.

6.7.1. Background

The kinin system and its role in inflammation were briefly explained in Chapter 1, the consequences of kinin activation include vascular permeability, vasodilatation and pain. They are ubiquitous mediators that produce many of the primary presentations of inflammation. It was the mode of activation of the kinins which was of particular interest in this study. Activation of the kinin generation system is achieved on contact with a variety of negatively charged surfaces, significantly contact lenses are anionic and may prove no exception.

The role of vitronectin in the closed eye and in contact lens spoilation has been under scrutiny recently, due to its important regulatory role in inflammation. It can inhibit complement mediated lysis and stabilise PAI-1, for example. Vitronectin has been shown here to adsorb onto the contact lens surface creating a vitronectin rich micro-climate, a significant observation due to its multi-regulatory role. In parallel with the studies and findings on vitronectin in relation to contact lens wear, its presence in tears and its mediation in inflammation, the role of kinin, if any, in tears was assessed.

Elsewhere in the body the kinin system has gained high interest due to its role in various disorders and in particular allergic responses. During allergic rhinitis response, HMW and LMW kininogens have been detected in nasal secretions.[122] Recent studies have also demonstrated kinin responses in bronchoalveolar fluids in asthmatics,[123] and bradykinin was shown to cause an increase in pulmonary inflation pressure, mucus secretion and breathlessness.[124] As yet their physiological role in these responses remains uncertain, it is not sure whether they are the causes or the consequence of the adverse host response. However these studies highlight an increasing significance of the kinins at mucosal surfaces and secretions, further pushing the importance of assessing their role (if any), at the ocular surfaces.

6.7.2. Kininogen as a Marker for Kinin Activity

High molecular weight kininogen was the glycoprotein of choice in the analysis of kinins in the ocular environment. This entity was used as a marker of kinin activity for the following reasons:

- Both antibody and antigen commercially available
- Affinity, and activation requirement, for negatively charged surfaces
- Key kinin family glycoprotein
- Implicated in a variety of disorders and allergic responses

6.7.3. Method of Analysis in the Study of the Kinins

Kininogen was not detected in the tears of open eye subjects in this study, this was to be expected due to the fact that if kinin was in open eye tears the levels would be way below the sensitivity levels of the assays employed. It is foreseen that if kininogen or any of the kinin family is discovered in the normal open eye tear, the concentrations would be minimal. However, crucially as a consequence of this non detection, optimisation of a kininogen immunodiffusion assay could not be achieved. Closed eye tear samples were scarce and understandably were not used to define an assay, therefore no work was done at this time on kininogen in closed eye tears.

The answer was to exploit the affinity of the kinin system for negatively charged surfaces and in particular the contact lens. The concept was that if the kinins did permeate into tears through plasma influx, or via the tissue bed, then the contact lens may provide a means for it to accumulate into sufficient quantities that made the protein detectable.

6.7.4. Method

According to the extraction studies protocol detailed earlier in the investigation of IgA, IgG, lactoferrin and albumin on Group IV daily wear Acuvue lenses, lenses worn for 1 day and 4 weeks were extracted and tested for kininogen. CIEs were employed due to their high sensitivity and predetermined ability to detect proteins in contact lens eluate.

6.7.4.1. Detection of Kininogen in the Eluate of *ex vivo* Group IV Contact Lenses by CIE: Plate-1

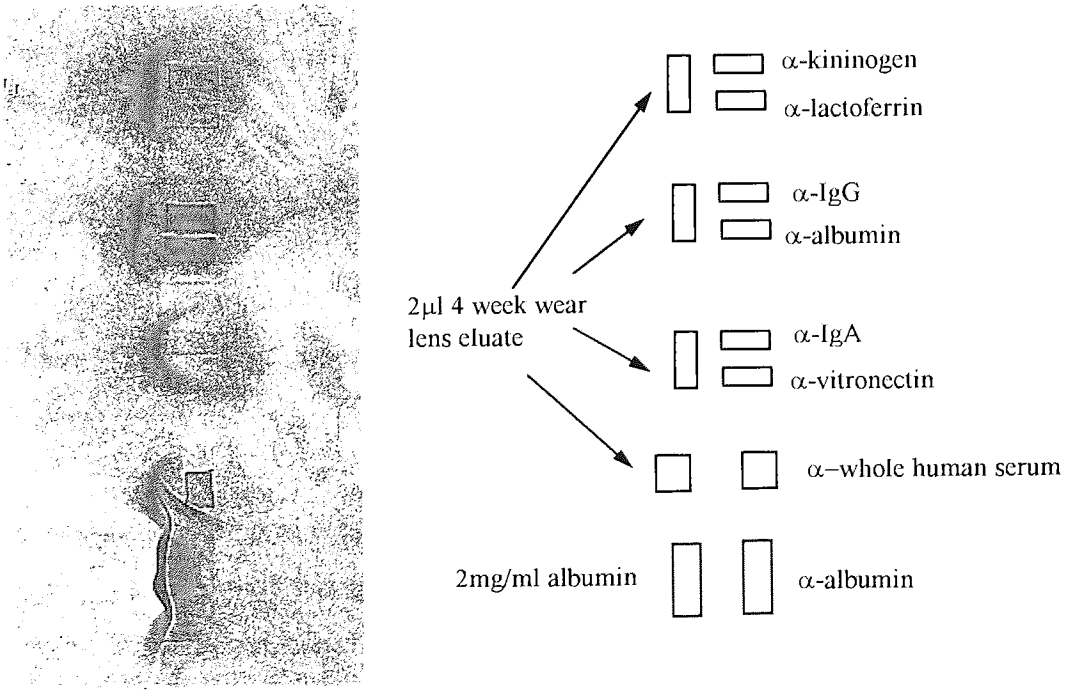


Figure 6.16. CIE of proteins from 4 weeks wear Group IV Acuvue eluate including kininogen: Plate-1

This gel was slightly ambiguous in its assessment of kininogen's presence in the eluate due to its proximity to lactoferrin in the adjacent antigen well. Consequently another gel was performed with kininogen and the other controls in separate wells to avoid cross contamination of results and thus negate false positive results. However, it would seem that kininogen was detected along with the other previously detected proteins in this 4 weeks wear eluate.

6.7.4.2. Detection of Kininogen in the Eluate of *ex vivo* Group IV Contact Lenses: Plate-2

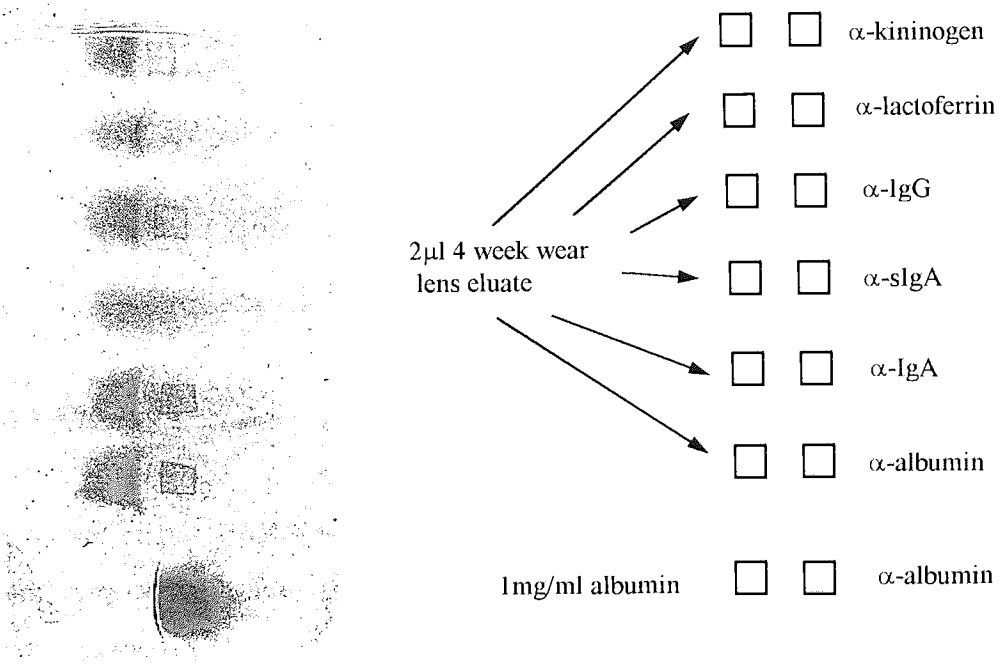


Figure 6.17. CIE of proteins from 4 weeks wear Group IV Acuvue eluate including kininogen: Plate-2

A band of identity was observed between the anti-kininogen antibody and the eluate. Again, the results of this gel would seem to suggest that kininogen was originally present in the ocular environment, adsorbed onto the contact lens, was removed by extraction and was thus detectable in the resultant eluate. An embossed version of this gel is provided in Appendix III to enhance the visualisation of the kininogen band.

No kininogen was detected in the eluates of the one day worn lenses, highlighting the build up that occurs over time for kininogen in correlation with the total protein deposition accumulation patterns.

6.7.5. Conclusion

Kininogen was present in the eluate of Acuvue Group IV lenses. Consequently it could be said that the kinins are present to some degree in tears. The implications of this discovery are many, in that like vitronectin on the surface of a lens, the accumulation and persistence of these glycoproteins in the ocular surface may be deleterious or advantageous to the host. A further understanding of the mode of actions of kinins in general is required in relation to adverse complications in host responses. However, it is quite reasonable to infer that the kinins in the ocular environment, especially with lens wear may prove to be significant in the allergic response. Additional and subsequent studies need to be performed in order to examine the kinins in tears more comprehensively.

6.8. Overall Summary

The aim of this chapter was to identify some of the parameters that affected the rate and type of deposition on soft contact hydrogel lenses. The main points observed were as follows:

- The initial experiment on the *ex vivo* lenses that compared the deposition characteristics of Acuvue, Surevue and Focus lenses detailed some interesting points regarding protein deposition on these Group IV lenses. The results showed a greater deposition on Surevue lenses followed by Acuvue and then Focus lenses. The Surevue and Acuvue lenses differ only in thickness, the water content and lens materials are the same, this would suggest that some protein moves into the lens matrix and more so in the case of Surevue. Further work by the group investigating these lenses by the surface analysis technique fluorescence found relatively similar quantities of total protein on the lens surface for both Surevue

and Acuvue. This would seem to substantiate the idea that the some of the tear proteins were permeating the lens matrix and to a greater extent in the thicker Surevue lenses. Work has already been done on Group IV lenses revealing that the small positively charged protein lysozyme, one of the primary Group IV depositors, can penetrate the lens matrix. Thus it is probable that the thicker Surevue lenses facilitates the greater influx of lysozyme and possibly other proteins. Previous research in this laboratory demonstrated that it was the charge rather than the size of lysozyme that produces its high deposition characteristics and increases its uptake.

- A comparison between the HEMA/MA compositions of both the Surevue and Acuvue lenses and the HEMA/MA/PVP of Focus revealed a difference in the deposition levels. As a general rule, the Focus lenses displayed the lowest levels of protein spoilation, the mean concentration of deposited protein for Focus was at 0.225mg, as opposed to 0.487mg and 0.545mg for Acuvue and Surevue lenses respectively. The influence of the addition of PVP in the lens material composition can be seen in these *ex vivo* lenses. The water content of the Focus is 55%; only 3% lower than the other two lens types and thus may not be a major influence on the outcome. The inclusion of PVP into the lens composition may be the main factor in increasing protein resistance or it may be due to the resultant decrease in the percentage MA in the matrix. However, it is clear that a compositional change affects the interaction between biomaterial and protein and in the long term the performance and durability of the contact lens.

- The effects of material composition were again evident with regard to the investigation into the spoilation of the Group II versus Group IV lenses, Medalist 66 and Acuvue respectively. Both these lens types possessing high water contents but the former is a non-ionic lens; the ionicity of the lens greatly influences the deposition characteristics of the lens. An approximate ten-fold increase in the protein levels deposited were observed going from the non-ionic to the ionic lenses.

- Time was observed to be an important factor in the deposition rate of proteins. Whereas the initial study assessed various parameters, the effect of wear over time was not taken into consideration, all the lenses were measured after a one month wear period. Consequently a study was set up to assess the rate of deposition over a 1 day through to a 28 day time interval. The effects were assessed on both Group II and Group IV lenses. The results showed that for the Group IV (Acuvue) lenses the protein deposition levels after an initial average 0.2mg of total protein on the lenses after 1 day wear increased to an average 0.45mg after 1 week. The average levels of deposition began to stabilise thereafter for the 2, 3 and 4 weeks wear. The Group II lenses did not appear to be influenced by time, the initial protein levels that were measured after 1 day wear were not greatly exceeded; the deposition levels remained static at approximately 0.03mg of protein. At such low levels of protein detected variations in deposition levels were somewhat insignificant.

- Patient influence is a big factor in affecting the resultant levels of protein measured as shown in Figures 6.3. - 6.7. all of which demonstrate the inter patient variation. One of the better examples is exhibited in Figure 6.3. which presents all the deposition concentrations displayed in a population of 21 patients for three lens types, where the standard deviation between patients can be as much as 25% about the mean. The left and right eye values in individual patients was also addressed where a difference in protein levels could be measured. The two points emphasise the importance in defining the patient population and noting the variety of results that can emerge simply due to patient influence. Care must be made in the analysis/quoting of individual patient's results.

The deposition findings were important in defining the overall characteristics of protein-material interactions but the results were indicative only of total protein values. The next step was to attempt to define the individual nature of the spoilant. Due to the fact that the immunodiffusion assays had previously been optimised for tear analysis, the obvious step was to attempt to employ the assays in the investigation of the lens deposits. Thus a means of efficiently extracting the protein from the lens

was required in order to take advantage and assess application of these assays. This was achieved by sampling many different methods of lens extraction. The most efficient method discovered here involved heating the lenses to 90°C for 3 hours in a mixture of urea, sodium dodecyl sulphate, dithiotheriol and tris[hydroxmethyl]aminomethane (method (d)).

The extraction efficiencies, by method (d), of the various lenses on trial, provided the following patterns of protein removal:

- Initially *in vitro* spoilt Acuvue lenses were used to assess the efficiencies of each extraction method. The results observed with the method (d) extractions proved interesting. Three different doping solutions were made up and used to spoil the Group IV lenses, (a), a pseudotear solution containing the main tear proteins provided an approximate 85% extraction efficiency, (b), a solution similar to (a) minus lysozyme which only allowed a 53% extraction efficiency and (c), a lysozyme only solution which gave an approximate 87% removal efficiency. This would seem to suggest that in the main lysozyme was easily removed but the other proteins were more difficult to extract, leaving behind an important deposition profile.

- Method (d) was then applied for use in the extraction of *ex vivo* lenses, the Acuvue and Medalist 66 lenses analysed in the time dependency study were examined. Results of the extraction proved that method (d) was less than efficient in the extraction of the low quantities of protein from the Medalist 66 lenses. The Acuvue lenses on the other hand were readily desorbed of protein providing similar percentage removal efficiencies as observed in the *in vitro* lens extractions.

The resulting eluates were tested for IgA, IgG, lactoferrin, albumin and kininogen where all five proteins were detected in the eluates, demonstrating their involvement to some extent in protein spoilation. Noting at this point, from the above percentage removal efficiencies, that the proteins detected in the eluate were not a full

representation of the true levels of the lens deposits due to the nature of the difficulty in removing all the protein from the lens.

All five proteins were detected in the eluates of the Group IV lenses, demonstrating the sensitivity of CIEs in the detection of protein in a 200µl eluate. The CIE results also illustrated the patterns of deposition displayed by each of the four proteins, IgG, IgA, lactoferrin and albumin on these daily wear lenses. The most distinctive and consistent observation to be made was the clear rise in levels after one day wear. An interesting follow on step would be to examine the deposition rate, and gauge the rise, from one day through to seven days inclusive, under the conditions set in this study.

Finally the fifth protein discovered and assessed in the eluates of the Acuvue lenses was HMW kininogen. A glycoprotein previously undetermined in tears and in lens wear. The uncovering of the presence of kininogen in relation to contact lens wear further emphasises the potential inflammatory responses that can occur during lens wear. In particular in allergic responses, the kinins may be at the fore in creating adverse complication. The reality of their presence remains to be unearthed and resolved. However, further work is required in this area, to substantiate the results and determine the significance.

Chapter 7

Discussion and Future Work

7.1. Discussion

Many techniques are currently available and employed in tear protein analysis and our knowledge of the complex nature of tears is increasing at a steady pace, but there remains many unanswered questions. The aim of this thesis was to investigate tear proteins in a variety of tear states with a view to understanding their presence in tears and their interactions with contact lenses. Many variables were encountered throughout this investigation including patient influence, tear collection methodologies, the effects of contact lens materials and the method of analysis. The methods of analysis of choice employed in this work was in the application and exploitation of the highly specific and sensitive probe - the antibody in immunoassays.

The advantages in applying antibodies as an investigative probe are numerous, for example, sensitivity, highly diverse in their applications and ease of interpretation. Specifically in this research, the antibody approach that was employed was in the form of the immunodiffusion assays which were assessed and developed for various tear protein investigations and their interactions with a variety of contact lens materials. It was their requirement for small sample volumes - volumes as low as 1μl could be investigated, that proved the primary advantage in the application and use of these antibody based assays.

One drawback that was encountered by antibody analysis was commercial antibody availability, as shown in regard to the relatively poorly understood tear lipocalins; these species of newly defined proteins need to be isolated and purified in order to raise their specific antibody, in order that the antibody can be employed to study the lipocalins in tears. Thus the immunoassays are limited by the availability and ease of production of the antibody and in the case of quantitative assays, the antigen additionally must be available; currently neither the tear lipocalins or its antibodies are yet available commercially.

Open eye tear samples were investigated in order to assess and develop the use of the immunodiffusion assays in tear analysis. A combination of the various immunodiffusion assays utilised provided an informative picture of particular tears with minimal sample volumes required. It was found that approximately 2μl was required for individual protein quantification by RID or RE. For detection and identification purposes a single 2μl sample could identify up to 6 proteins by Ouchterlony and a kaleidoscope of proteins by IEP. For greater sensitivity an approximate 1-2μl tear sample was adequate to identify and detect specific individual proteins by CIE.

The immunodiffusion assays provided clear, reliable and reproducible results in the analysis of the main tear proteins. The mean open eye concentrations for the proteins investigated were comparable with those generally perceived, with the exception of albumin. Continually, during the course of various studies involving open eye tear albumin determination, levels were found to be lower than those commonly quoted, i.e., a 1.3mg/ml average, with an approximate mean concentration of 0.05mg/ml constantly determined. The RE assay employed in its measurement was very sensitive, as shown with regard the closed eye work, and care was taken during tear collection, and thus it is suggested that the mean levels of albumin in basal tears may actually be somewhat lower than previously thought.

The macro-SDS-PAGE system was also utilised in open eye tear analysis but was found to be unpredictable, unreliable, and with a requirement for sample volumes in excess of 7μl, was deemed unsuitable.

During the determination of the open eye tear samples the phenomenon of the automatically stimulated tear (AST) subjects was recognised, representing a population of patients who automatically presented a certain level of reflex tears, irrespective of the use of the non-invasive microcapillary method to reduce this phenomenon. This state involved a rush of excess tearing initiated on attempting to extract a tear sample, on occasion even prior to starting sampling! The AST sample obtained was an automatic

nervous reaction encountered during sample collection. It was reasonable to assume that this tear rush reduced the levels of proteins detectable, creating a dilution factor reflecting a lower total protein concentration than would be a fair representation of the true tear protein levels of that patient in the normal tear.

In Chapter 2 whilst assessing the optimum tear collection method this point was addressed; the results of an AST sample versus a normal tear investigated by CIE highlighted a visible difference between these two patient types with regard to protein concentrations. Quantitatively, during the course of the open eye tear analysis, the AST samples were continuously observed to display lower protein levels, notable for both tear and plasma derived proteins. The significance of the AST samples in the main was their effect on overall mean results, especially in small population trials. This set of subjects, if present in a study, would lower the mean concentrations calculated, and possibly create a false minimum result. Thus, it was found to be important to address and identify these AST subjects and their numbers in particular studies.

Whereas the open eye tear samples were relatively simple to obtain and examine, due to the plentiful supply and ease of sampling, the assessment of closed eye tears proved somewhat difficult and troublesome. As discussed in Chapter 5, taking a 'true' closed eye tear sample is currently, practically impossible. This tear state, as the name suggests, would require the collection of these tears whilst the eye was closed, creating an unworkable situation. A compromise was required and this was achieved by taking samples immediately upon eye opening and waking. As a consequence some ambiguity as to the true nature of this tear sampled must be accepted. A true definition of the tears sampled needs to be made clear. The time taken between waking and collection must be extremely important and influential on the results observed. The speed with which the eye reverts to an open eye state is unknown and the mechanisms of change are unclear. Consequently, the results of the protein levels measured, presented in this thesis, under the term closed eye tears must be understood as being the closest working representation. These tears, which are extracted on eye opening, may prove to some extent

representative of a different tear state. A state of recovery of the eye going from a closed internal eye state to an open external eye state with constant tear flow dynamics.

This problem aside, another obstacle met was the fact of the difficulty in taking the 'on-waking' tear which, due to expense parameters, required the volunteer to be capable of, at home, self-sampling. Consequently, the use of self-sampling further debated the issue of the nature of the closed eye tear sample and whether it was representative of such. The time and duration of each 'closed eye tear' collection would have varied somewhat from sample to sample, thus presumably affecting the outcome of results to some extent. The use of this self sampling method resulted in the loss of control of the tear collection methodology and suggested a lack of standardisation. Additionally, from a purely practical perspective, the difficulties in self-collection, on immediate waking, are obvious and added to the collection problems.

Finally, another complication unearthed was related to the sample volume, and that which could be obtained on waking. From personal self-sampling experience, the eye on waking was extremely dry and required approximately ten minutes and above to provide even one extractable microlitre. By this point, it is assumed, that the eye would have been washed/flushed with reflex tearing which would wash and dilute the eye of evidence of the closed eye state.

In short, the main problem experienced in the field of closed eye tear analysis was the lack of sampling control and this and the other problems incurred were beyond the scope and control of this project. However, where the immunodiffusion assays were developed with open eye tear samples, it was decided to attempt to examine the 'closed eye tears' exploiting their low sample volume requirements. The tears tested for this area of analysis, referred to as closed eye tear samples, as pointed out were not technically indicative of such, but in reality, were possibly 'diluted' versions of the true close eye tear. Nevertheless, the results found for these samples, representative of the closed eye

tear, displayed clear increases in the plasma derived/leaked proteins and also substantiated the IgA rich tears previously observed during the closed eye phenomenon.

The CEE tears investigated for IgA content in Chapter 5, displayed a variety of results and differences in change going from the open eye to the closed eye IgA levels in the five patients analysed. This variance in protein concentration increase between patients may be indicative of the sensitive dynamics of change occurring in the eye immediately on eye opening/waking. The time delay/duration of sampling may the foremost factor affecting the results observed and may not be solely due to patient influence. The nature of the 'at home, unsupervised collection' resulted in the lack of standardisation but also meant that the collection was not accurately timed and thus the above statement is speculative. It is quite possible that whenever closed eye tear samples can be truely extracted and investigated, the results of the protein analysis will show similar yet more exaggerated protein level changes.

The closed eye environment was described as a state of sub-clinical inflammation, rich in IgA and a variety of pro-inflammatory and immunocompetent regulators. The question was then raised as to how the ocular surface is protected from damage due to its exposure to a potentially explosive inflammatory environment. The answer was suggested in the form of vitronectin, with its multifunctional capabilities, which demonstrated a variety of anti-inflammatory functions.

At the start of Chapter 3, the diverse functions of vitronectin were described. Its involvement in many cascades and systems and thus its interactions with a variety of plasma proteins, suggested it was very important in plasma/serum functioning, but its relevance in the eye may have seemed less important. This was not the case, with growing evidence of more and more plasma proteins 'invading' the tear film, especially in the closed eye environment. Increasingly, more and more plasma-derived proteins are being discovered (and those not found present can not be ruled out), especially in contact lens wear which assumes some closed eye similarities, e.g. decrease in blink action,

reduction in atmospheric oxygen availability and a change in the tear protein profile. Contact lens wear can induce mild or major red eye, involving vascularisation of the cornea, thus increasing the permeability of the blood/tear barrier and creating more susceptibility to plasma/serum leakage.

Vitronectin was shown to have an affinity for contact lenses, it was observed to absorb out of solution or the ocular environment onto the surface of both *in vitro* and on *ex vivo* lenses. This absorption was influenced by a number of factors including, water content and ionicity of the lens, wear modality and patient influence. Fibronectin, which shares many functional similarities, was also investigated but it was found under these experimental conditions that its role was minor and non-influential. The main points ascertained regarding the adherence of vitronectin to the lens were as follows:

- There existed a direct correlation between the concentration of vitronectin in solution and the amount absorbed onto the lens surface. A concentration of vitronectin above which no more adsorption would be observed was not obtained or studied, as this was out of the concentration parameters of this study.

- Vitronectin has a greater affinity for high water content ionic lenses, an increase in the water content clearly demonstrated a rise in cell mediated adhesion. However, the ionic property of the lens appeared to be the more dominant factor, high water content Group IV lenses displayed greater vitronectin adsorption levels than high water content Group II lenses.

- Vitronectin accumulated on the surface of Group IV Acuvue lenses over time. There was a marked increase in the levels of vitronectin going from one day to fourteen days wear, as shown by means of a comparison of the one day versus daily wear lenses.

- A comparison of Acuvue DW and EW *ex vivo* lenses, which were worn for a total of 198 and 168 hours respectively, demonstrated greater vitronectin levels during

extended wear, noting that the extended wear lenses would be worn in the CEE state, where there is more available vitronectin from tears and the corneal bed.

- A preferential adsorption on the surface of the lens by vitronectin was also recognised; the pattern of note was a post lens micro-climate towards the periphery. This adhering glycoprotein may act alone or, through its recognition of a variety of ligands, may bind and act in conjunction with a variety of other proteins. This, then further highlights the importance of the micro-climate that vitronectin may create at the periphery of a contact lens, particularly in extended wear lenses. The micro-climate created in lens wear may be beneficial or deleterious to the host.

As a consequence of the above observations the interaction of vitronectin with bacteria as described in Chapter 3 is an interesting one. Vitronectin may serve to bind and present a potential pathogen to immunocompetent cells, e.g., polymorphonucleocytes, for clearance. On the other hand, the pathogen may utilise and bind to vitronectin as a means of establishing its presence in the ocular surface, which may prevent the immune system from detecting its presence. Whichever theory may prove to be true, the consequence of bacterial adherence to vitronectin in the presence of a contact lens would be further enhanced in the resultant micro-climate. During overnight extended wear the consequences of vitronectin mediated bacterial adhesion may prove very important, for example, persistent bacterial presence on the surface of a contact lens may cause an excessive inflammatory response.

The vitronectin assay proved an important and useful on-lens assay in the analysis of this protein's deposition on the surface of a lens and tear interactions with the lenses, due to vitronectin's affinity and ability to bind to both the lens surface and a variety of ligands, including certain cell integrins. This was an extremely useful non-denaturing assay. Unfortunately, the dual cell and ligand binding property ability was not a feature of the tear proteins like IgA, IgG, lactoferrin and albumin under investigation in lens spoilation, and therefore they could not be applied in an assay. However, an alternative method for

analysing these proteins in lens spoilation was attempted, with a view to exploiting the immunodiffusion assays in the analysis of the individual protein deposition characteristics. Initially the spoilation of lenses by proteins was examined as a total protein outcome. This was achieved by means of UV spectrophotometry; a quantitative technique with the ability to measure the total surface and bulk protein deposition.

These UV deposition studies demonstrated the highly influential significance of lens material composition and stressed the significance of patient variability and the need to define particular patient trends especially when using individual patients in various assays. The UV studies investigating a variety of patients were very useful in aiding the selection of patients for further investigation. The following key points were observed through UV spectrophotometry total protein measurements on *ex vivo* lenses.

A comparison of three Group IV lens types illustrated the intra-group deposition variances, the effects of lens thickness and material composition.

- Surevue, which differs only in lens thickness from Acuvue lenses, demonstrated a greater susceptibility to spoilation by proteins. The thicker lens allowing a greater accumulation of protein.

- Both Surevue and Acuvue allowed a greater level of deposition than Focus, a comparison of a HEMA/MA composition for the first two lens types against the Focus HEMA/MA/PVP make up. The water content was not an influencing factor here as all the lenses possess a 55-58% water content.

The time dependency study by UV spectrophotometry on both Group IV and Group II lenses provided interesting information on the build up of total protein over time up to 28 days daily wear.

- The affect of material composition was again evident, an approximate 10-fold increase in deposition was observed going from the Medalist 66 (II) to Acuvue (IV) lenses. This inter group difference between the non-ionic Group II lenses and the ionic Group IV lenses was significant.

- The deposition characteristics over time, from 1 day wear to 28 days wear, were found to be different for the Group II and IV lenses. The Group II lenses did not appear to be greatly influenced by wear duration, the initial protein spoilation levels measured at 1 day were not greatly exceeded at 28 days. The Group IV lenses on the other hand displayed time dependency. Protein levels measured at 1 day were greatly augmented after 7 days wear but thereafter the levels remained more static.

The UV studies in general provided the following information:

- The influence and variances from patient to patient was very evident. Additionally, differences between measured protein levels on the left and right eye lenses of individual patients were highly variable.

As stated above, the analysis of one tear component, vitronectin, was achieved using an on-lens assay - in order to look at other on-lens deposited tear components, a method to extract the protein from the lens and into solution was ascertained. One such method was obtained, defining the most efficient, easy and reproducible method for the extraction of proteins from the lenses, tested on a variety of *in vitro* spoilt lenses. The extraction method chosen involved heating the lenses to 90°C for 3 hours in a mixture of urea, sodium dodecyl sulphate, dithiotheriol and tris[hydroxmethyl]aminomethane (method (d)).

The extraction trials were performed using different doping solutions on Group IV Acuvue lenses, one which contained the main tear proteins (PTS-1), one with the main tear proteins minus lysozyme (PTS-2) and one with only lysozyme. PTS-1 allowed an approximate 78% removal, PTS-2 gave an approximate 53% removal and the lysozyme only solution allowed an approximately 87% removal. These results showed that almost all the lysozyme deposited was extracted, but with possibly only a half of the other total protein was readily removed from the lenses.

Lenses from both the Group II and IV time dependency spoilation study were extracted by method (d) to analyse the difference in its performance on each of the two Group lenses in removal of proteins, and to look at the deposition rates of individual proteins deposited over time. In the case of extraction of the Medalist 66 lenses there was only a slight reduction in deposited protein; this would suggest extraction method (d) had only a minor effect on the removal of deposited proteins from the Group II *ex vivo* lenses. Noting the already low levels of protein deposition on these lenses made the extraction more difficult. The Group IV *ex vivo* lenses on the other hand displayed similar high removal percentages (~85%) as observed in the *in vitro* trials. The percentage removal efficiency was irrespective of wear time or original protein quantity, but all these Acuvue lenses were heavily spoilt as a result of one day to four weeks wear.

The eluates of both the Group II and IV lenses post-extraction were then analysed for individual protein profiles to investigate , the identity of individual proteins and to attempt to assess the rate of deposition. The eluates were initially run on SDS-PAGE, but the results achieved by this method were poor. No protein bands were detected at all for the Medalist 66 lenses and only lysozyme bands were observed in the Acuvue extracts.

The eluates were then examined by the detection assay CIE in an attempt to give a more in depth and sensitive analysis of the eluates. Five proteins were investigated, IgA, IgG, lactoferrin, albumin and kininogen. The latter will be dealt with individually after this section. Protein in only the Acuvue eluates were detectable by this method, none of these

proteins were present at a detectable level in the eluates of the Medalist 66 lenses extracted.

The results of the analysis of the eluates of the Acuvue lenses demonstrated that, as a general rule, both the plasma and tear derived proteins were deposited and extracted quite similarly. The detection of all four proteins in the eluates of the Group IV lenses was prominent after 2 weeks wear, in the cases of lactoferrin and IgG, a further increase in the band intensity, and therefore protein concentration, was observed in the 4 weeks wear eluates. The presence of lactoferrin, IgG and IgA was also detected after 1 week wear. A questionable line of detection was found for IgG after 1 day wear in some of the eluates tested.

These results provided a little insight into the deposition of the four proteins on Group IV lenses and proved that all four of these proteins are involved in the spoilation of these lenses to some extent. The key point that can be accepted was that each protein accumulated over time mainly between 1 day and 7 days wear, demonstrating a constant build up that is not greatly effected by the daily cleaning of the lenses. The different rates of deposition cannot be directly compared between proteins due to the fact that each are present in tears at varying concentrations, in order to do so, their relative concentrations in tears must be considered. However, the concluding outcome of these results would seem to suggest that as a general rule, once the lenses are worn for 7 days or more the protein accumulation is greatly evident. The next step would be to ascertain where between the 1 to 7 days the accumulation significantly rises or whether is it simply a gradual rise.

These results would seem to suggest that 1 day wear offers the lowest spoilation, naturally, but once the lenses are worn beyond 7 days the spoliation levels were not greatly increased and thus if the lenses are worn for 7 the deposition affects and consequences of wearing the lenses for the whole month would seem minimal.

The next step in the analysis of the Group IV lens eluates was in the investigation of kininogen. Previously, no work had been done on the analysis of kinins in tears. However, the recent discoveries of the kinins in allergic reactions at other mucosal sites, their potent inflammatory mediation and their ability to be activated on contact with negatively charged surfaces made its detection or non-detection important and thus its presence was assessed.

Preliminary work discovered that kininogen was found to be involved in lens spoilation and was adsorbed onto the lens surface. This would suggest that the kinins are present in the ocular environment, but presently the consequences of which are unknown.

The kinins may prove to be important in contact lens induced inflammation because they can be activated on contact with negatively charged substances and contact lenses may prove no exception. The role of kinins in a pro-inflammatory capacity has recently seen research in the development of kinin antagonists for therapeutic intervention and it is becoming an exciting new area in the control of inflammation. Currently studies are at an early developmental stage in the body as a whole, but their persistence allergic responses makes them prime targets in the consideration of antagonistic therapeutic agents.

The work done on the kinin family of inflammatory mediators in tears and contact lens wear was only a beginning, and further analysis is required, but the point of their possible presence was introduced here and cannot be disregarded.

7.2. Future Work

1. *Determination of tear protein concentrations as diagnostic tools.* All the tear samples investigated in the development and application of the immunodiffusion assay were from healthy subjects in order to define the parameters of each assay. The next step would be to assess their use as diagnostic tools to ascertain patterns of these protein levels in immunocompromised subjects, or those presenting a variety of complications, and to attempt to investigate if any trends were forthcoming that may occur with specific disorders. The development and standardisation of analytical techniques to monitor tear protein levels remains of high priority. Tear protein analysis is not only significant in the understanding of the pathogenesis of a number of diseases, but it has and will prove an important diagnostic tool, e.g., in the use of a particular protein or mixture of proteins as an indication of the on-set, cause or manifestation of a disease. The proteins involved may be analysed for concentration changes or for conformational changes which may become markers in particular disorders. One or more correlative proteins levels may prove diagnostically important in the detection of the onset of the ocular problems. For example, a decrease in concentration of tear specific gland proteins like lactoferrin and lysozyme are currently parameters for the diagnosis of lacrimal gland dysfunction in conditions such as Sjörgens Syndrome and keratoconjunctivitis.

2. *The effects of contact lens wear on the tear protein profile.* Another area that may prove interesting would be to analyse the long term effects of contact lens wear on the tear protein profile. It has been observed that albumin, IgG, IgA and lactoferrin accumulate over time on Group IV Acuvue contact lenses. An interesting step would be to compare the levels of these proteins, and others in the future, in the tears of the patients at the beginning and at the end of the wear time. Tear samples could be taken at the start, during the course of lens wear and at the end of a wear period, looking at the dynamics of the protein shifts. Alternately, or in conjunction with, tears could be collected over a certain time period while the lenses were in place to investigate the

regulation (or lack of) of the eye on the levels of certain proteins in the tears during the lens wear. This may give an insight into how the host monitors protein levels in the circulating tears; if there is a compensation influx of proteins released into the eye during contact lens wear to make up for the fact that the contact lenses are denuding the eye of essential tear components, or if the levels of secreted proteins are uninfluenced by the presence of the contact lens. Analysis of contact lens absorbed proteins versus circulating protein quantities with and with out contact lens wear may hold the key. For example, lysozyme is known to be greatly taken up by Group IV lenses initially on lens insertion and during the course of wear, it would be interesting to assess the levels of free lysozyme in the tear fluid over a given time during the course of an average eight hour wear time. Do the levels of lysozyme released increase to compensate for those preferentially adsorbed by the lens, or is the release rate unaffected? Further, if the eye was shown to compensate for the lens insertion, how is this mechanism affected after years of wear, in problematic versus non-problematic wear.

3. *Mechanisms of change going from closed to open eye tears.* With regard to the closed eye environment, a time scale picture should be drawn up in order to assess the rate of recovery of the eye in reverting from the closed eye environment to the open eye environment. The first step in this experiment would involve optimising the tear collection itself. A rapid non-stimulating method which could collect 1-2μl would be ideal. It would be a difficult task considering the time scale involved, as presumably the eye takes no more than five minutes to revert to an open eye state. If a rate or trend could be observed over, for example a 30 second interval scale, it would assist in the correlation of closed eye results, defining the closed to open eye dynamics. These investigations may corroborate the previously suggested idea that the variables in the IgA closed eye results were due to samples being taken at varying stages of the eye recovering from the stagnant closed eye tear to a dynamic open eye tear, as opposed to primarily patient influence or other factors. However, as stated throughout, the general problems in assessing the exchange to open eye on waking are those

encountered on taking a closed eye tear sample ordinarily, but are increased due to the requirement for a staggered set of samples. Additionally, taking tears at the non-stimulatory (~1μl/min) mark would mean collecting approximately 0.5μl per 30 seconds which is an acutely low volume with which to work. Increasing the time intervals to 1 minute may be too long to note the precise changes and would yet still result in low working volumes.

4. *Application of immunofluorescence in on-lens assay studies.* While the extraction studies were performed in order to ascertain the nature of the protein spoilant, the difficulties encountered in removing the proteins from the lens must be considered. Although high removal percentages were evident on some lenses, the work done on the PTS-2 may suggest of that extracted only approximately 50% of non-lysozymal spoilation is removed, thus preventing an analysis of the whole picture of spoilation. Additionally, the low deposition profiles of the Group II lenses proved troublesome in the application of extraction methods. Thus the use of immunofluorescence in, conjunction with or solely, in the analysis of on-lens spoilation both quantitatively and qualitatively is an important way forward. In conjunction with the extraction studies, immunofluorescence could be used to analyse and identify the protein remaining on the lens surface after extraction, highlighting those proteins with a greater affinity for the lens. The extraction method of choice in this thesis was a harsh method and protein denaturation was an unavoidable consequence, the use of immunofluorescence with relevant antibody tags would overcome this problem.

5. *Enhancement of CIE quantitative analysis.* The work on the extracted lenses was initially primarily carried out to obtain the most efficient extraction method and to ascertain whether the eluted protein could be detected by immunoassay, namely counter immunoelectrophoresis. Analysis of the time dependant study lenses was consequently undertaken in order to assess the changes in deposited protein on the lens over time. The drawback of this method of investigation was that the bands were basically bands of detection and were not quantitative. An arbitrary scale of band

assessment on the CIEs was set up. This may have been crude, but it was important and useful in aiding the interpretation of results. Ideally the development of densitometry or another band intensity measurement method would be the optimum in the analysis of the CIE precipitation bands. These methods could then be employed in order to translate the detection bands, at least semi-quantitatively.

6. *Further investigations into the presence of the kinin family of inflammatory mediators in tears.* The examination of the kinins in tears, in this thesis, was preliminary and only a start off point to the wide range of investigations that could be performed on these important inflammatory mediators. Their presence in the closed and open eye tears, yet needs to be determined. The use of the aforementioned, immunofluorescence technique to analyse the accumulation of kininogen on lens surfaces may also prove useful. The growing detection, understanding and implications of the kinins in a variety of disorders throughout the body, further the need for this analysis. Of particular importance is the increasing detection and implications of the kinins in allergic responses. Examination of tears for kinin presence in allergic reactions, with and without lens wear, in the eye, may prove important in the development of therapeutic targets. Additionally, the use of contact lenses in the study of the kinins may prove very useful, in the eluate or by the use of cells as a probe in a similar assay as that presented in Chapter 3.

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Appendix I

Buffers and Solutions

APPENDIX I

Buffers and Solutions

1. PAGE monomer solution

(30%T, 2.7%C_{bis})

Acrylamide (BDH 44299)..... 58.4g

Bis (Sigma M-7279)..... 1.6g

distilled H_2O (dH_2O)..... to 200ml

Store at 4°C in the dark

2. Separating gel buffer

(1.5M Tris-Cl, pH8.8)

Tris (Sigma T-1378)..... 36.3g

Adjust to pH 6.8 with HCl

dH_2O..... to 200ml

3. Stacking gel buffer

(0.5M Tris-Cl, pH6.8)

Tris..... 3.0g

Adjust to pH 6.8 with HCl

dH_2O..... to 50ml

4. 10% SDS

SDS (BDH 10807)..... 10g

dH_2O..... to 100ml

5. APS Initiator

(10% ammonium persulphate)

Ammonium persulphate (BDH 10032)..... 0.5g

dH_2O..... to 5.0ml

6. Running gel overlay

(0.375M Tris-Cl, 0.1%SDS, pH 8.8)

Tris..... 25ml solution (2)

SDS..... 1.0ml solution (4)

dH_2O..... to 100ml

7. Treatment buffer

(0.125M Tris-Cl, 4%SDS, 20%Glycerol, 10% 2-mercaptoethanol, pH 6.8)

Tris..... 2.5ml solution (3)

SDS..... 4.0ml solution (4)

Glycerol (Fisons G/0600/08)..... 2.0ml

2-mercapatoethanol (2-ME) (Sigma M-3148)..... 1.0ml

dH_2O..... to 10.0ml

* Divide in aliquots and store in freezer

8. Tank buffer

(0.025M Tris, 0.192M glycine, 0.1%SDS, pH 8.3)

Tris..... 2g

Glycine (Sigma G-8898)..... 57.6g

SDS..... 40ml solution (4)

dH_2O..... to 4.0 litres

9. Water-saturated n-butanol

n-butanol..... 50ml

dH_2O..... 5ml

10. Coomassie Brilliant Blue stain

Methanol..... 90mls

dH_2O..... 90mls

Glacial acetic acid..... 10mls

Coomassie Brilliant Blue (BDH 44329)..... 1.2g

11. Destain

Methanol	30mls
dH_20	80mls
Glacial acetic acid	10mls

12. Fixative

Methanol	25%
Glacial acetic acid	10%

13. Gel storage solution

Methanol	25%

14. 2 x TBE

Tris	21.8g
Boric acid (BDH 10058)	11g
EDTA (Sigma ED-2SS)	1.86g
dH2O	to 1 litre

15. 10 x Saline

Sodium chloride (Fisons S/3160)	90g
dH_2O	to 1 litre

16. 1 x Barbital Buffer (Fluka 11706)

0.05mol 5,5-diethylbarbituric acid sodium salt	
0.01mol 5,5-diethylbarbituric acid	reconstitute with 1 litre of dH_2O

Appendix II

Ultra Violet Spectrophotometry Results

APPENDIX II

Ultra Violet Spectrophotometry Results

Absorbance to Concentration Conversion Table for Total Protein

Absorbance	Concentration (mg)	Absorbance	Concentration (mg)
0.01	0.000	0.3	0.126
0.02	0.003	0.4	0.173
0.03	0.007	0.5	0.220
0.04	0.012	0.6	0.263
0.05	0.017	0.7	0.311
0.06	0.021	0.8	0.351
0.07	0.025	0.9	0.395
0.08	0.030	1.0	0.441
0.09	0.034	1.1	0.488
0.1	0.039	1.2	0.535
0.11	0.043	1.3	0.575
0.12	0.048	1.4	0.621
0.13	0.052	1.5	0.667
0.14	0.057	1.6	0.706
0.15	0.061	1.7	0.759
0.16	0.065	1.8	0.789
0.17	0.070	1.9	0.842
0.18	0.074	2.0	0.889
0.19	0.079	2.1	0.932
0.20	0.083		

Background Blank Lens Absorbances

Surevue Blanks			
Negative Powers	Mean Absorbance	Positive Powers	Mean Absorbance
5.00	0.075	1.00	0.107
4.50	0.074	1.50	0.081
4.00	0.097	2.00	0.111
3.50	0.071	2.50	0.100
3.25	0.063	3.00	0.101
2.50	0.079	3.50	0.097
2.00	0.072	4.00	0.095
1.50	0.083	4.50	0.106
1.00	0.095	5.00	0.086

Acuvue Blanks			
Negative Powers	Mean Absorbance	Positive Powers	Mean Absorbance
5.00	0.059	1.00	0.032
4.50	0.065	1.50	0.025
4.00	0.069	2.00	0.079
3.50	0.072	2.50	0.072
3.00	0.047	3.00	0.038
2.50	0.052	3.50	0.090
2.00	0.052	4.00	0.101
1.50	0.069	4.50	0.090
1.00	0.064	5.00	0.121

Focus Blanks			
Negative Powers	Mean Absorbance	Positive Powers	Mean Absorbance
5.00	0.227	1.00	0.193
4.00	0.253	1.50	0.277
3.50	0.215	2.00	0.237
2.50	0.216	2.50	0.209
2.00	0.212	3.00	0.209
1.50	0.237	3.50	0.251
1.00	0.218	4.00	0.238
		5.00	0.253

Frequency 55			
Negative Powers	Mean Absorbance	Positive Powers	Mean Absorbance
5.50	0.200	1.00	0.173
5.00	0.151	2.00	0.221
4.50	0.128	2.50	0.210
4.00	0.158	3.50	0.212
3.00	0.102	4.00	0.294
2.50	0.129	4.50	0.206
2.00	0.134	5.50	0.243
1.50	0.108	6.00	0.198

1 Day Acuvue			
Negative Powers	Mean Absorbance	Positive Powers	Mean Absorbance
5.25	0.021	1.00	0.018
4.50	0.023	1.50	0.027
4.25	0.023	2.00	0.023
3.50	0.028	2.50	0.023
3.00	0.027	3.00	0.025
2.50	0.024	3.50	0.023
2.00	0.028	4.00	0.023
1.50	0.014	4.50	0.026
1.00	0.019	5.00	0.024

Medalist 66			
Negative Powers	Mean Absorbance	Negative Powers	Mean Absorbance
4.00	0.141	2.00	0.115
3.50	0.123	1.50	0.115
3.00	0.127	1.00	0.115
2.50	0.100		

Mean background subtracted absorbances for blank lenses measured by UV

Lens Name	Mean Background
Surevue	0.089
Acuvue	0.067
Medalist 66	0.119
1 Day Acuvue	0.023
Focus	0.229
Frequency	N/A

Time Dependency Concentration (mg) Results: Acuvue Lenses

Patient No.	1 Day	1 Week	2 Weeks	3 Weeks	4 Weeks
1	0.155	0.416	0.342	0.454	0.378
2	0.290	0.417	0.46	0.600	0.526
3	0.332	0.365	0.395	0.406	0.411
4	0.276	0.395	0.439	0.411	0.339
5	0.185	0.583	0.36	0.387	0.365
6	0.155	0.356	0.633	0.339	0.305
7	0.192	0.440	0.407	0.410	0.503
8	0.062	0.405	0.415	0.394	0.312
9	0.206	0.808	0.531	0.462	0.466
10	0.042	0.477	0.428	0.518	0.381
11	0.185	0.376	0.374	0.304	0.397
12	0.200	0.454	0.471	0.388	0.466
13	0.636	0.629	0.537	0.928	0.480
14	0.179	0.269	0.405	0.303	0.377
1	0.151	0.702	0.414	0.588	0.466
5	0.293	0.589	0.553	0.696	0.733
16	0.320	0.372	0.325	0.242	0.862
17	0.232	0.839	0.593	0.545	0.575
18	0.229	0.492	0.521	0.351	0.528
19	0.201	0.398	0.378	0.368	0.791
20	0.256	0.341	0.825	0.372	0.562
21	0.319	0.466	0.537	0.370	0.488
Mean	0.232	0.481	0.471	0.447	0.487
Max	0.636	0.839	0.825	0.928	0.862
Min	0.042	0.269	0.325	0.242	0.305
StDev	0.118	0.151	0.116	0.152	0.148

Time Dependency Concentration (mg) Results: Medalist 66 Lenses

Patient No.	1 Day	1 Week	2 Weeks	3 Weeks	4 Weeks
1	0.024	0.039	0.032	0.036	0.039
2	0.028	0.03	0.035	0.031	0.028
3	0.034	0.035	0.036	0.037	0.04
4	0.029	0.029	0.043	0.033	0.040
5	0.028	0.031	0.029	0.03	0.031
6	0.032	0.028	0.030	0.0290	0.031
7	0.031	0.032	0.032	0.036	0.036
8	0.04	0.032	0.032	0.033	0.041
9	0.027	0.024	0.03	0.032	0.029
10	0.032	0.029	0.098	0.032	0.034
11	0.029	0.031	0.031	0.036	0.045
12	0.035	0.034	0.03	0.032	0.035
13	0.03	0.033	0.044	0.038	0.062
14	0.028	0.031	0.022	0.031	0.036
15	0.037	0.032	0.053	0.043	0.046
16	0.032	0.027	0.043	0.038	0.033
17	0.026	0.037	0.036	0.034	0.038
18	0.031	0.036	0.033	0.034	0.048
19	0.039	0.027	0.032	0.033	0.03
20	0.028	0.031	0.034	0.041	0.04
21	0.029	0.031	0.033	0.036	0.033
22	0.034	0.03	0.032	0.037	0.023
Mean	0.031	0.031	0.037	0.035	0.037
Max	0.04	0.039	0.098	0.043	0.062
Min	0.024	0.024	0.022	0.029	0.023
StDev	0.0041	0.0035	0.015	0.0035	0.0083

Appendix III

Embossed Versions of Immunodiffusion Gels

Appendix III

Embossed Versions of Immunodiffusion Gels

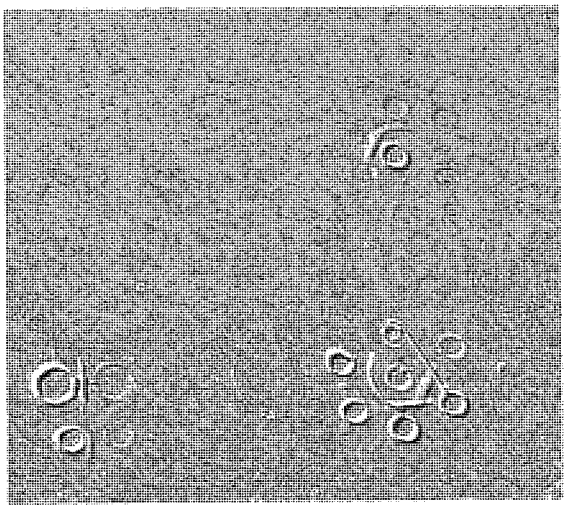

Figure 4.8. Ouchterlony of tear sample, kininogen and lysozyme

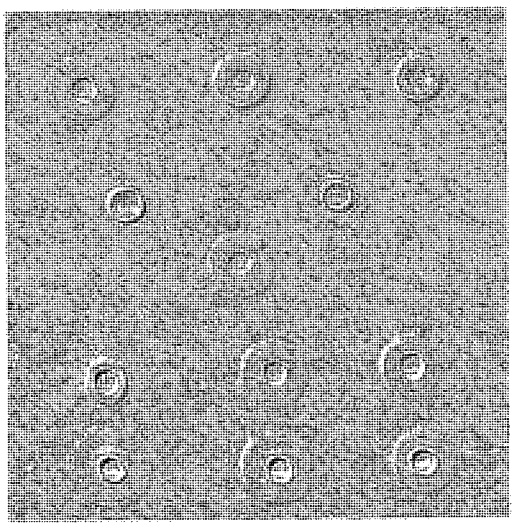

Figure 4.10. IgA RID gel of open eye tear samples. 1-5 = IgA standards, 6 = TS-1, 7 = TS-2, 8 = TS-3, 9 = TS-4 (R), 10 = TS-4 (L), 11 = TS-6 (R), 12 = TS-6 (L)

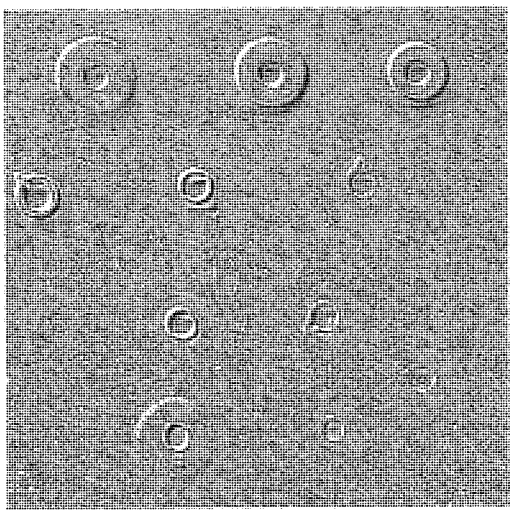

Figure 4.12. Analysis of open eye tear samples for lactoferrin by RID. 1-5 = lactoferrin standards, 6 = empty, 7 = TS-2, 8 = TS-1, 9 = TS-5, 10 = TS-4, 11 = TS-9

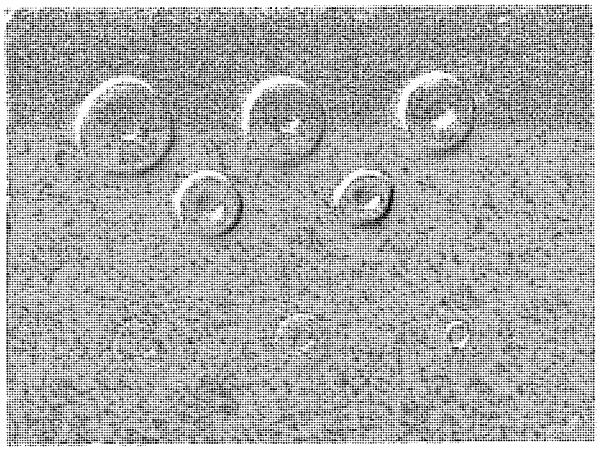

Figure 4.14. Analysis of open eye tear samples for IgG by RID. 1-5 = IgG standards, 6 = TS-1, 7 = TS-2, 8 = TS-1: Plate-1

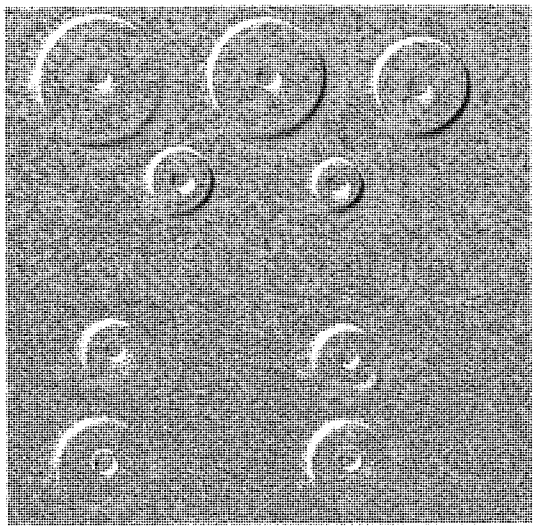

Figure 4.16. Analysis of open eye tear samples for IgG by RID. 1-5 = IgG standards, 6 = TS-5, 7 = TS-6, 8 = TS-7, 9 = TS-8: Plate-2

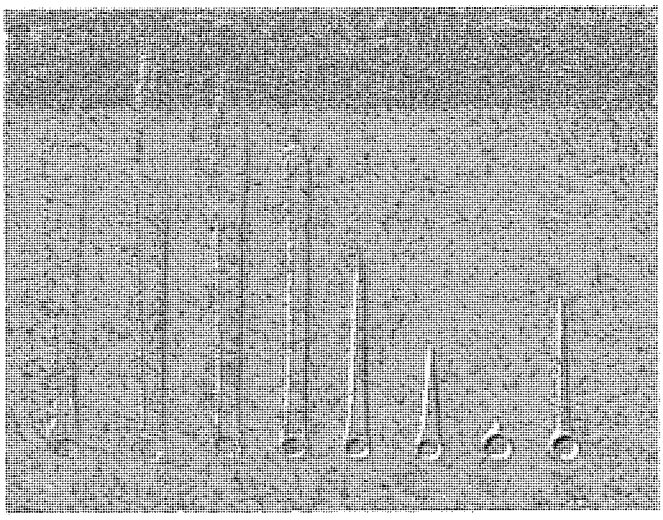

Figure 4.18. Analysis of open eye tear samples for albumin by rocket electrophoresis. 1-5 = Albumin standards, 6 = TS-9, 7 = TS-1, 8 = TS-10

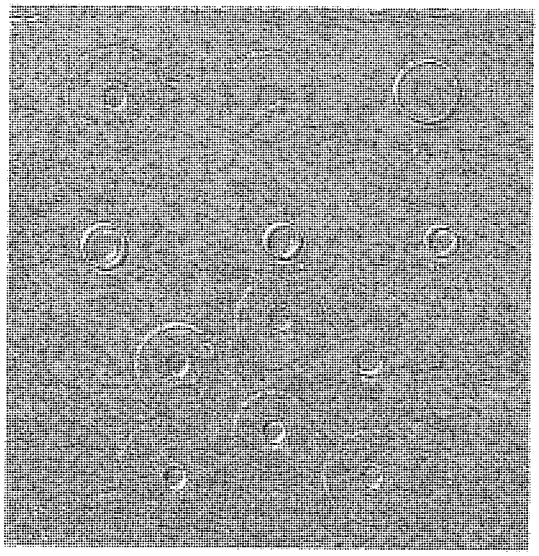

Figure 5.1. Analysis of closed eye tear samples for IgA by RID: Plate-1. 1-5 = IgA standards, 6 = empty, 7 = TS-11 (0), 8 = TS-12 (0), 9 = TS-12 (C), 10 = TS-13 (O), 11 = TS-13 (C), 12 = empty.

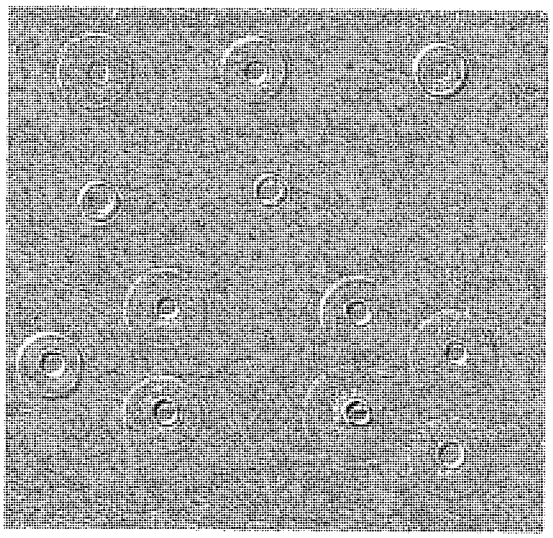

Figure 5.3. Analysis of closed eye tear samples for IgA by RID: Plate-2. 1-5 = IgA standards, 6 = empty, 7 = TS-1 (0), 8 = TS-14 (C), 9 = TS-14 (O), 10 = TS-4 (O), 11 = TS-4 (C), 12 = TS-15 (C), 13 = TS-15 (O)

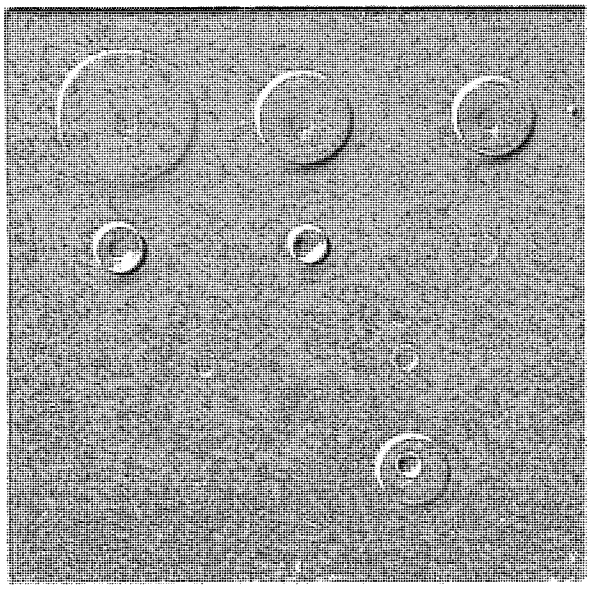

Figure 5.5. Analysis of closed eye tear samples for IgA by RID. 1-5 = IgG standards, 6 = empty, 7 = TS-1 (0), 8 = empty, 9 = TS-4 (O), 10 = TS-4 (C)

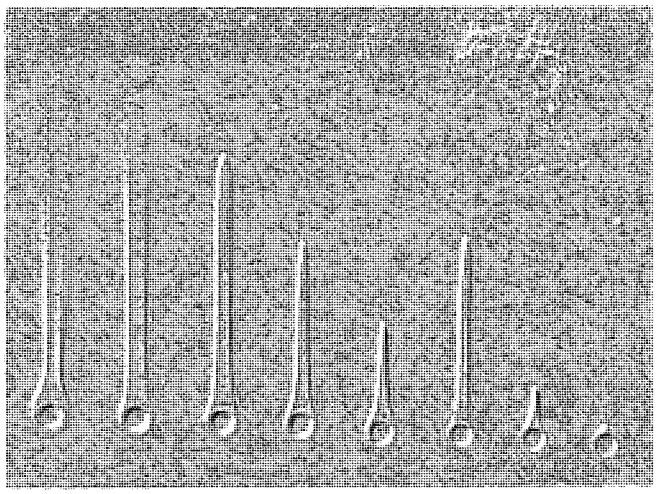

Figure 5.7. Analysis of closed eye tear samples for albumin by RE: Plate-1. 1-5 = albumin standards, 6 = TS-16 (O), 7 = TS-16 (C), 8 = TS-1 (O)

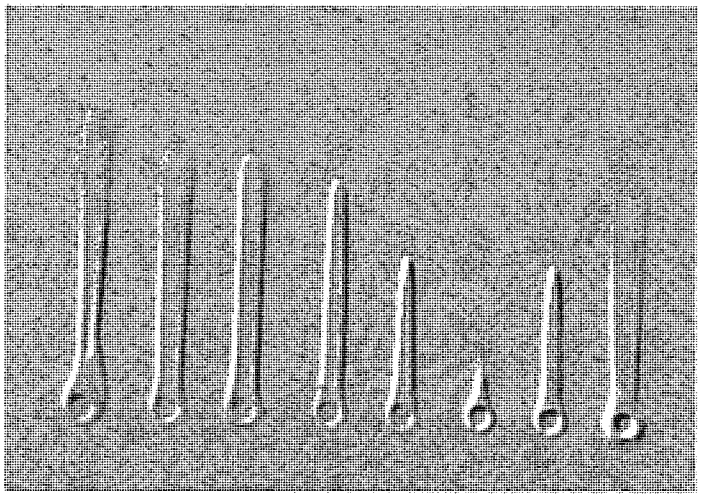

Figure 5.9. Analysis of open versus closed eye tear samples for albumin by RE: Plate-2. 1-5 = Albumin standards, 6 = TS-1 (O), 7 = TS-4 (O), 8 = TS-4 (C)

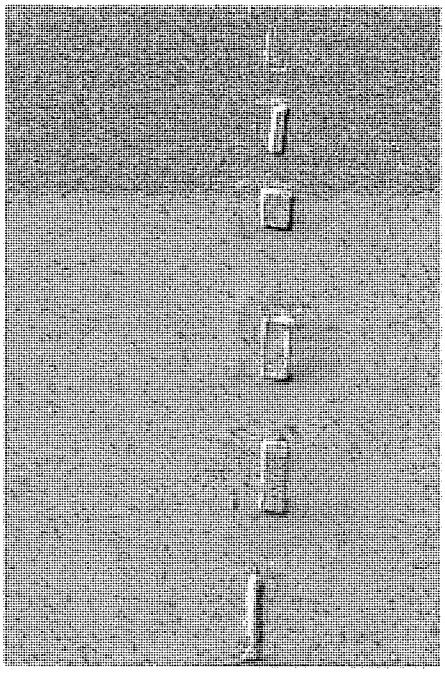

Figure 6.11. CIE of eluate comparing albumin levels on 1 day - 4 weeks wear Acuvue

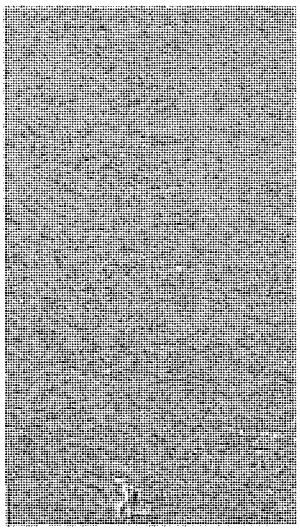

Figure 6.12. CIE of eluate comparing lactoferrin levels on 1 day - 4 weeks wear Acuvue

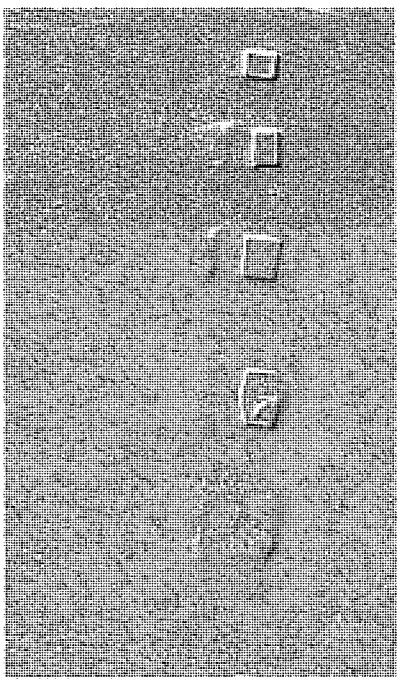

Figure 6.13. CIE of eluate comparing IgA levels on 1 day - 4 weeks Acuvue wear

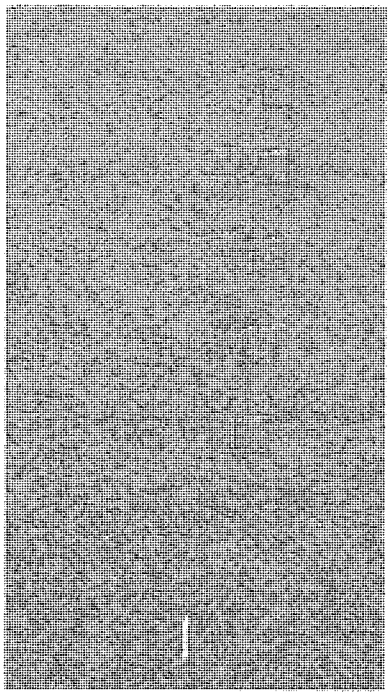

Figure 6.14. CIE of eluate comparing IgG levels on 1 day - 4 weeks Acuvue wear

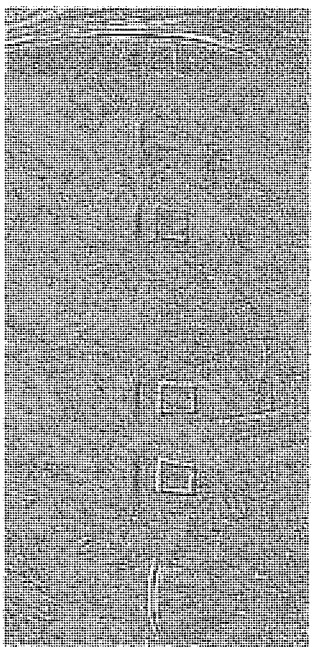

Figure 6.17. CIE of proteins from 4 weeks wear Group IV Acuvue eluate including kininogen: Plate-2